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THE ROLE OF GDF15 IN OVARIAN CANCER

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A

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by

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Dedication

I dedicate this dissertation to my family. Thanks for always being there for me.

Dedico esta disertación a mi familia. Gracias por siempre estar ahí por me.

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First of all, I would like to thank Dr. Kwong-Kwok Wong for his guidance. If we had an idea or experiment that we wanted to pursue, even if it was an area or technique that our lab was not knowledgeable about, if it was feasible and we had the means, you encouraged us to go for it. I would also like to thank the current and past lab members for their advice, support, assistance and friendship: Yvonne Tsang-Lee, Jong-Sun (Sunny) Choi, Suet Yan Kwan, Zhifei Zu, Erin Crane, and Huijuan Song.

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THE ROLE OF GDF15 IN OVARIAN CANCER

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Growth Differentiation Factor 15 (GDF15) is induced in situations such as stress, inflammation, treatment with non-steroidal anti-inflammatory drugs, as well as other therapeutic agents. As a secreted protein, GDF15 is seen as a potential biomarker in several types of cancer as well as in other diseases such as cardiovascular diseases, diabetes, and rheumatoid arthritis. In ovarian cancer, high GDF15 serum levels correspond to poor survival. It has further been shown to be expressed at higher levels in serum in ovarian cancer patients post-chemotherapy than pre-chemotherapy.

The overall 5-year survival for ovarian cancer is 46%, as a result of late diagnosis when treatment is mostly ineffective. Following initial treatment, 50-75% of patients will develop chemoresistance. Therefore, there is a great interest in identifying markers and therapeutic targets to improve treatment outcome. In this study, we aimed to determine the role that GDF15 plays in the chemoresponse to cisplatin in ovarian cancer. A microarray study identified GDF15 as being among the most highly induced genes following cisplatin treatment of an ovarian cancer cell line. This observation was further verified both *in vitro* and *in vivo*. We also found GDF15 induction by platinum agents to be p53 dependent. In addition, *in vivo* studies of a mouse orthotopic model revealed that GDF15 knockdown tumors were larger than control tumors. The tumors in which GDF15

had been knocked down were smaller following cisplatin treatment. Furthermore, *in vivo* tumors formed with A2780 ovarian cancer cells in which GDF15 expression was suppressed, demonstrated a reduced percentage of stromal cells compared to tumors formed with control A2780 cells. The stromal percentage of the GDF15 knockdown tumors did not change following cisplatin treatment unlike the control tumors. This study shows for the first time that GDF15 affects tumor composition. In addition, RPPA and RNA-seq was conducted on the mouse tumors to identify downstream targets of GDF15.

In summary, this study showed that induction of GDF15 by platinum agents is p53 dependent. This study further showed the effect GDF15 has in ovarian cancer, specifically the tumor composition. This study suggests that targeting GDF15 could be beneficial for patients with p53 wild type ovarian tumors that are often resistant to standard platinum-taxane chemotherapy. Further studies to identify the GDF15 receptor and downstream pathways are necessary.

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Abbreviations

AMH	Anti-Müllerian Hormone
ATM	Ataxia Telangiectasia Mutated
ATR	ATM- and Rad3-related protein
BIM	BCL-2-L-11
BMPs	Bone Morphogenic Proteins
COL6A1	Collagen, Type VI, Alpha 1
CRABP1	Cellular Retinoic Acid Binding Protein 1
ERK	Extracellular Signal-Regulated Kinase
FDR p-value correction	False Discovery Rate p-value Correction
FN1	Fibronectin 1
GDF15	Growth Differentiation Factor 15
GFY `	Golgi-Associated, Olfactory Signaling Receptor
IC	Inhibitory Concentration
IPA	Ingenuity Pathway Analysis
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-Activated Protein Kinase
MMR	Mismatch Repair
MMP1	Matrix Metallopeptidase 1
MMP10	Matrix Metallopeptidase 10
NER	Nucleotide Excision Repair
PARP1	Poly (ADP-Ribose) Polymerase 1
RPKM	Reads Per Kilobase Per Million Mapped Reads
RPPA	Reverse Phase Protein Array

TCGA	The Cancer Genome Atlas
TGF-β	Transforming Growth Factor-β

Chapter 1: Introduction

Ovarian Cancer

Ovarian cancer is the fifth most common cause of cancer related death among women [1]. For 2016, it is estimated that there will be 22,280 new cases and 14,280 deaths in the United States [1]. The five-year survival rate for ovarian cancer is 46%. If ovarian cancer is found in the early stages when it is still localized to the primary site, the survival rate is 92%. Unfortunately, only 15% of ovarian cancers are diagnosed while the cancer is still localized [2]. The majority of ovarian cancer patients are diagnosed when their cancer is in the advanced stage due to the fact that early stage ovarian cancer usually does not cause symptoms. The symptoms caused by more advanced ovarian cancer are nonspecific and often mistaken for gastrointestinal or reproductive diseases [3, 4]. Symptoms of ovarian cancer may include: weight loss, a frequent need to urinate, discomfort in the pelvis area, abdominal bloating or swelling, and changes in bowel habits [3].

There are three types of ovarian cancer, epithelial which makes up 90% of ovarian cancers, germ cell (4%) and gonadal-stromal cell (6%) [4]. Epithelial ovarian cancer can be further classified into four subtypes: (low grade and high grade) serous, clear cell, endometrioid, and mucinous [5]. These four subtypes are divided into two categories: Type 1 (clear cell, endometrioid, mucinous, and low grade serous) and Type 2 (high grade of serous, endometrioid, and undifferentiated) [6].

Type 1 tumors are thought to develop through a defined sequence starting with benign tumor lesions [7]. They grow slower and tend to be resistant to the standard ovarian cancer treatment although they may respond to hormonal treatment. Type 2 ovarian cancers are more common and while they are more aggressive they tend to

respond to the standard of care. Type 1 ovarian cancers are diagnosed in the early stages while type 2 cancers tend to be diagnosed in the later stages [6].

There are also differences between these two tumor types at the molecular level (Table 1). Among the molecular alterations on Type 1 ovarian cancers, low-grade serous carcinomas have wild type p53 and BRCA1/2 [6]. However, about two-thirds have BRAF (24%), KRAS (33%), and ERBB2 (9.5%) mutations although these are mutually exclusive [8]. In mucinous tumors, KRAS is frequently mutated [9]. ARID1A is frequently mutated in clear cell (46-57%) and endometrioid (30%) cancers [10, 11]. Clear cell carcinomas also have PI3K (33%) mutations and loss of PTEN (40%) suggesting that the PI3K pathway plays an important role in the tumorigenesis of this subtype of ovarian cancer [12-14]. Endometrioid ovarian tumors have 38-50% mutation rate for CTNNB1 and 66% mutation/LOH rate for PTEN [15].

Type 2 ovarian cancer consists of the high-grade serous subtype, which accounts for the majority of epithelial ovarian cancers. This particular subtype has a high p53 mutation rate [16]. An analysis from the cBioPortal using the TCGA ovarian serous provisional data set of tumor samples with sequencing and CNA data revealed that 87% of the samples had p53 mutations [17, 18]. Twenty percent of high-grade serous tumors have germline or somatic BRCA1/2 mutations and an additional 11% have hypermethylation of BRCA1. The TCGA study further revealed BRCA1 methylation/mutation to be mutually exclusive. In addition, the TCGA data study revealed that in 67% and 45% of cases, the RB1 and PI3K/RAS pathways were altered when looking at mutations, copy number, and gene expression combined [16].

Type (I/2)	Subtype	Frequency [7]	Mutations
I	Clear cell	12-13%	ARID1A (46-57%) [10] PIK3CA (33%) [12-14]
I	Endometrioid	9-11%	ARID1A (30%) [11] PIK3CA (39%) PTEN (18%) CTNNB1 (38-50%) [15]
I	Mucinous	3%	KRAS (60%) [9]
I	Low grade serous	*	BRAF (24%) KRAS (33%) ERBB2 (9.5%) [8]
II	High grade serous	*	TP53 (87%) [17, 19] BRCA1/2 (20%) [16]

Table 1: Ovarian cancer subtypes.

*Serous tumors account for about 70% of ovarian cancers. Of these only about 10% are low-grade serous and the remainder are high-grade [20].

Treatment

The standard treatment for ovarian cancer is usually cytoreductive surgery followed by six cycles of combination chemotherapy consisting of a platinum agent (cisplatin/carboplatin) and a taxane (paclitaxel), regardless of the subtype [6]. When cytoreductive surgery is not considered feasible, surgery may be performed after neoadjuvant chemotherapy. Data from two clinical trials comparing neoadjuvant chemotherapy and primary debulking surgery revealed that while there was less residual disease following neoadjuvant chemotherapy, which was associated with less treatment-related morbidity, there was no association with increased survival [21-23].

One of the major problems in ovarian cancer treatment is that more than 70% of patients will experience disease recurrence within 12-18 months [6].

Patient response to treatment

Based on a patient's response to treatment, they can be categorized in one of the following groups: platinum-refractory, platinum-sensitive, and platinum-resistant (Figure 1) [6]. Patients that are platinum-refractory do not respond to standard treatment. Patients are considered to be platinum-resistant if less than six months after treatment their tumors recur and platinum-sensitive if they relapse more than six months after treatment. More than 70% of patients will recur. Retreatment of a platinum-taxane regiment for platinum–sensitive patients with disease recurrence results in a 20-50% response. Second-line treatment for platinum-sensitive disease is usually a combination of carboplatin with paclitaxel, liposomal doxorubicin, or gemcitabine. Platinum-resistant patients whose disease recurs in less than six months after treatment are treated to weekly paclitaxel, liposomal doxorubicin, or topotecan, which result in a 10-30% response [6].

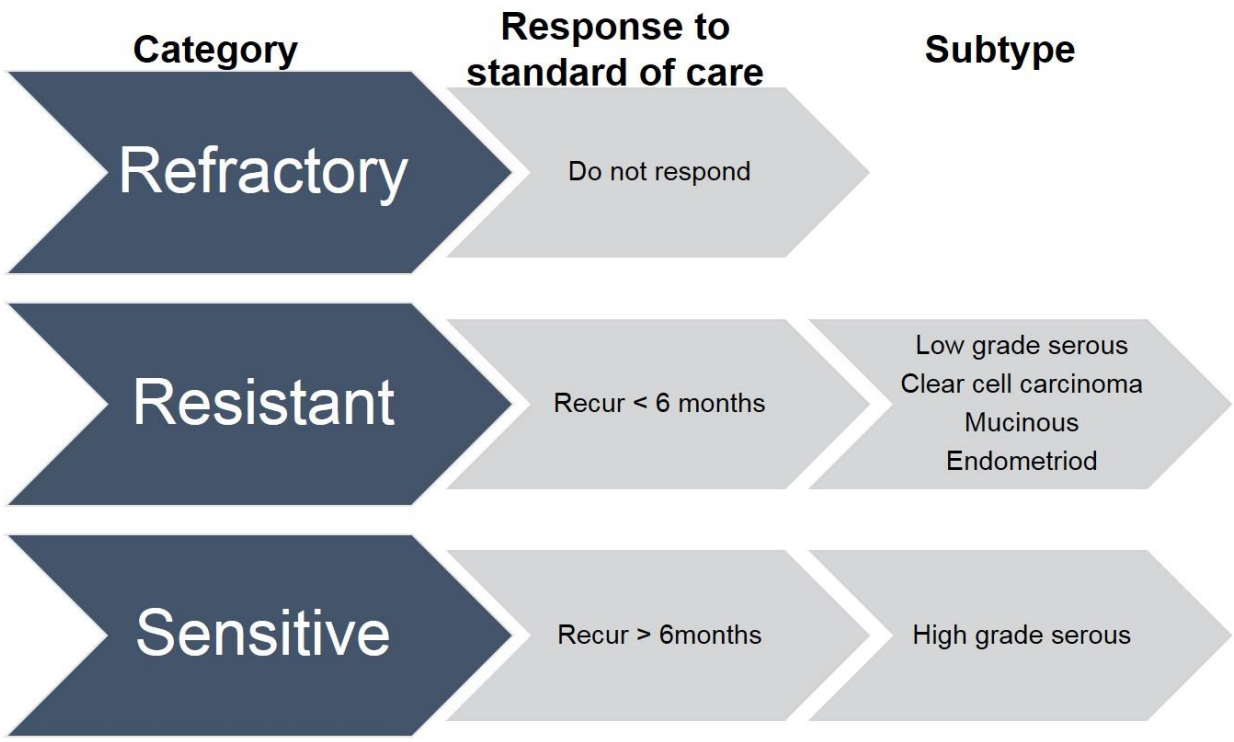

Figure 1: Patient treatment response categories.

Chemoresistance

Chemotherapy is among the most common types of cancer treatment [24]. Unfortunately, drug resistance to chemotherapeutic agents is a common occurrence. There are two types of drug resistance: intrinsic and acquired. Intrinsic resistance is defined by the presence of pre-existing factors within the tumor cells which render the therapeutic agent obsolete, whereas acquired resistance occurs when the tumor originally responds to the treatment and develop resistance through activation of alternative signaling pathways or mutations [25]. It is also accepted that chemoresistance may develop through selection of a population of resistant cells originally present in the tumor [25]. Over the years, several mechanisms have been

shown to be involved in drug resistance, which will be described in this section (Figure 2).

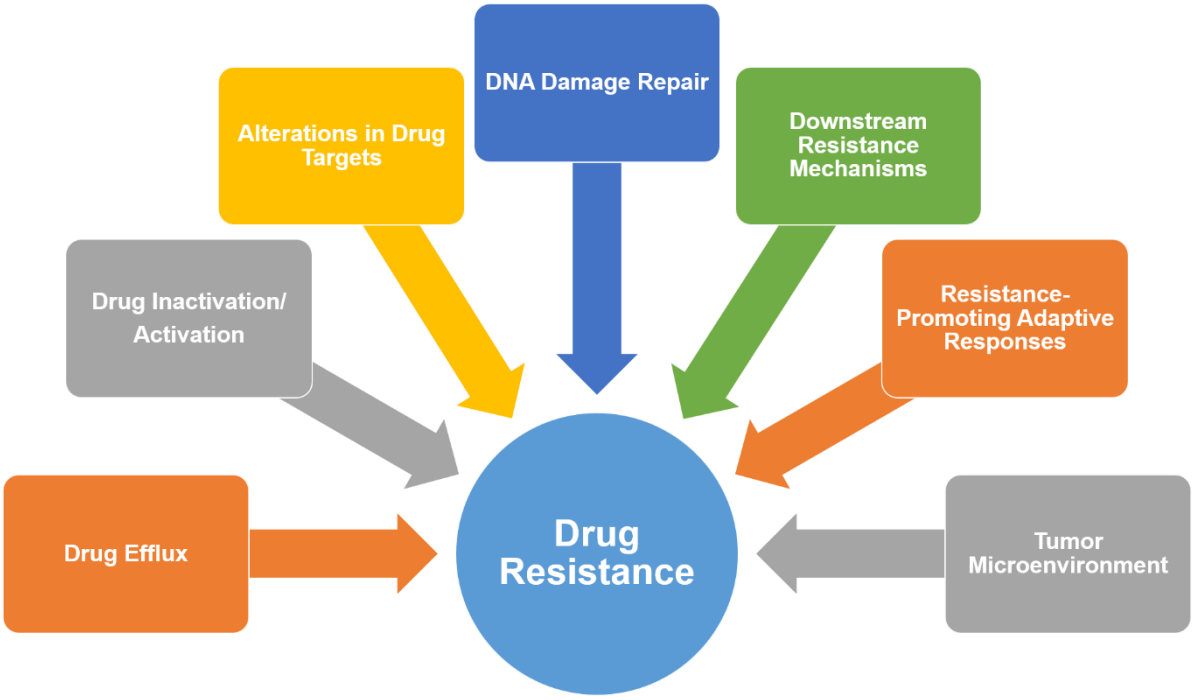

Figure 2: Mechanisms of drug resistance.

Drug efflux

Drug efflux is among the mechanisms that cancer cells develop drug resistance. Cell membrane transporter proteins such as the ATP-binding cassette (ABC) transporter transmembrane family of proteins regulate the flow through the plasma membrane of varying chemotherapeutic agents without specificity in structural differences. The multi-drug resistance protein 1 (MDR1) was the first ABC transporter to be identified. Its overexpression has been associated with chemotherapeutic outcome in several types of cancers such as colon, kidney, liver, neuroblastoma, and pancreatic cancers [26].

Drug inactivation/activation

Another mechanism of drug resistance is drug inactivation/activation, which is dependent on the drug. For example, capecitabine, a therapeutic agent that keeps cells from making DNA and RNA, must be converted to its active form, 5-fluorouracil (5-FU), by thymidine phosphorylase. Capecitabine resistance develops as a result of the inactivation of thymidine phosphorylase by methylation. Another example is Irinotecan, a topoisomerase I inhibitor which binds to topoisomerase I-DNA complex preventing ligation of the DNA strand. This results in double strand DNA breaks, which leads to replication fork arrest and cell death [27]. Through an enzymatic reaction, Irinotecan is converted to 7-ethyl-10-hydroxycamtothecin (SN-38), which is a more active form. SN-38 has been shown to be deactivated by UDP glucoronosyltranferase 1 (UGT1A1) [28]. Gagnon *et al*. showed that colon cancer cell lines expressing low levels of UGT1A1 had hypermethylation of the UGT1A1 promoter and similarly when these cells were treated with the demethylating agent 5-Aza-dC expression of this gene was restored [29].

Alterations in drug targets

Alterations in the drug target through mutations or changes in gene expression are also another way in which drug resistance may develop. Examples of these are the epidermal growth factor receptor (EGFR) inhibitors gefitinib and erlotinib. In non-small cell lung cancers containing activating mutations in the EGFR tyrosine kinase domain, high response rates have been observed with these inhibitors yet within a year most patients acquire resistance. A secondary EGFR mutation (T790M) has been observed in 50% of cases in which resistance was developed [30-32].

Drug resistance can also occur through the alteration of signaling pathways. An example of this occurs with trastuzumab (Herceptin). Trastuzumab is a humanized

monoclonal antibody targeting the extracellular domain of HER-2 [33]**.** HER-2 is overexpressed in 20-30% of breast cancers as a result of amplification. This alteration leads to a decrease in overall survival and disease-free survival [34, 35]**.** Whether it is as a single agent or in combination with other chemotherapeutic drugs, trastuzumab has been shown to improve response [36-39]. Despite this, 15% of patients receiving trastuzumab will progress and develop metastatic disease [40]. Activation of the PI3K pathway through either PTEN loss or mutation of PIK3CA has been shown to lead to a shorter progression free survival in trastuzumab treated breast cancer patients compared to patients without PTEN loss or PIK3CA mutation [41] [42]. In addition, it has also been shown that when IGF1R was inhibited through either siRNA or the IGF1R inhibitor (NVP-AEW541) in trastuzumab resistant breast cancer cell lines (SKBR3/Tr and BT474/Tr), an increase in response to trastuzumab was observed [43].

DNA damage repair

DNA damage repair also plays a role in chemoresistance. Multiple chemotherapeutic agents induce DNA damage whether it is directly as is the case of platinum agents such as cisplatin which forms inter and intra strand crosslinks with DNA and will be discussed later in this chapter. DNA damage can also occur indirectly. An example of this is topoisomerase inhibitors, like Irinotecan (discussed in Drug inactivation/activation section) [25].

When DNA damage occurs, the cells will attempt to repair the damage through cell cycle arrest and if damage is too extensive lead to cell death. The type of DNA lesion caused by the chemotherapeutic agents (single strand/double strand breaks, DNA crosslinks, base damage, replication lesions, and bulky adducts) determines which DNA repair pathways will repair the lesions [44]. Resistance develops as a result of a failed

response by the DNA damage response mechanisms [45]. The failure for cancer cells to respond to the DNA repair pathways can be a result of alterations of oncogenes/tumor suppressors or a defect in a DNA repair pathway [25]. For example, platinum induced DNA damage is mainly repaired through the nucleotide excision repair pathway (NER). It has previously been shown that defects in the NER pathway lead to cisplatin hypersensitivity. Overexpression of excision repair cross-complementing 1 protein (ERCC1) and *xeroderma pigmentosum* group A (XPA) has been connected to cisplatin resistance [46].

Another example is that of tumors deficient in homologous recombination. BRCA1 and BRCA2 play important roles in homologous recombination. Germline mutations in these two genes account for 5-10% of breast and 10-18% of ovarian cancers [47]. Cells with mutation in BRCA1 or BRCA2 have been shown to be sensitive to PARP1 inhibitors as well as DNA crosslinking agents like cisplatin and carboplatin. Yet studies have shown that resistance can develop in BRCA2 tumors as a result of secondary intragenic mutations that restore the reading frame of wild type BRCA2 [48, 49].

Downstream resistance mechanisms

Chemotherapeutic response should ideally result in the induction of cell death yet there are intrinsic adaptive responses that are triggered, which promote cancer cell survival [25]. Two important mechanisms involved in cell death that become deregulated in cancer are apoptosis and autophagy.

The extrinsic (death receptor) and the intrinsic (mitochondrial) pathways are the two major apoptotic pathways. Each of these pathways require specific signals that in turn activate their own initiator caspase, which results in the activation of the executioner

caspase 3 and leads to cell death [50]. The balance between pro-apoptotic and anti-apoptotic proteins plays an important role in the regulation of cell death. Studies have shown that an imbalance of these proteins results in the dysregulation of apoptosis [51]. An example of this is Bcl-2. Raffo *et al*. showed that overexpression of Bcl-2 in the prostate cancer cell line LNCaP increased the cell's resistance to apoptotic stimuli *in vitro* and promoted tumor formation by the cell line *in vivo* [52]. Likewise, overexpression of Bcl-x_L cells prevented the cells treated with various chemotherapeutic agents from undergoing apoptosis [53].

Autophagy is another mechanism involved in cell death. It is a lysosomal degradation pathway. In order to maintain cellular biosynthesis and cell viability as a result of metabolic stresses, like nutrient deprivation, this pathway degrades proteins and cellular organelles. In cancer, autophagy has been shown to inhibit tumor initiation. Conversely, it has also been shown to contribute to drug resistance by promoting cell survival caused by therapeutic agents [25]. An example of this is the therapeutic agent, Chloroquine, which is an inhibitor of autophagy. Amaravadi *et al*. showed in a lymphoma mouse model that inhibition of autophagy with either chloroquine or knockdown of ATG5, an essential autophagy gene, enhanced tumor cell death caused by p53 activation or alkylating drug [54].

Resistance-promoting adaptive responses

Another form for drug resistance results from the ability of cells to adapt through activation of prosurvival signaling, oncogenic bypass and pathway redundancy, as well as epithelial-mesenchymal transition (EMT). An example of prosurvival signaling is that of EGFR targeted therapies. Addition of EGFR-targeted therapies can sensitize various types of cancer to agents such as irinotecan, paclitaxel, and 5-FU [25]. An example of

oncogenic bypass is that of *KRAS*-mutant colorectal cancer. *KRAS*-wild type colorectal cancers benefit from the addition of EGFR targeted therapies to irinotecan while *KRAS*-mutant colorectal cancers do not benefit from EGFR inhibitors as KRAS is not dependent of EGFR activation [25]. An example of pathway redundancy is that of BRAF. Vemurafenib is an inhibitor to BRAF, in particular to its mutated form BRAF-V600E. Despite its clinical response rates in melanoma, secondary resistance develops in about 50% of patients. Some resistance mechanisms that have been identified are the activation of alternative RAF isoforms as well as acquired mutations in *KRAS*, *NRAS* and *MEK1* [25]. Lastly, EMT can also be involved in drug resistance. There is evidence of EMT in tumor samples of non-small cell lung cancer patients resistant to EGFR inhibitors [55].

Tumor Microenvironment

The tumor microenvironment consists of the ECM (extracellular matrix), cancer associated fibroblasts, endothelial cells, pericytes, adipocytes, mesenchymal stem cells, as well as immune and inflammatory cells [25, 56]. Crosstalk between stromal cells and tumor cells provide an environment in which the tumors cells can prosper as well as provide protection to the tumor cells from cytotoxic agents [25]. An example of how the tumor microenvironment promotes drug resistance is a study of Burkitt's lymphoma. In a mouse model of Burkitt's lymphoma, Gilbert *et al.* showed that paracrine factors in the microenvironment influence lymphoma cell survival following exposure to doxorubicin. The mice treated with doxorubicin, secreted interleukin-6 and Timp-1 from the thymus creating a "chemo-resistant niche". This "chemoresistant niche" led to the survival of residual lymphoma cells and eventual relapse [57]. Further evidence demonstrating how the stroma affects tumor cells can be found in a study that looked at the influence of 23

different stromal cell types when co-cultured with 45 cancer cells in response to 35 anticancer agents. This study revealed that the anticancer agents used were ineffective at causing cell death when the cancer cells were cultured in the presence of stromal cells as opposed to cancer cells only. This led to the conclusion that the stroma mediated drug resistance [58].

Cisplatin

While *cis*-diamminedichloroplatinum (II) commonly known as cisplatin, was first synthesized by Michele Peyrone in 1844 and its chemical structure was first identified by Alfred Wagner in 1893. It was not until the 1960s when interest developed as a result of work conducted by Rosenberg *et al*. when it was shown that platinum mesh electrodes could inhibit cell division in *Escherichia coli* [59, 60]. Cisplatin was first approved in 1978 by the FDA (Food and Drug Administration) for the treatment of testicular and bladder cancer [61]. Since then it has been shown to be one of the most effective and widely used agents in different types of cancer such as brain, breast, head and neck, kidney, leukemia, lung, ovarian, and testicular cancers [62]. Although it is widely used as a result of its therapeutic outcome associated with disease stabilization or partial responses some patients are intrinsically resistant to cisplatin-based therapies, while others develop chemoresistance despite having tumors that were originally sensitive to cisplatin. The development of cisplatin chemoresistance is frequently seen in ovarian cancer patients [63]. Another limiting factor of cisplatin are its side effects that include neurotoxicity, ototoxicity, myelosuppression, renal toxicity, nausea, and vomiting [62].

Cisplatin structure

Cisplatin is a water soluble square planar coordination complex that contains two chloride atoms and two ammonia moieties all of which surround a central platinum atom

(Figure 3) [62]. Due to its neutral status, cisplatin has to be activated in order for its interaction with DNA to occur. Cisplatin is activated through a series of spontaneous aquation reactions in which the cis-chloro ligands are replaced with water molecules creating reactive species. Its primary mode of cytotoxicity is attributed to its interaction with nucleophilic N7-sites of purine bases in DNA, which result in DNA-DNA and DNA-protein interstrand and intrastrand crosslinks [64]. ApG and GpG intrastrand adducts account for 85-90% of bound cisplatin and are responsible for its cytotoxic effects [65]. These different types of cisplatin-induced DNA damage are recognized by several DNA repair pathways.

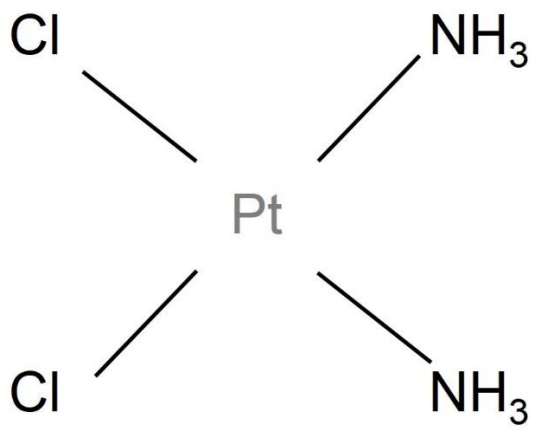

Figure 3: Cisplatin structure.

Cisplatin mechanism of action

Understanding the effect that a particular therapeutic agent has at the molecular level is important. In this section the effect of cisplatin on the cancer cells will be discussed.

Oxidative stress

Mitochondrial oxidative metabolism as well as cellular responses to cytokines, bacterial infection, and xenobiotics generate reactive oxygen species (ROS). Oxidative stress occurs when there is an imbalance of ROS [66]. During oxidative stress, the excess in ROS results in damage to DNA, cellular proteins, and lipids, which can contribute to tumorigenesis [59]. Cisplatin induces oxidative stress. As mentioned previously, cisplatin is activated through aquation reactions that replace one or both chlorines with water molecules. These molecules can then interact with a number of endogenous nucleophiles found in the cytoplasm such as reduced glutathione (GSH), metallothioneins, methionine, and other proteins through their cysteins, generating reactive oxygen species [59].

DNA adducts and damage recognition

The primary target of cisplatin is believed to be DNA [67]. Cisplatin binds to DNA through the platinum atom. The platinum atom forms covalent bonds with the N7 position of purine bases in the DNA. These covalent bonds form 1,2- or 1,3-intrastrand crosslinks [67]. These distortions are recognized by multiple DNA repair pathways. Intrastrand crosslinks are the major lesions of DNA damage caused by cisplatin. These lesions are repaired mainly by the nucleotide excision repair (NER) pathway yet the mismatch repair (MMR) pathway can also recognize DNA damage induced by cisplatin. If the extent of DNA damage is limited, the cells go into cell cycle arrest to allow for DNA damage to be repaired but if the damage is beyond repair the cells will go through apoptosis [61].

Cell cycle arrest

Cisplatin induced DNA damage can lead to cell cycle arrest for repair. A study by Donaldson *et al*. showed that synchronized cells were at their most sensitive at G1 prior

to the start of DNA synthesis and least sensitive during entry into S phase following cisplatin treatment [68]. Yet, at physiologically relevant cisplatin levels DNA synthesis is not inhibited. Instead cisplatin treated cells go through S phase and arrest at G2 leading to cell death [69, 70].

Apoptosis

Genotoxic stress as a result of cisplatin activates multiple signaling pathways that lead to apoptosis or chemoresistance [59]. p53 stability and transcriptional activity is regulated by the ATM (ataxia telangiectasia mutated protein) and ATR (ATM- and Rad3-related protein) kinases. These kinases activate p53 through phosphorylation. Of the two, the ATR kinase is preferentially activated by cisplatin [65]. The mitogen activated protein kinase (MAPK) signaling pathways ERK, JNK, and p38 have all been shown to be activated by cisplatin. ERK has been shown to activate p53 [71]. Further, the p38α substrate, $p18_{Hamlet}$, not only increases upon genotoxic stress but it also induces transcriptional activation of NOXA and PUMA, which are known p53 target genes [72]. In addition, p73 can also mediate apoptotic response. Of the MAPK signaling pathways, JNK has been shown to be required in p73-mediated apoptosis following cisplatin exposure [73]. Cisplatin activates c-Abl through recognition by the mismatch repair pathway (MMR) [59, 74]. In the nucleus c-Abl forms a complex with and phosphorylates MEK kinase 1. This complex activates JNKs [59].

Gene expression

As cisplatin enters the cells, self-defense mechanisms are activated resulting in genetic and epigenetic changes that alter gene expression [75]. Cisplatin resistance that will be discussed in the next section is a major issue. In order to understand these gene expression alterations, numerous studies have been conducted in various cancer types

comparing untreated vs cisplatin treated cells at different time points and/or concentrations. Other studies have compared cisplatin sensitive cell lines with their cisplatin resistant derivatives. Regardless of how the experiments are conducted, the goal is the same, to identify gene expression changes resulting from cisplatin to circumvent resistance.

Cisplatin Resistance

One of the main limitations of cisplatin is the high rate of chemoresistance. Despite efforts at developing other second-generation platinum compounds cisplatin remains the only therapeutic option in specific clinical settings [61]. Therefore, over the past 30 plus years, efforts have been made to develop ways to circumvent platinum resistance. There are several known cisplatin resistance mechanism, which Galluzi *et al*. have categorized as follows: pre-target, on-target, post-target, and off-target [61].

Pre-target resistance

Reduction of intracellular accumulation of cisplatin is one of the pre-target mechanisms that cancer cells use, which can occur by either inhibition in drug uptake or through drug efflux. Several groups have shown that limited intracellular accumulation of cisplatin is sometimes a result of reduced uptake [76, 77]. As mentioned previously, drug efflux plays a role in drug resistance. Although there are a number of transporters and ion pumps that can control cisplatin uptake, copper transporter 1 (CTR1) has been shown to be the major influx transporter for platinum agents such as cisplatin, carboplatin and oxaliplatin by several laboratories. Both *in vitro* and *in vivo*, knockout of CTR1 leads to resistance to platinum agents [67, 78]. In addition, ATP7A and ATP7B, which like CTR1 are also cooper transporters, have been shown to be overexpressed in resistant cancer cells compared to their parental cell lines [79, 80]. Komatsu *et al*.

further showed that when ATP7B was overexpressed in the human epidermiod KB cell line, these cells acquired resistance to not just cisplatin but also cooper by a nine and two fold increase. ATP7B overexpression also led to an increase in cisplatin efflux compared to the control cells [80]. ATP7B has even been shown to not just be a predictive marker of recurrence in ovarian cancer patients treated with cisplatin-based chemotherapy but also as a chemoresistance marker in moderately-/poorly-differentiated ovarian cancer patients treated with cisplatin-based chemotherapy [81]. The fact that several cooper transporters have been shown to regulate cisplatin uptake implies that there may be similarities in the way that cooper and platinum agents are transported [78].

Another pre-target mechanism of resistance is increased deactivation of cisplatin. Once inside the cells, the amount of active cisplatin is limited by its binding to nucleophilic species such as glutathione (GSH), metallothioneins, methionine, and other cysteine-rich proteins which inactivate it [61].

On-target resistance

On-target resistance mechanisms occur at the DNA level. Cisplatin forms inter- and intra-strand DNA adducts which results in apoptosis. Cancer cells have developed the ability to increase the repair of DNA adducts and to also tolerate these unrepaired lesions. The nucleotide excision repair (NER) pathway is the major pathway for platinum adduct removal thus repairing the DNA damage [65]. Studies in testicular cell lines and tumors as well as in non-small cell lung cancer and lung adenocarcinoma cell lines have shown that overexpression of XPA or ERCC1 correlate with cisplatin resistance [67]. Cisplatin-induced DNA damage can also be detected, although not repaired by the MMR pathway. This data suggests that mutation or downregulation of MSH2 and MLH1 are

involved in the MMR pathway, which can lead to acquired cisplatin resistance [61]. In addition, the inter-strand adducts induced by cisplatin can also cause double strand breaks which would be repaired by homologous recombination (HR). BRCA1 and BRCA2, genes involved during this process are frequently mutated in familial breast and ovarian cancers leading to HR-deficient cancers. Cancers deficient in homologous recombination tend to be sensitive to crosslinking agents such as cisplatin [61]. Secondary mutations in BRCA2 reestablish its function, which lead to the development of resistance [48, 49, 61].

While there is great interest in identifying alternative platinum agents to circumvent cisplatin's limitation, resistance may be difficult to overcome. Jennerwein *et al.* showed that L1210/PDD cells had increased DNA repair of R,R-DACH-Pt damaged plasmid even though they were not resistant to this DACH analogue. They concluded that the enhanced DNA repair in the, L1210/PDD cell line, which is resistant to cisplatin, was independent of the type of damage introduced [82].

Post-target resistance

As mentioned previously, there are downstream resistance mechanisms that the cells use to promote cell survival. For cisplatin defects, these downstream mechanisms can be in the signaling pathways that would normally lead to an apoptotic response to DNA damage and/or defects in the cell death pathways. An example of this is the inactivation of p53. p53 occurs in 50% of all cancers [61]. A study by O'Connor *et al.,* in which the 60 National Cancer Institute (NCI) were characterized for p53 and treated with 123 anticancer agents revealed that the cell lines with p53 mutations tended to be less sensitive to alkylating agents, DNA/RNA antimetabolites, and topoisomerase inhibitors than the p53 wild-type cell lines [83]. In addition, testicular cancer germ cell tumors are

sensitive to cisplatin. Further, a study in 22 testicular tumors found no p53 mutations [84]. While the examples given are in line with p53's role in cell death, there is evidence that p53 wild-type tumors can also be resistant. An example of this can be found in ovarian cancer. While the most common type of ovarian cancer is the high-grade serous subtype, of which 87% possess p53 mutations, the remaining subtypes (clear cell carcinoma, endometrioid, and mucinous) tend to be p53 wild-type [6, 17, 19]. These p53 wild-type cancers tend to be resistant to the standard treatment (platinum/taxane) [6]. Further, our lab has shown that from the TCGA ovarian high grade serous carcinoma study, the p53 wild-type tumors had a poorer survival and were more chemoresistant than the tumors with p53 mutations [85]. Induction of cell death requires stabilization and activation of p53. Martinez-Rivera and Siddik suggest that p53 resistance may develop directly through factors that either reduce or abrogate p53 activity. It is also suggested that p53 resistance may be indirectly regulated by deregulation of downstream pathways [86].

In addition to p53 inactivation, deregulation of factors involved in intrinsic and extrinsic apoptotic pathway have been shown to modulate response to cisplatin. MAPK family members have also been shown to contribute to cisplatin resistance [61]. Brozovic *et al*. showed that cisplatin resistant cells have attenuated MAPK signaling compared to cisplatin sensitive cells. They concluded that in cisplatin induced cell death sustained activation of SAPK/JNK and p38 maybe a general mechanism [87].

Off-target resistance

There is also evidence suggesting that cisplatin resistance may also develop from alterations in signaling pathways not directly affected by cisplatin [61]. An example of this is dual-specificity tyrosine-phosphorylation regulated kinase 1B (MIRK). This kinase

is upregulated in several solid tumors and has a role in prosurvival. MIRK has been shown to increase the expression of antioxidant enzymes such as superoxide dismutase 2 and superoxide dismutase 3 by decreasing reactive oxygen species [88]. In lung cancer cells, knockdown of MIRK led to an increase in apoptosis as well as sensitization of cells to cisplatin [19].

GDF15

Growth Differentiation Factor 15 (GDF15) also known as Macrophage Inhibitory Cytokine-1 (MIC-1), nonsteroidal anti-inflammatory drug activated gene (NAG1), Placental Transformation Growth Factor-β (PTGF-β), Placental Bone Morphogenic protein (PLAB), and Prostate Derived Factor (PDF), was identified by several groups using different cloning strategies (Reviewed in [89-92]). In humans, GDF15 is located in chromosome 19p12.1-13.1 and has only two exons. Human GDF15 is expressed at low levels in the colon, kidney, and prostate while abundantly expressed in the placenta [93-96]. While the GDF15 mouse gene is closely related to the human gene, GDF15 distribution in the mouse differs from that of human. A sequence comparison between the human and mouse GDF15 promoters revealed a 39% homology within a 700 bp region [91]. GDF15 is synthesized as a 308 amino acid (aa) protein precursor consisting of a 29 aa signal peptide, 167 aa pro-domain with an N-linked glycosylation site at position 70. Following dimerization of the full length GDF15 protein precursor, the dimeric pro-protein is cleaved at the amino acid target sequence RXXR resulting in a 112 amino acid C-terminal dimeric mature protein, which is secreted. This secreted dimer has been detected in sera as well as in the media of cultured cells. While the role that the various states in which GDF15 exists in the cells remains to be studied, a recent study by Min *et al*. revealed that full length GDF15 can accumulate in the nucleus and

that its retention in the nucleus resulted in the absence of the secretion of its mature protein. This study further showed that GDF15 inhibits the expression of genes targeted by TGF-β at the transcriptional level [97].

GDF15 and TGF-β

GDF15 is a member of the transforming growth factor- β (TGF-β) superfamily. The TGF-β family consists of TGF-β, activins, anti-Müllerian hormone (AMH), bone morphogenic proteins (BMPs), GDFs, inhibins, myostatins, and NODAL [98]. Signaling for TGF-β ligands essentially follow the same path. Briefly, the ligands require two types of serine/threonine receptors (a type I and a type II). Some ligands may require an additional co-receptor for optimal binding to the type I-type II receptor complex. The activated receptor complex consists of a constitutively active type II receptor. This receptor phosphorylates several serines and threonines in a highly conserved glycine and serine rich domain near the membrane spanning region of the type I receptor providing a binding site for downstream substrates of which the most understood so far are the SMADs [98].

Although the receptor(s) for GDF15 is not known, it has been suggested that GDF15 may signal through TGF-β receptors. Tan *et al*. showed that conditioned medium from GDF15 overexpressing cells suppressed cell proliferation of cancer cell lines (MvlLu and Du145) with an intact TGF-β signaling pathway but did not suppress cell proliferation of cancer cell lines with a defective TGF-β signaling pathway such as TβRI mutant cell line R1B/L17 [99], TβRII mutant cell line RKO [100], and Smad4-null cell line MDA-MB468. This data suggests that the growth inhibition by GDF15 requires a functional TGF-β receptor/Smad4 signaling pathway [101].

GDF15 in disease

Elevated levels of GDF15 are associated with all-cause mortality, and GDF15 has been proposed as a marker for several diseases. A Swedish study showed GDF15 as a predictor of all-cause mortality in the general population independent of age, BMI, smoking history, telomere length, IL-6 and CRP serum levels [102]. A separate study in older community-dwelling adults demonstrated that increased levels of GDF15 are independently associated with an increased risk of all-cause mortality [103].

Several studies have looked at GDF15 in cardiovascular diseases as well and suggested it be used as a marker. Among the first to look at GDF15 in cardiovascular disease was a study by Brown *et al*. In their study, Brown *et al*. showed that the baseline plasma concentration of GDF15 that experienced a cardiovascular event increased with the risk or experiencing said event [104]. A study of chronic heart failure patients showed that increasing levels of GDF15 was associated with an increased the risk of death and this was independent of other variables such as age, body mass index, heart failure etiology, concomitant medical therapy, and the levels of hemoglobin and uric acid [105]. Additional studies looking at acute pulmonary thrombosis and idiopathic pulmonary arterial hypertension yielded the similar results. GDF15 could predict risk [106].

In addition, GDF15 has also been implicated in other areas such as being upregulated in airway epithelium following cigarette smoke exposure as well as metabolic disorders (obesity, insulin resistance, weight loss, and anorexia), and inflammation, stress response, bone formation, hematopoietic development, and adipose tissue function [90, 107].

GDF15 and cancer

Expression of GDF15 in cancer

GDF15 is thought to play a role in several types of cancer. GDF15 levels are higher in tumor compared to normal tissue. In particular GDF15 expression has been shown to increase during the progression of breast, cervical epithelial, colorectal, gastrointestinal, glioma, melanoma, oral squamous cell carcinomas, ovarian, pancreatic, and prostate cancers [90]. Its high expression is associated with poor survival. GDF15's detection in the blood makes it of great interest in the clinic, as a potential biomarker. For example, Koopmann *et al*. showed that in pancreatic cancer patients, GDF15 serum levels could differentiate between patients with pancreatic and other periampullary adenocarcinomas from those with non-cancerous lesions as well as the currently used marker CA19-9. In addition, when both GDF15 and CA19-9 were combined, sensitivity and specificity was increased compared to using either target alone [108]. Furthermore, GDF15 was associated with worse outcome in a study of the cerebrospinal fluid of 33 glioblastoma patients [109].

The role that GDF15 plays has been shown to be very context and cell type specific. For instance, it was been shown to be anti-tumorigenic in some cases and pro-tumorigenic in others [106].

Mutations in GDF15

The previous section discussed GDF15 expression but what about mutations? An analysis of various cancer types found in the cBioPortal revealed that with the exception of the primary central nervous system lymphoma and uterine carcinosarcoma, GDF15 mutations are not very frequent [17, 18]. Although GDF15 mutations are rare, a recent study in oral squamous cell carcinoma revealed that out of 46 patients 41.3% had

GDF15 mutations. The patients with the mutations had a poorer outcome compared to those with wild type GDF15 [110]. Another study showed that the H6D GDF15 mutation in colorectal cancer patients increased susceptibility to distant metastasis and poor prognosis [111]. Based on the cBioPortal, no GDF15 mutations have been found in ovarian cancer [17, 18].

Anti-tumorigenic role of GDF15

Several studies have shown that GDF15 promotes growth arrest and apoptosis. Overexpression of GDF15 in the HCT-116 colon cancer cell line decreased the clonogenic activity of these cells *in vitro*. In the same study, a xenograft mouse model in which GDF15 was knocked down in the HCT-116 cell line revealed an increase in tumor growth compared to the control [112]. Further supporting an anti-tumorigenic effect for GDF15 is a study by Baek *et al*., in which transgenic mice overexpressing human GDF15 and treated with azoxymethane (AZO), a colonic carcinogen known to induce early neoplastic lesions, developed statistically significant smaller number of tumors compared to the control, suggesting GDF15 suppresses formation of early neoplastic lesions in the colon [113].

Pro-tumorigenic role of GDF15

While the above examples show an anti-tumorigenic role for GDF15, studies have also been published supporting a pro-tumorigenic role. GDF15 knockdown in a xenograft mouse model of melanoma cell lines (D04, A2058, and C32) had a reduction in tumor growth compared to the control [114]. GDF15 has also been implicated in migration and invasion. In the gastric cancer cell line SNU-216, overexpression or treatment with recombinant GDF15 induced invasion through the upregulation of urokinase-type plasminogen (uPA) partly through the activation of ERK1/2 [115]. GDF15

promoted migration and invasion in prostate cancer cells by reorganizing actin through the focal adhesion kinase-Ras homologous A (FAK-RhoA) signaling pathway. In this paper, Senapati *et al*. further showed an increase in metastatic tumors in an orthotopic mouse model of the PC3 prostate cancer cell line overexpressing GDF15 [116].

GDF15 has also been shown to modulate the immune system. A syngeneic mouse model of glioma in which GDF15 was knocked down revealed that GDF15 knockdown tumors were smaller. GDF15 knockdown in this model also prolonged survival and resulted in an increase in T cell and macrophage infiltration compared to control tumors [117].

GDF15 and chemoresistance

The rapid induction of GDF15 by chemotherapeutic agents as well as non-steroidal anti-inflammatory drugs suggest GDF15 may play an important role in drug response and should be further studied. A study in which lung cancer cell lines (NCI-H226 and NCI-H2170 were treated with cisplatin and whose gene expression was analyzed through a cDNA microarray revealed a correlation between cisplatin resistance and increased GDF15 levels [118]. In another study from a phase II clinical trial in which high-risk localized prostate cancer patients were treated with neoadjuvant therapy consisting of docetaxel and mitoxantrone followed by prostatectomy identified significantly, increased levels of GDF15 in chemoresistant prostate cancer cells were observed, suggesting that GDF15 may contribute to docetaxel and mitoxantrone resistance [119]. Furthermore, increased GDF15 serum levels in patients with hormone-refractory prostate cancer following chemotherapy were associated with increase tumor progression and a shorter survival after treatment [120].

GDF15 and ovarian cancer

In ovarian cancer, GDF15 has been shown to be upregulated in the plasma of ovarian cancer patients compared to healthy controls and benign ovarian tumors. Furthermore, ovarian cancer patients with high preoperative GDF15 had a lower survival time (15 months) when compared to patients that had lower preoperative GDF15 (28 months). Staff *et al.*, also showed that in serous ovarian tumors GDF15 was found within the tumor but not in the endothelial or stromal compartments [121]. In another study, Staff's group showed that GDF15 was higher in post-chemotherapy effusions compared to pre-chemotherapy effusions [122]. In the same study it was shown that high expression of GDF15 in tumor cells correlated with poor survival in the pre-chemotherapy samples. There was no correlation in the post-chemotherapy samples [122].

Hypothesis and Specific Aims

GDF15 is rapidly induced under different kinds of stress. My preliminary data (see Chapter 3) also showed that GDF15 is induced following cisplatin treatment. Thus, I hypothesize that the induction of GDF15 is a defense mechanism that cells use in response to chemotherapeutic agents. My study has two specific aims as follows:

1. Examine GDF15 expression and its role following induction of cisplatin in ovarian cancer.
2. Investigate how GDF15 affects the tumor microenvironment.

Chapter 2: Materials and Methods

Cell lines

The following ovarian cancer cell lines were used in this study: A2780, ALST, ES2, HEYA8, KOC7C, OVCAR3, OVCA5, OVCA420, OVCA432, RMG1, SKOV3, SMOV2, and TOV21G. For *in vivo* experiments RMG1-luc and A2780-luc cell lines containing a luciferase vector were used. All cell lines were cultured in RPMI media (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 37°C with 5% CO_2. The A2780 and ES2 cell lines were a gift from Dr. Rosemarie Schmandt; the A2780-luc, TOV21G, RMG1, and OVCA5 cell lines were a gift from Dr. Samuel Mok; and the KOC7C and SMOV2 cell lines were a gift from Dr. Naoto Ueno; all of these are faculty at The University of Texas MD Anderson Cancer Center.

Chemotherapeutic Agents

Cisplatin (Pfizer), carboplatin (Hospira), and paclitaxel (Hospira) were all obtained from the M.D. Anderson Cancer Center pharmacy in solution.

Gene expression profiling

The A2780 cell line was treated with either 5 or 10 µM cisplatin (Teva Parenteral Medicines, Irvine, CA) and total RNA was extracted at 0, 0.5, 1, 2, and 16 hours using the mirVana miRNA isolation kit (Ambion, Austin, TX). The DNA-free kit (Ambion) was used to remove the genomic DNA. To prepare the samples for hybridization to the GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA), we used the MessageAmp Premier RNA amplification kit following the manufacturer's protocol.

Briefly, 100 ng of total RNA was reverse transcribed to synthesize first strand cDNA. After second strand cDNA synthesis, an in vitro transcription reaction resulted in biotin labeled aRNA. Then 12 µg of the sample was fragmented and hybridized to a U133 Plus 2.0 array in a GeneChip hybridization oven 640 model at 45°C for 16 hours. The arrays were washed and stained using a Fluidics Station 450 and scanned with a confocal laser GeneChip Scanner 3000. Analysis was conducted using the guided workflow option in the GeneSpring GX software. Additionally, we downloaded a dataset from the Gene Expression Omnibus database (Accession # GSE8057) from Pendyala *et al*. in which the A2780 cell line was treated with cisplatin at different concentrations and timepoints. This dataset was analyzed using OmniViz software (Instem Life Science Systems, Stafforshire, United Kingdom).

Real-time polymerase chain reaction

To determine expression of genes of interest, total RNA was extracted using the PureLink RNA mini kit (Invitrogen), and the manufacturer's protocol, was followed including DNAse treatment. In the case of the RNA extracted from mouse tissue, the frozen tissue was cut and transitioned for at least 16 hours in RNAlater-Ice (ThermoFisher Scientific) at -20°C. Following tissue transition, the manufacturer's protocol for Total RNA isolation procedure from the mirVana miRNA isolation kit was followed. The DNA-free kit was then used to remove the genomic DNA. Once RNA was extracted using either of the above mentioned kits, the high-capacity cDNA reverse transcription (Applied Biosystems, Carlsbad, CA) was used. One µg of RNA was reverse transcribed according to the manufacturer's protocol. The real-time polymerase chain reaction (PCR) was performed as follows: 10 µl of master mix (7.5 µL of 2X iQ Supermix (Bio-Rad, Hercules, CA), 1.75 µL molecular grade water, 0.75 µl of 20x TaqMan gene

expression assay mix (Applied Biosystems) was added to 5 µl of diluted (1:25) cDNA. Real-time PCR was performed in a C1000 thermal cycler (Bio-Rad). The following TaqMan assays were used for: CDKN1A (Hs00355782_m1), FDXR (Hs00244586_m1), GDF15 (Hs00171132_m1), MMP1 (Hs00899658_m1), MMP10 (Hs00233987_m1), TP53I3 (Hs00936520_m1), Cela2a (Mm00468213_m1),Clps (Mm00517960_m1), Pnlip (Mm00813468_m1), Prss2 (Mm00657001_m1), and Prss3 (Mm02393636_gH). Either cyclophilin A (PPIA, 4333763F) or beta glucuronidase (GUSB, 4333767F) were used as controls for human and hypoxanthine phosphoribosyltransferase 1 (Hprt, Mm00446968_m1) for mouse genes. All TaqMan assays used were bought from Applied Biosystems.

Western blot analysis

Total protein was extracted with RIPA buffer containing protease inhibitors. Thirty µg of protein was subjected to electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After being blocked for 1 hour in 5% milk in phosphate-buffered saline with 0.05% Tween 20 (PBST), the membrane was incubated overnight at 4°C with the primary antibody, rinsed 3 times with PBST for 10 minutes, and incubated at room temperature for 1 hour with the secondary antibody. After being rinsed 3 times for 10 minutes, the membrane was scanned using the Odyssey imaging machine (LI-COR Biosciences, Lincoln, NE). The primary antibodies used were anti-NAG-1/PTGF β, rabbit polyclonal (anti-GDF15) (1:1000) Millipore, Billerica, MA), anti-PARP-1/2 (H-250), rabbit polyclonal (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and β-actin (1:3000, Sigma-Aldrich). The secondary antibodies used were goat anti-mouse and goat anti-rabbit from LI-COR Biosciences at a 1:10,000 dilution.

Stable GDF15 knockdown in cell lines

GDF15 mission shRNA lentiviral transduction particles (catalog # TRNCN0000058388, TRNCN0000058389, Sigma-Aldrich, St. Louis, MO) were transfected into the A2780 6-well plate 24 hours after plating. Puromycin (InvivoGen, San Diego, CA) was used for stable selection at a concentration of 1 µg/ml. The stable cell lines were used in the mice studies.

***in vivo* studies**

GDF15 secretion in the blood mouse experiment

A total of 50 (5 to 6 week old female) nude mice were injected intraperitoneally with either 2 x 10^6 A2780 cells or 5 X 10^6 RMG1 cells containing a luciferase reporter construct, once Institutional Animal Care and Use Committee (IACUC) approval was granted. The mice were then monitored weekly for tumor growth via the injection of D-luciferin (Caliper Life Sciences, Hopkinton, MA) and the use of the Xenogen imaging system (Caliper Life Sciences). Once the mice developed tumors, 25 mice were administered either PBS (5 mice), 2.5 mg/kg cisplatin (10 mice), or 5 mg/kg cisplatin (10 mice) via intraperitoneal injection. Either 24 or 48 hours after treatment 5 mice were randomly selected to be euthanized at each timepoint for each concentration according to IACUC guidelines. Tumor samples and blood were collected from the mice. A week after the first 25 mice were treated, the experiment was repeated with the remaining mice containing tumors of that particular cell line.

GDF15 knockdown mouse experiment

Five to six week old nude mice were injected intraperitoneally with 2 x 10^6 A2780 following Institutional Animal Care and Use Committee (IACUC) approval. Eighteen and

21 days after injection mice were treated to 5 mg/kg cisplatin though intraperitoneal injection. Forty-eight hours after last cisplatin treatment mice were euthanized according to IACUC guidelines. Tumor samples and blood were collected for each mouse.

Enzyme-linked immunosorbent assay (ELISA)

When the mice were euthanized, blood was collected in a serum tube. The blood was allowed to clot for at least 30 minutes at room temperature and was then centrifuged at full speed for 10-15 minutes at 4°C. After centrifugation, the serum was transferred to a centrifuge tube and stored at -80°C until all samples were collected. The human GDF15 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used to measure GDF15, according to the manufacturers' protocol. Absorbance was measured in the BMG Labtech plate reader (Chicago, IL). The 540 nm absorbance was used along with the 450 nm absorbance for correction. GraphPad Prism 5 (GraphPad Software) was used to analyze the data.

Immunohistochemistry (IHC)

Immunohistochemistry was conducted on paraffin embedded tissue from the GDF15 knockdown mouse experiment. The slides were deparaffinized and dehydrated. Antigen retrieval was conducted in a decloaking chamber (Biocare Medical, Concord,CA) at 121°C for 3 minutes and 95°C for 1 minute in citrate buffer (Poly Scientific, Bay Shore, NY). Slides were stained with Ki67 mouse monoclonal antibody (catalog # 550609, BD Pharmingen, San Jose, CA) at a dilution of 1:50 and counterstained with hematoxylin in the Lab Vision Autostainer 360 (Thermo Scientific, Waltham, CA). The mouse-on-mouse HRP-polymer (Biocare Medical, Concord, CA) was also used during the procedure.

Slides stained for Ki67 were then analyzed with the assistance of the Flow Cytometry and Cellular Imaging Facility at M.D. Anderson Cancer Center. Images of the stained slides were taken using the Vectra 2 microscope (PerkinElmer, Waltham, MA) along with the inForm 2.2.5800.23313 software program (PerkinElmer, Waltham, MA). Thirty randomly chosen fields where taken at 4X magnification that had more than 5% tissue as well as 30 randomly chosen fields at 20X with at least 30% tumors tissue. Once the images were taken, the software was set up to detect tissue segmentation (tumor, other tissue, non-tissue/white space, red blood cell, random (necrotic tissue)) and cell segmentation, and staining intensity. Images that did not contain any tissue or tissue that did not include any tumor cells were rejected and not included in the analysis. For Ki67, we only looked at the staining in the tumor tissue. The h-score of this analysis was used to determine differences between the control and the knockdown groups. When looking at tumor composition, the tissue segmentation data was analyzed.

RPPA

Protein was extracted from 20-40 µg of mouse tumor samples in RIPA buffer obtained from the Functional Proteomics Reverse Phase Protein Array (RPPA) Core Facility at M.D. Anderson Cancer Center. Frozen tissue was homogenized in 200 ul of ice cold RIPA buffer. Samples were then spun down for 5 minutes at 2600 rpm and frozen for at least an hour before continuing with protein isolation. Samples were thawed out and spun down at full speed for 20 minutes at 4°C. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Samples were then mixed in 4x SDS buffer without bromophenol blue and boiled for 5 minutes. Samples were then submitted to the RPPA core facility for further processing at a concentration of 1 µg/µl. Briefly, serial diluted protein lysates were arrayed on

nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon BioSystems). Each slide was probed with a previously validated primary antibody and a biotin-conjugated secondary antibody. Amplification of the original obtained signal was done using the Dako Cytomation-catalyzed system (Dako) and visualization conducted using a DAB calorimetric reaction. To generate spot intensity, the slides were scanned, analyzed, and quantified using a customerized-software Microvigene (VigeneTech Inc.). The "Supercurve Fitting" logistic model developed by the Department of Bioinformatics and Computational Biology in M.D. Anderson Cancer Center was used to fit each dilution curve (http://bioinformatics.mdanderson.org/OOMPA). Data points were normalized for protein loading and transformed to linear values "normalized linear".

mRNA-seq

Total RNA was extracted from frozen mouse tumors. Briefly, 20-40 mg of frozen tissue was cut and soaked in RNA*later*-ICE (Ambion) for at least 16 hours. Tissue was then homogenized for 30 seconds in lysis buffer from the miRVana miRNA isolation kit (Ambion). The total RNA isolation procedure from the kit was followed. Genomic DNA was removed using the DNA-*free* DNA Removal Kit (Ambion). RNA samples were then submitted to the M.D. Anderson Cancer Center Sequencing and Microarray Facility for next generation sequencing using Illumina's HiSeq 2000. FASTQ files were analyzed using the CLC Genomics Workbench version 6.0 software program (Qiagen, Redwood City, CA).

TCGA analysis

From the cBioPortal (www.cbioportal.org), RNA expression (RNA-seq V2RSEM) for genes of interest, copy number alterations/amplification and mutation data was downloaded for the ovarian serous cystadenocarcinoma (TCGA, provisional) tumor

samples with sequencing and CNA data (311 samples) and the uterine corpus endometrioid carcinoma (TCGA, Nature) for all complete tumor samples [123]. Spearman correlation analysis was conducted in GraphPad Prism comparing GDF15 to the genes of interests for both the ovarian and the endometrioid data. For the ovarian data set all samples regardless of p53 status were included whereas in the endometrioid data set only samples with p53 wild type were included in the analysis.

Statistical analysis

The Kruskal-Wallis test was used for analysis of the GDF15 secretion in the blood mouse experiment for both the mice sera and the Real-time PCR analysis of that experiment. For all the analysis conducted as a result of the GDF15 knockdown experiment the Mann-Whitney U test was used for analysis. Unless otherwise stated the error bars represent the standard error. For all correlation analysis, the Spearman rank correlation test was used. GraphPad Prism version 6 (GraphPad Software, Inc. La Jolla, CA) was the software program used to conduct the analyses.

Chapter 3: GDF15 promotes ovarian cancer tumorigenesis

Rationale

More than 70% of ovarian cancer patients will experience disease recurrence following initial treatment. Patient response to follow up treatment is poor with only 20-50% responding to second-line treatment in the platinum-sensitive group and 10-30% in the platinum resistant group [6]. Therefore, there is a great interest in identifying biomarkers or therapeutic targets to improve response to treatment in ovarian cancer. In an attempt to identify genes involved in chemoresponse, we identified GDF15 as being one of the most upregulated genes following cisplatin treatment of a cisplatin sensitive ovarian cancer cell line. We hypothesized that cisplatin-sensitive ovarian cancer cells, once treated with cisplatin, should express genes which reflect drug response. GDF15 was chosen for further study because as a secreted protein it would be detectable in blood, and be of great use as a biomarker. Given that GDF15 is a stress response gene, the goal of this chapter was to address specific aim 1 which is to examine GDF15 expression and its role following induction by cisplatin in ovarian cancer.

Results

Differentially expressed genes following cisplatin treatment of a cisplatin sensitive ovarian cancer cell line

In order to identify genes involved in cisplatin resistance, gene expression profiling of the A2780 cell line was conducted following treatment with either 5 or 10 μM cisplatin and RNA collected at different timepoints (0, 30 minutes, 1, 2, and 16 hours).

Figure 4 shows the most differentially cisplatin inducible genes from our study. As further validation, the GEO dataset (GSE8057) was analyzed using the OmniViz software (Figure 5). The GEO dataset also consisted of the A2780 cell line, yet unlike our data which was treated continuously with cisplatin until RNA was collected. Brun *et al.*, treated the cells for 2 hours with cisplatin at its IC_{90} (25 µM) and collected the RNA at 0, 2, 6, 16, and 24 hours after treatment as well as cells treated with IC_{10} (2.6 µM), IC_{25} (4.0 µM), IC_{50} (12 µM) and IC_{90} (25 µM) [124]. Despite the differences in treatment, we found several genes in common. Of these similarities we chose four genes (CDKN1A, FDXR, GDF15, and TP53I3) for further validation at the RNA and protein level. These four genes were induced in a time and dose dependent manner following cisplatin treatment (Figure 6).

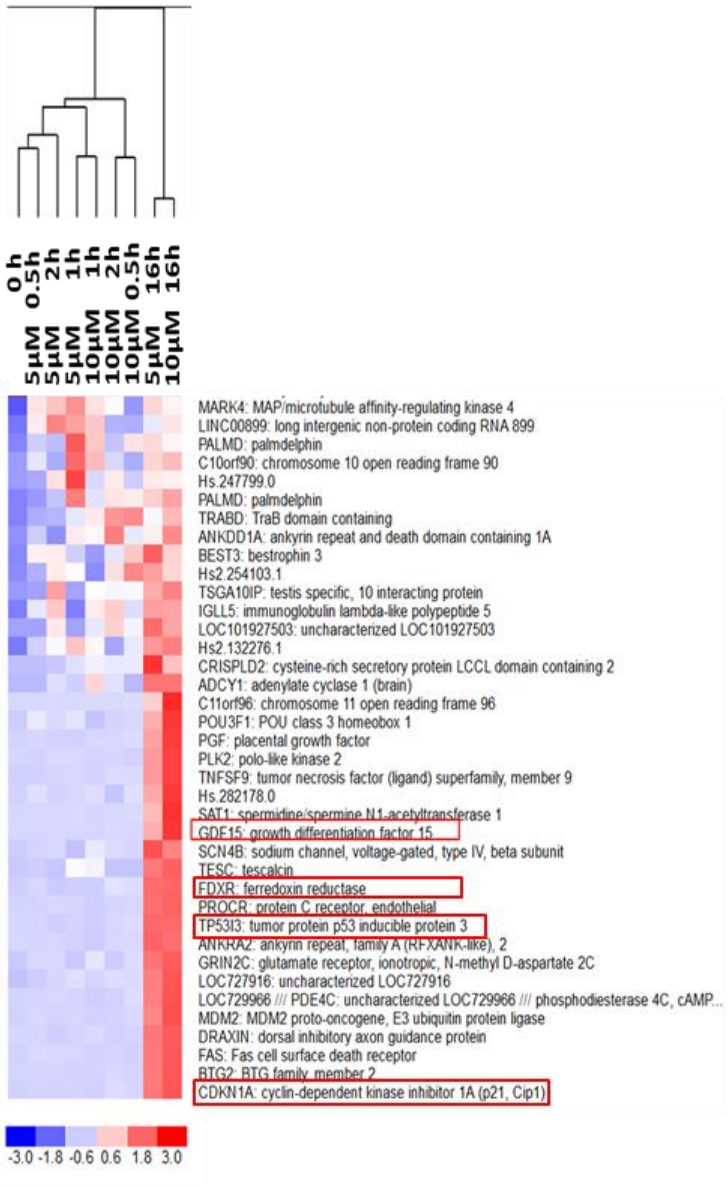

Figure 4: Most differentially cisplatin induced genes in the A2780 cell line.

Visualization of differentially expressed genes with greater than a 4 fold difference between the untreated sample at 0 hr and cisplatin treated samples (5 μM and 10 μM) at different timepoints. The genes surrounded by red boxes are the genes chosen for further validation.

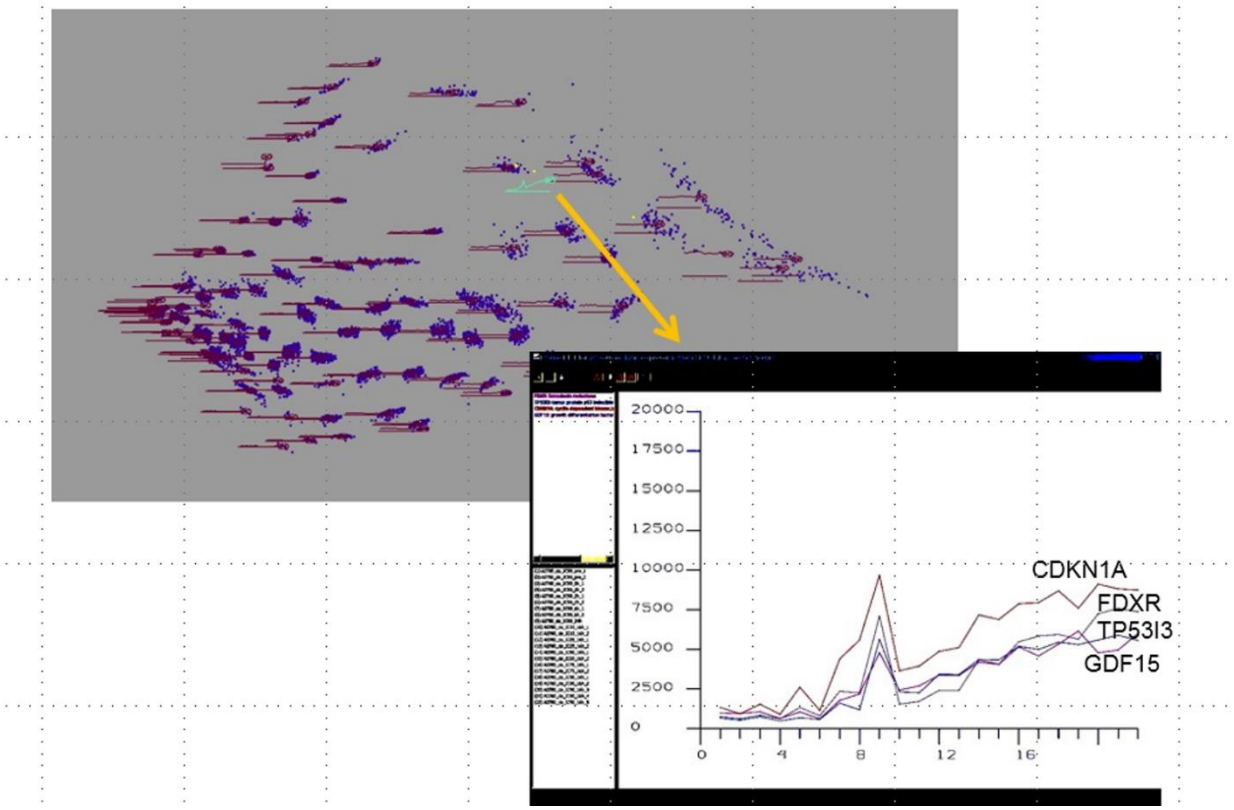

Figure 5: Most differentially expressed genes in GEO DataSet-GSE8057.

Visualization using the OmniViz software of expression profiles from the A2780 cell line treated at different times and varying doses of cisplatin. The clusters of dots represent genes with similar expression profiles. The cluster of genes in mint green in the gray box in enlarged to reveal the expression profile of the four genes of interest (CDKN1A, FDXR, GDF15, and TP53I3).

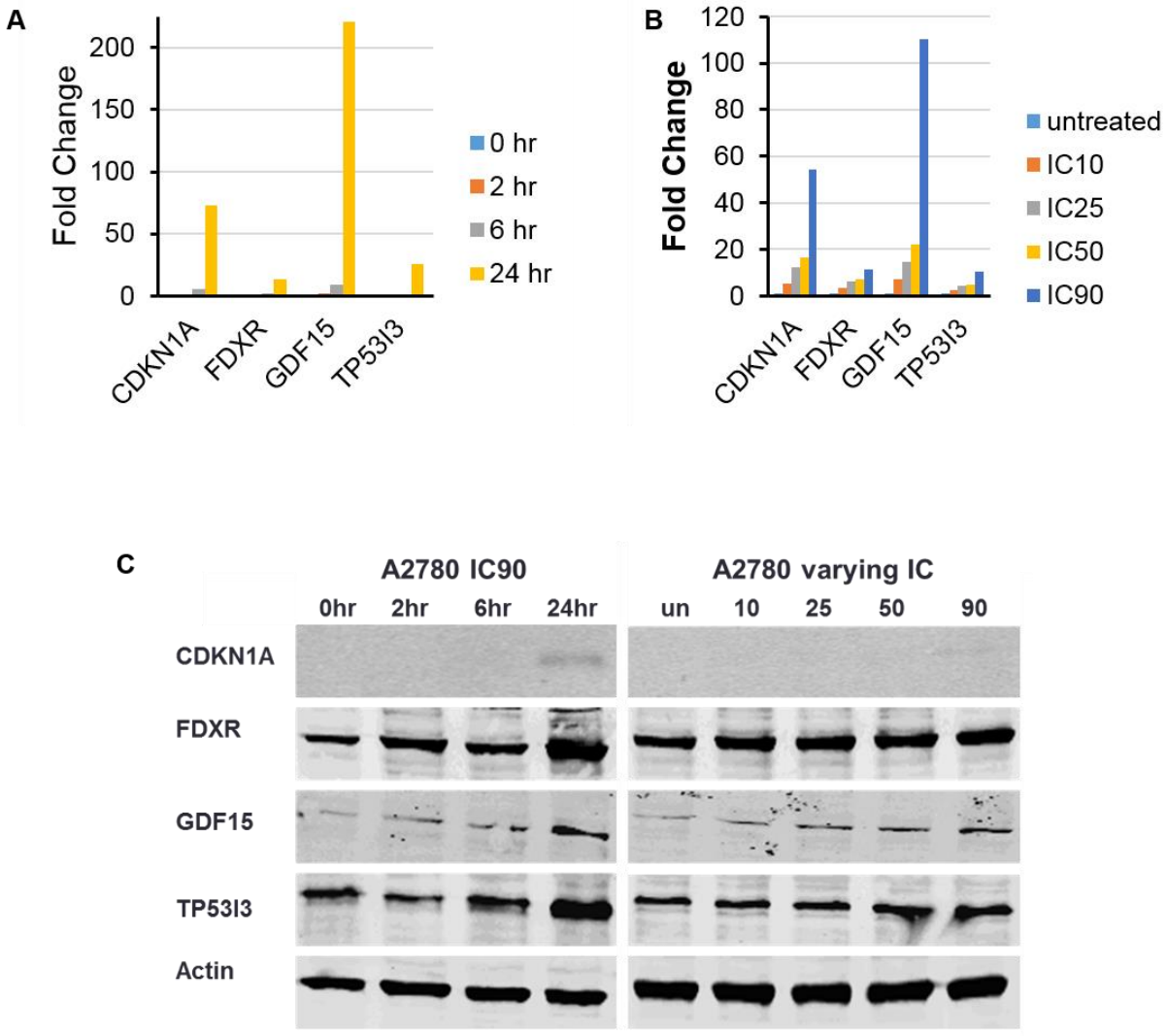

Figure 6: Gene induction by cisplatin is time and dose dependent.

(A) Real-time PCR of A2780 cell line treated with cisplatin IC90. (B) A2780 cell line treat with cisplatin at varying ICs for 16 hours. (C) Western blot analysis of (A and B).

GDF15 induction following treatment with therapeutic agents

Out of the four validated genes, GDF15 was chosen for further analysis as it had the highest induction following cisplatin treatment. Furthermore, as a secreted protein, GDF15 would be easy to use as a serum biomarker. To determine whether our observation was not just specific to the A2780 cell line, thirteen ovarian cancer cell lines were further treated with 2.5 µM cisplatin. RNA and protein analysis of these samples revealed GDF15 upregulation occurred only in p53 wild-type cell lines suggesting that this was a p53 dependent event (Figure 7).

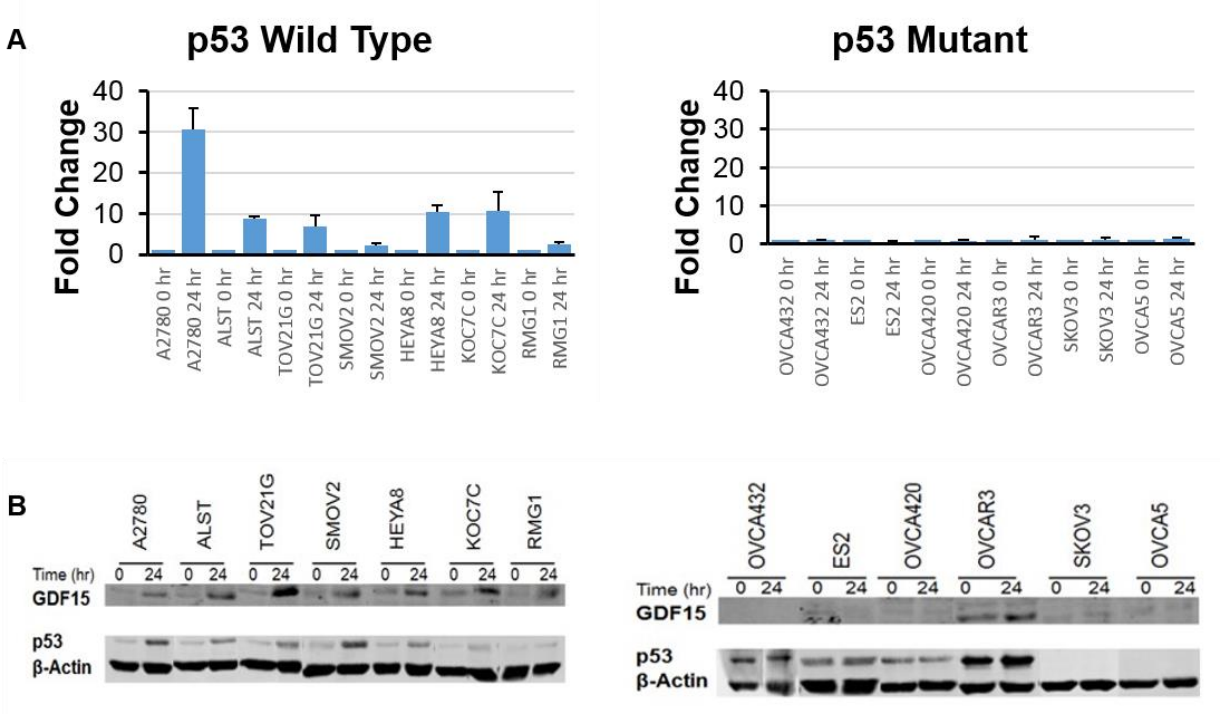

Figure 7: Induction of GDF15 in ovarian cancer cell lines following cisplatin treatment. Thirteen ovarian cancer cell lines were treated with 2.5 µM cisplatin. p53 wild-type cell lines. (A) RNA and (B) protein. p53 mutant cell lines (C) RNA and (D) protein.

Ovarian cancer treatment consists of surgery followed by a combination of chemotherapeutic agents, typically a taxane like paclitaxel and a DNA damaging agent such as cisplatin or carboplatin. Therefore, six ovarian cancer cell lines (three p53 wild-type and three p53 mutant) were further treated with varying doses of either carboplatin or paclitaxel (Figure 8). Similar to the cell lines treated with cisplatin, the p53 wild-type cell lines showed an upregulation of GDF15 expression following treatment with carboplatin while there was no effect on GDF15 expression in the p53 mutant cell lines. When the cells were treated with paclitaxel there was an induction of GDF15 only in the SMOV2 cell line.

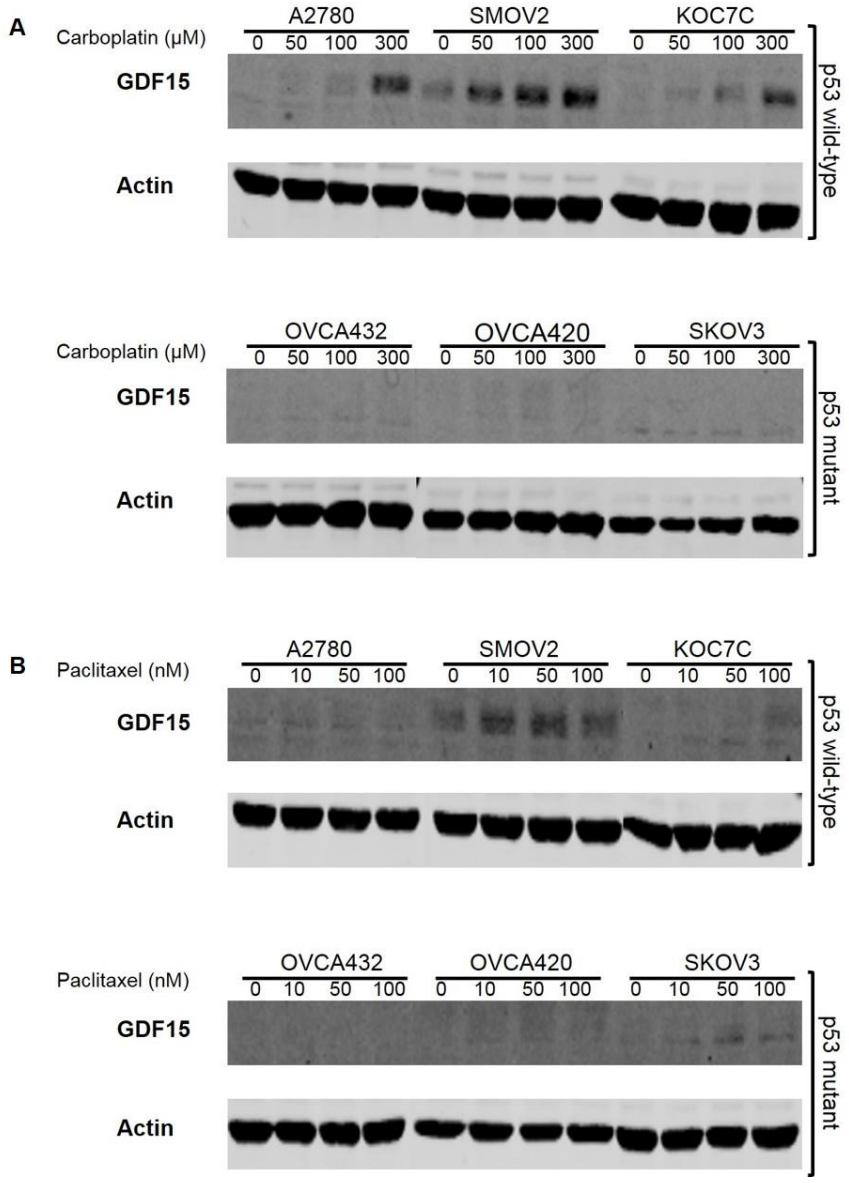

Figure 8: GDF15 expression in ovarian cancer cell lines treated with varying doses of carboplatin and paclitaxel.

(A) Carboplatin treated cells and (B) Paclitaxel treated cells.

Induction of GDF15 requires wild-type TP53

Studies from other groups have shown GDF15 to be both p53 dependent and independent. From our data it seems as though GDF15 is dependent on p53 at least following cisplatin and carboplatin treatment. To further test this observation, p53 was transfected into a p53 null cell line (SKOV3). Following p53 overexpression in the cell line, an upregulation of GDF15 was observed when compared to the control. Furthermore, knockdown of p53 in a p53 wild-type cell line (A2780) suppressed the induction of GDF15 by cisplatin as compared to the control (Figure 9). Further supporting our observation that GDF15 is p53 dependent.

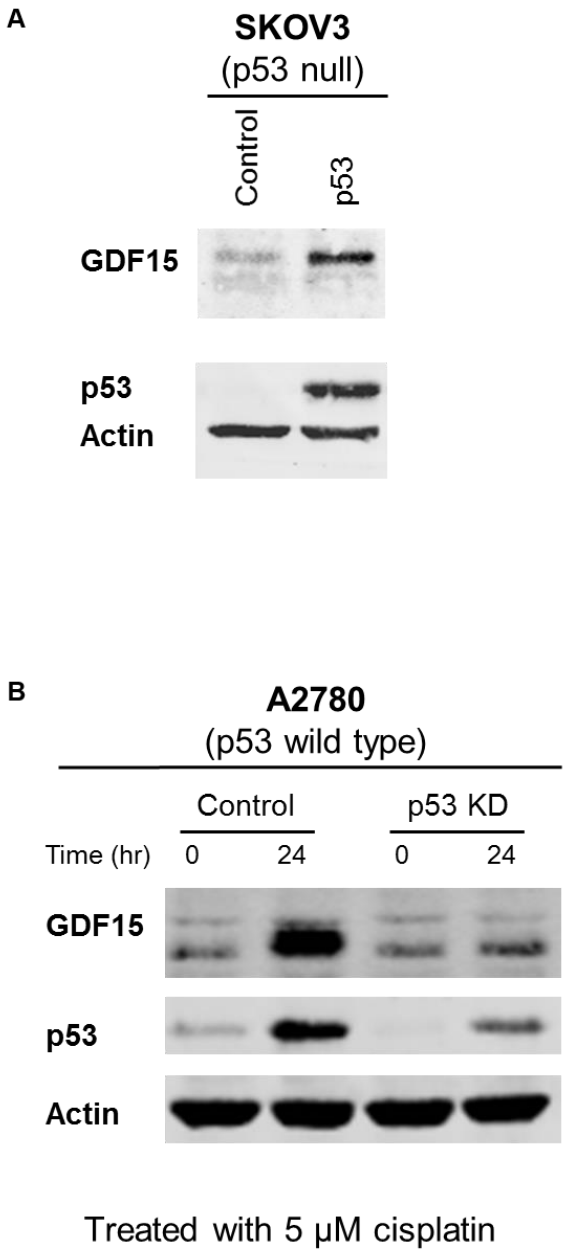

Figure 9: GDF15 induction is p53 dependent.

(A) p53 overexpression in a p53 null SKOV3 cell line induced GDF15 expression (B) p53 knockdown in a p53 wild type cell line A2780 suppressed GDF15 induction by cisplatin.

GDF15 is secreted into the blood in vivo after mice with tumor burden are treated with cisplatin

Because GDF15 is a secreted protein, the expression of GDF15 in the blood following cisplatin treatment was further tested. Two cell lines were chosen for this experiment: A2780 (a cisplatin sensitive cell line) and RMG1 (a cisplatin resistant cell line). Once mice had formed tumors, they were treated with either PBS, 2.5, or 5 mg/kg cisplatin (Figure 10). The tumors and blood collected from these mice were collected 24 and 48 hours after treatment. As expected, mice treated with cisplatin had an upregulation of GDF15 both at the RNA level and in the mice sera (Figure 11). This upregulation was higher in the mice injected with the A2780 cell line compared to the RMG1 cell line.

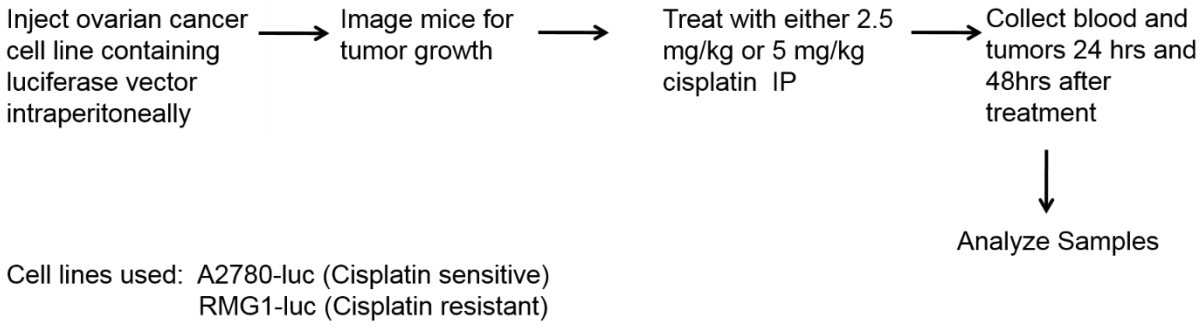

Figure 10: Mouse model studying GDF15 induction following cisplatin treatment.

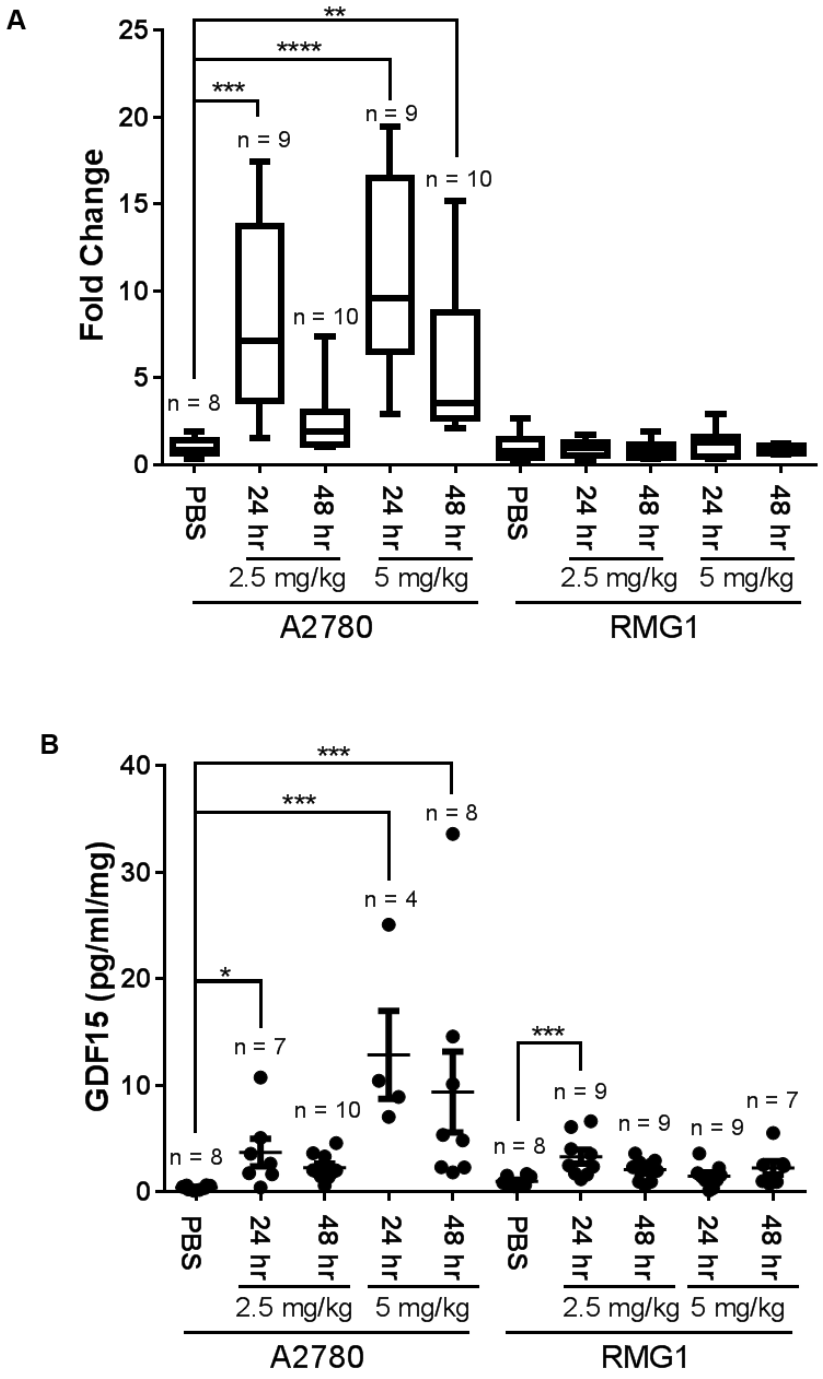

Figure 11: GDF15 is induced following cisplatin treatment *in vivo*.

(A) Mouse tumors. p-value: ** = 0.004, *** = 0.0007, and **** = <0.0001, (B) Mice sera. p-value: * = 0.03, ** = 0.003, and *** = 0.0002

GDF15 affects tumor growth but not proliferation

To study the role that GDF15 plays in ovarian cancer in response to cisplatin, mice were injected IP with either control, or one of two GDF15 knockdown (GDF15 KD) stable cell lines from the A2780. Note: since we lost mice due to sickness we grouped both GDF15 KD groups into one. (Figure 12). Once tumors developed the mice were treated twice with either 5 mg/kg cisplatin within a five day period or PBS. Forty-eight hours after the last treatment blood and tumors were obtained. Figure 13 shows the efficiency of our knockdown.

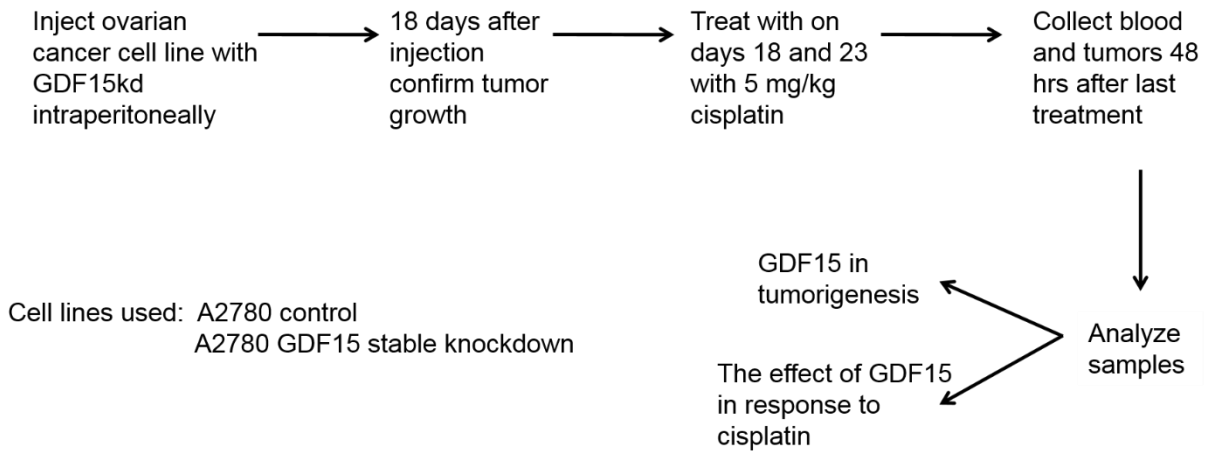

Figure 12: Mouse model to study the effects of GDF15 knockdown.

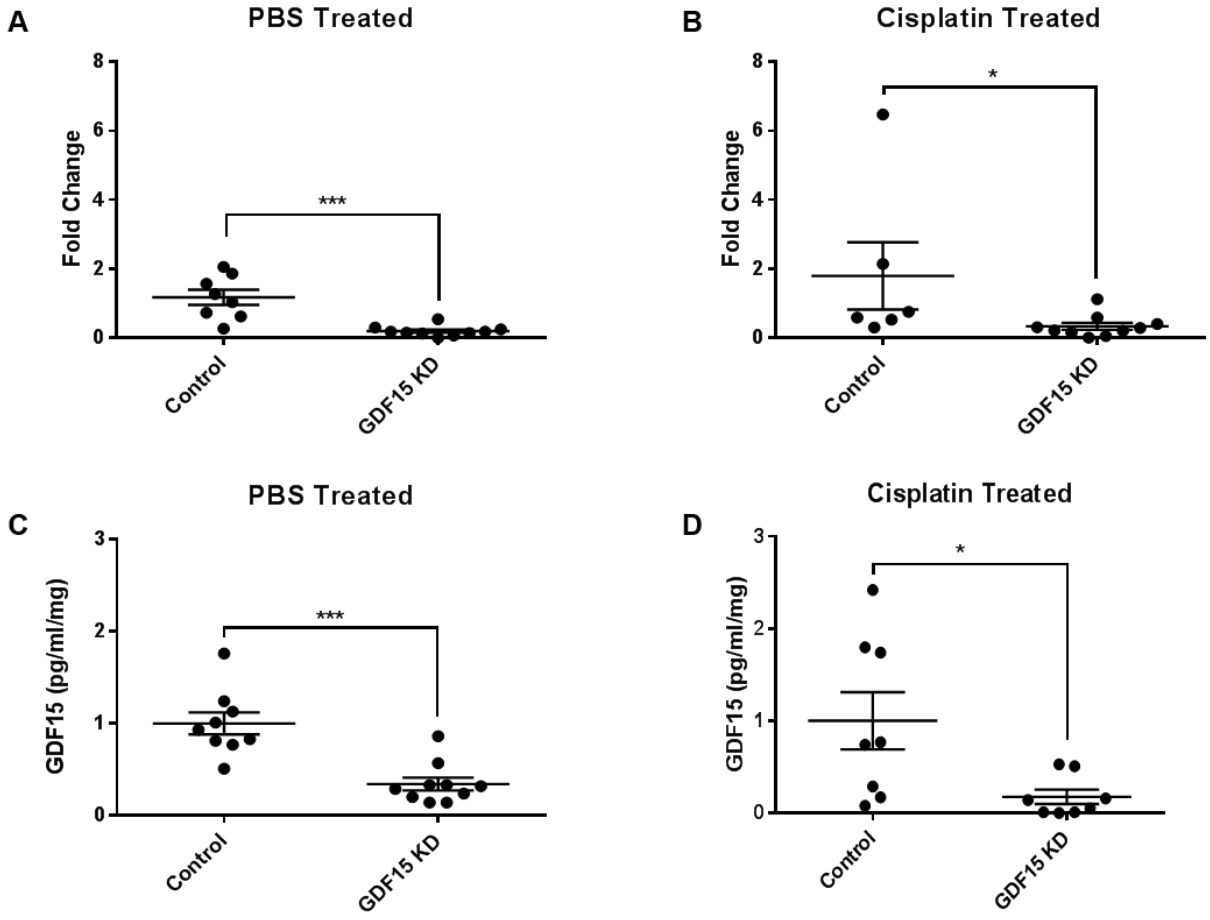

Figure 13: GDF15 knockdown validation in mice.

(A and B) GDF15 expression in mouse tumors, (C) normalized to Control PBS treated – p-value = 0.0002, (B) normalized to Cisplatin treated – p-value = 0.02 (C and D) GDF15 levels in mice sera, (C) normalized to Control PBS treated - p-value = 0.0004, (D) normalized to Cisplatin treated – p-value = 0.01, ,(A)

Following confirmation of GDF15 knockdown, we looked to see if there were any differences between GDF15 KD tumors and control tumors. Our analysis on tumor weight revealed that GDF15 KD tumors were larger than control tumors. This indicated that GDF15 affected tumor growth (Figure 14). To look at proliferation, IHC was conducted on the tumor samples. Ki67 staining revealed no differences in cell proliferation in PBS treated tumors control vs GDF15 knockdown (GDF15 KD) tumors (Figure 15 A).

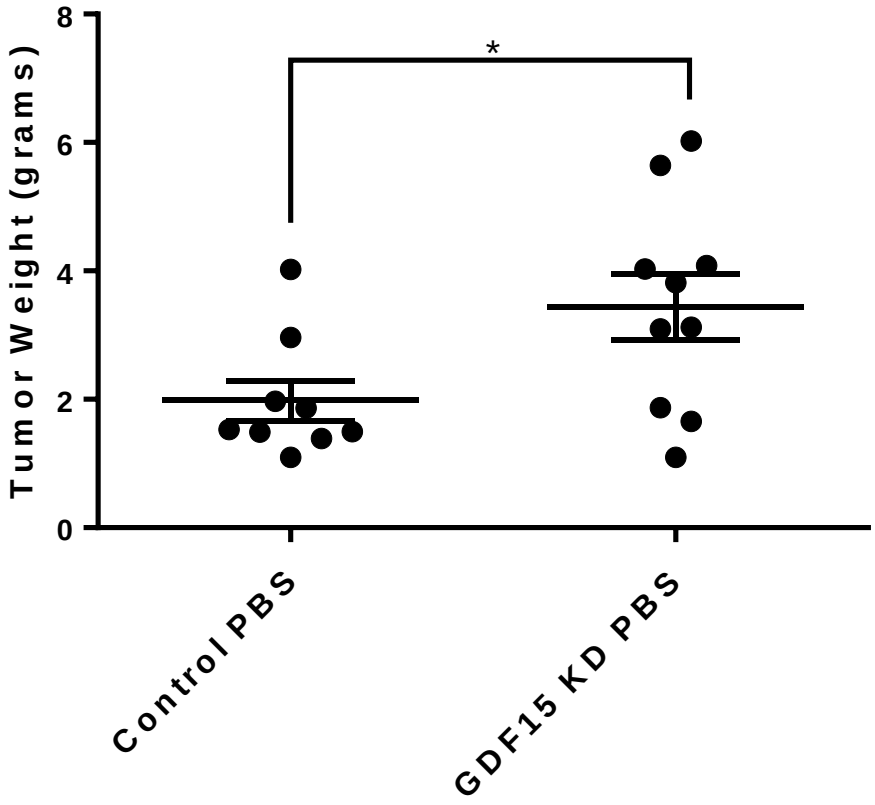

Figure 14: GDF15 KD affects tumor growth.

p-value: *= 0.03

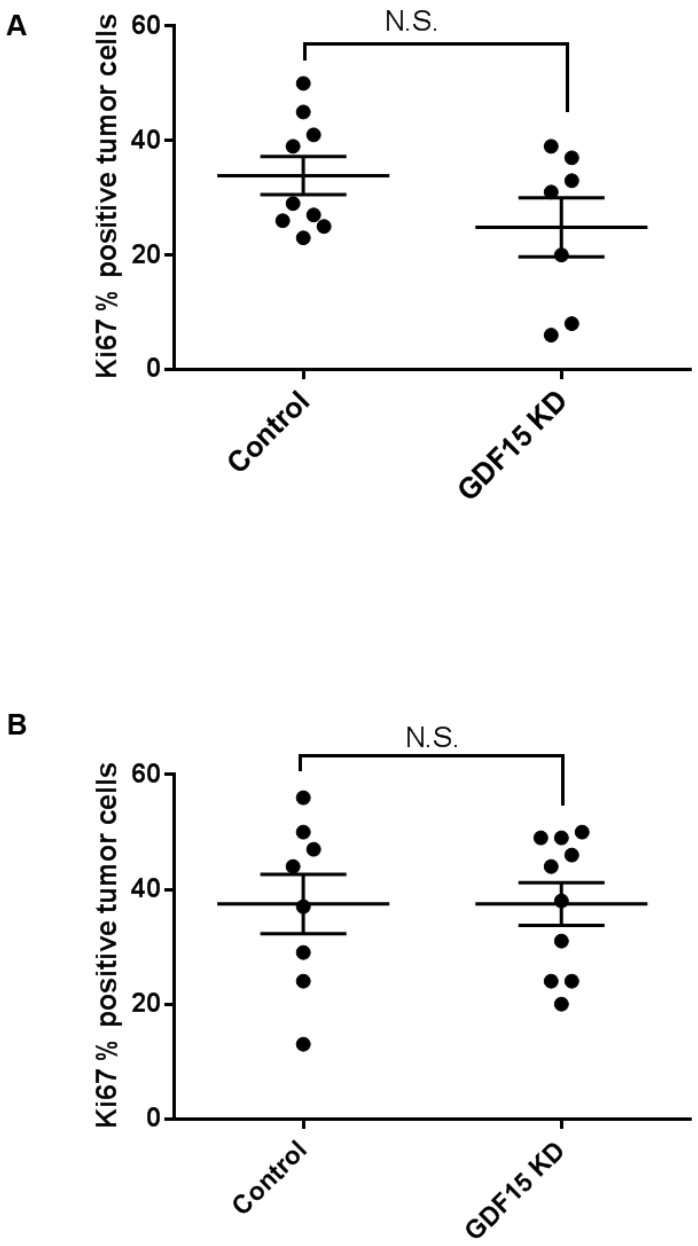

Figure 15: GDF15 KD does not affect cell proliferation.

(A) PBS treated tumors, (B) cisplatin treated tumors. N.S. = not statistically significant.

GDF15 KD tumors are smaller following cisplatin treatment than control tumors

As expected, cisplatin treated tumors in both groups were smaller than PBS treated tumors of their respective groups. To account for the tumor size difference between the control and the GDF15 KD tumors when determining if GDF15 made the tumors more sensitive or resistant, the cisplatin treated tumors were normalized to their own PBS treated tumors (Figure 16). This analysis revealed that the reduction in the size of GDF15 KD tumors after cisplatin treatment were more than that of the control tumors.

Since tumor growth is a balance between cell proliferation and apoptosis we next looked to see if there was a difference in cell proliferation. Ki67 IHC of tumor samples treated with cisplatin was used to examine whether there were any differences in cell proliferation following cisplatin treatment. Comparing the cisplatin treated tumors to the GDF15 KD tumors showed that there was no effect on cell proliferation following cisplatin treatment (Figure 15 B).

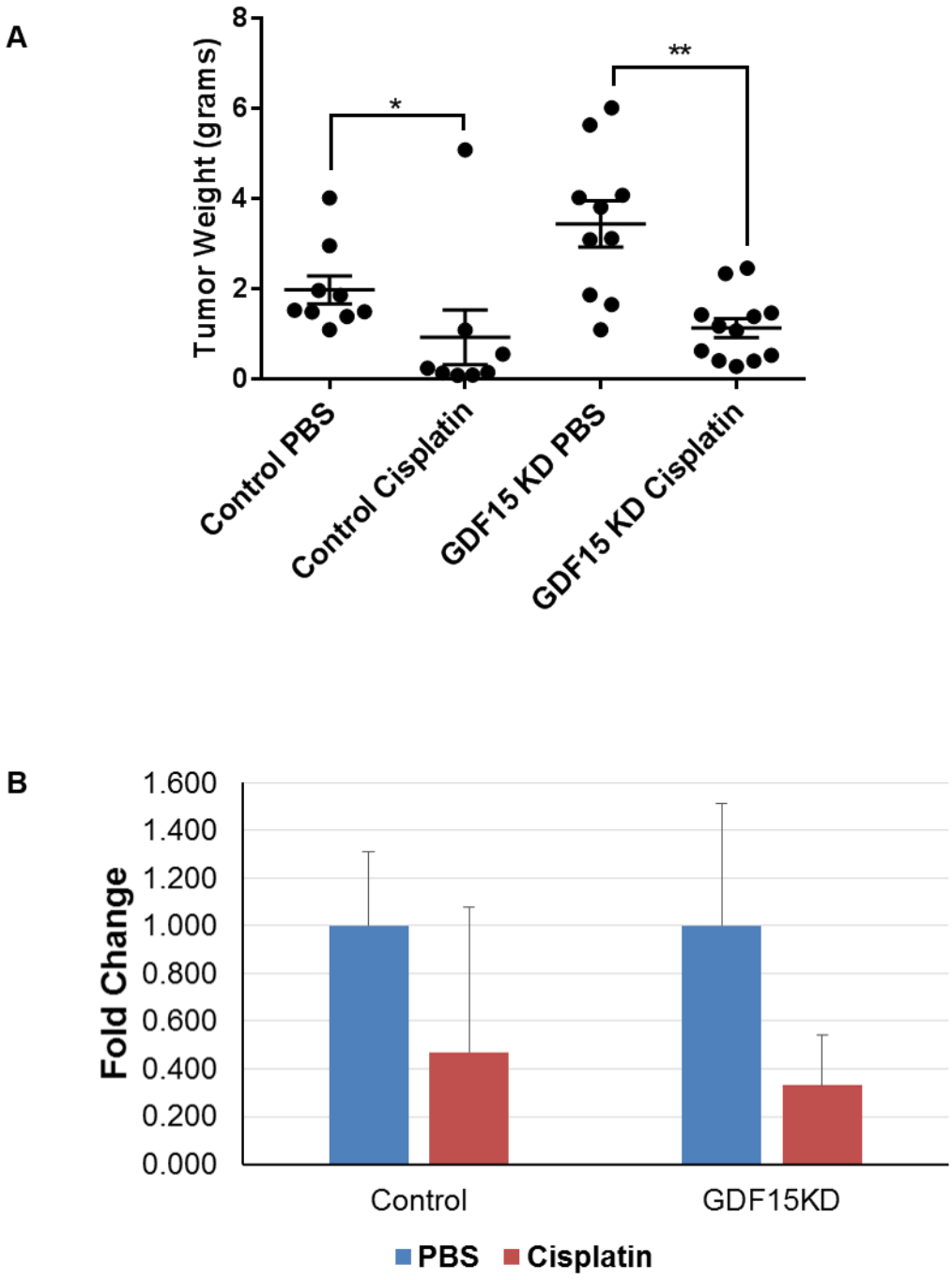

Figure 16: GDF15 KD makes tumors more resistant to cisplatin.

(A) Tumor weight of control and GDF15 KD PBS and cisplatin treated groups, p-value: *= 0.01, **= 0.004. (B) Each group normalized to their own PBS treated.

GDF15 RPPA data of mouse tumor samples

To study the pathways that GDF15 may be regulating, protein was extracted from 16 mouse tumor samples (3 control PBS, 3 control cisplatin, 2 GDF15 KD1 PBS, 2 GDF15 KD1 cisplatin, 3 GDF15 KD2 PBS, and 3 GDF15 KD2 cisplatin) and submitted for RPPA analysis. The average of each group was taken and comparisons were then made by comparing each group with their own PBS treated – (i) the GDF15 knockdown PBS treated with the control treated, and (ii) the GDF15 knockdown cisplatin treated with the control cisplatin treated. When each group of cisplatin treated mice was compared to their own group of PBS treated mice, RPPA data revealed a decrease in PARP1 while there was an increase in BIM (Figure 17). Interestingly, when both of the GDF15 knockdown groups treated with cisplatin were compared to the control group treated with cisplatin, the only protein that was differentially expressed was BIM.

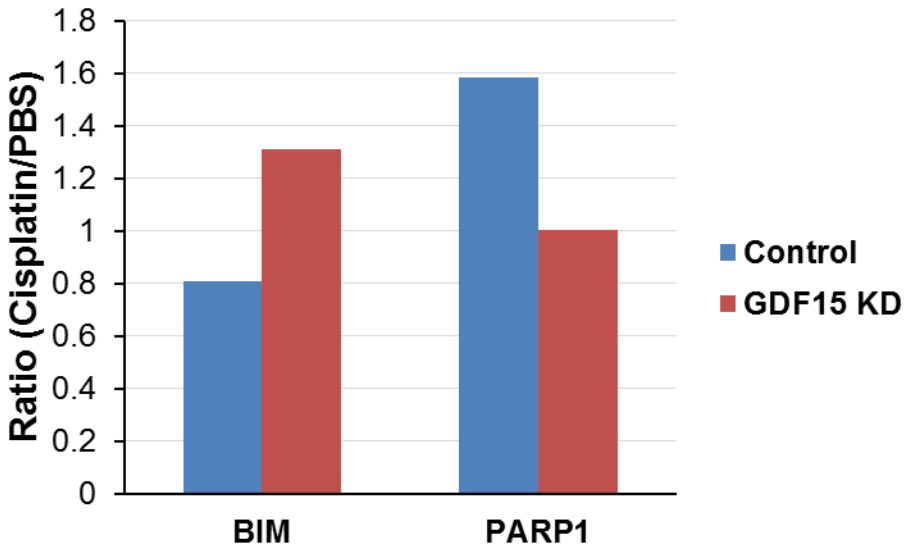

Figure 17: Apoptotic proteins in RPPA data.

Proteins comparing each group individually PBS vs cisplatin treatment.

Conclusion

In this chapter, we identified and validated four genes to be induced by cisplatin in a time and dose dependent manner (CDKN1A, FDXR, GDF15, TP53I3). We further showed that GDF15 is induced by platinum agents and that this induction is p53 dependent. Induction of GDF15 was also observed *in vivo* following cisplatin treatment. In addition, an *in vivo* orthotopic mouse model in which GDF15 was knocked down revealed that GDF15 affects tumor growth but it does not affect cell proliferation. Lastly, GDF15 KD tumors were smaller following cisplatin treatment than control tumors. As further support of this observation, RPPA data of GDF15 KD tumors had an increase in BIM (a pro-apoptotic protein) and a decrease in PARP1 (participates in the repair of DNA damage).

Chapter 4: GDF15 affects tumor component and its response to cisplatin

Rationale

As discussed in chapter 1, the tumor microenvironment consists of different cell types not just tumor cells. The crosstalk between tumor cells and stromal cells plays a role in how tumors respond to therapeutic agents as well as in tumor progression. As a secreted protein, GDF15 is expected to have both autocrine and paracrine signaling. The goal of this chapter was to address specific aim 2, which was to investigate how GDF15 affects the tumor microenvironment.

Results

GDF15 knockdown affects tumor composition independent of cisplatin treatment

As a secreted protein, GDF15 has both autocrine and paracrine signaling. To determine whether GDF15 affected tumor composition, Vectra2 images from the Ki67 staining were used for cellular segmentation analysis of tumor cells and non-tumor cells within the tumor. We evaluated whether there were any changes between tumor and stromal tissue composition based on GDF15 levels. Quantification of these compartments showed the stromal component of the tumors to be lower in the GDF15 knockdown tumors compared to the control both in the PBS treated and cisplatin treated groups (Figure 18). The tumor component in these tumors was higher in the GDF15 knockdown tumors when compared to the control.

Next, the cisplatin tumor component was normalized to the PBS treated group and likewise the cisplatin treated stromal component was normalized to the PBS stromal component. Interestingly, this analysis revealed that while in the control cisplatin treated

tumors there was an increase in the stromal component, in the GDF15 KD tumors the stromal component of the tumors was not affected suggesting that GDF15 KD affects tumor component. These data suggested that stromal cell component from control tumors appeared to be more resistant to cisplatin than the tumor cell component.

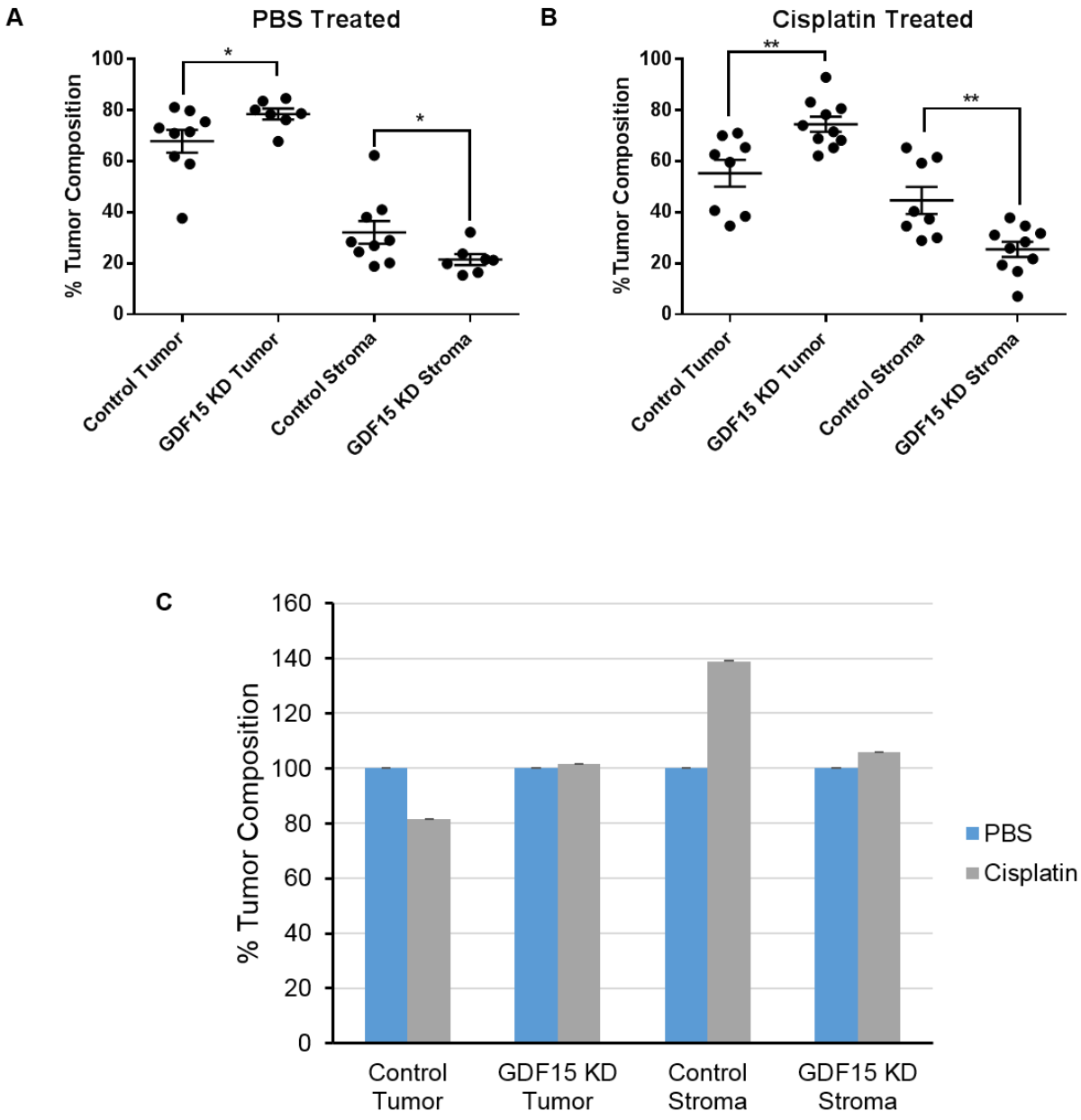

Figure 18: GDF15 affects tumor composition independent of cisplatin treatment. Tumor and stroma percentage in GDF15 KD mouse tumors. (A) PBS treated, (B) cisplatin treated, and (C) Each group normalized to their own PBS treated group.

GDF15 mouse tumor RNA-Seq data aligned to the human genome

To study the genes that are regulated by GDF15, RNA-seq was conducted on eight tumor samples. Four of the samples were from the control group (two PBS treated and two cisplatin treated), the remaining four samples were from the GDF15 knockdown groups (two PBS treated and two cisplatin treated). Using the CLC Genomics Workbench software program the sequences were aligned to both the human genome and the mouse genome. Gene expression analysis by aligning the RNAseq data to the human genome reflected the changes in gene expression of the A2780 human cancer cell. On the other hand, aligning the RNAseq data to the mouse genome reflected the changes in the mouse stromal cells within the tumors. This way we could study for the autocrine and paracrine effects of GDF15. Comparisons of gene expression were performed between the following groups: PBS vs Cisplatin treated, Control PBS vs. KD PBS, and Control Cisplatin vs KD Cisplatin (Table 2). We found no changes in gene expression that were statistically significant between the Control PBS vs. KD PBS groups. We attribute this to the fact that, in general, GDF15 is expressed at very low levels and it is only when under cisplatin as well as many other agents, and stress, acute tissue injury that this protein is induced.

Feature ID	Fold change	FDR p-value correction	Control Cis1 RPKM	Control Cis2 RPKM	KD Cis1 RPKM	KD CIS2 RPKM
GFY	6.57	0.01	3.07	5.84	29.03	30.74
MMP1	-14.21	0.01	33.12	14.71	2.31	0.8
MMP10	-5.43	0.01	40.69	56.9	7.46	10.3
CRABP1	14.82	0.05	8.02	59.44	763.95	273.38

Table 2: RNA-seq data of human genes in cisplatin treated control vs. kd mouse tumors.

MMP1 and MMP10 in mouse tumors

From the RNA-seq analysis of the human genome, MMP1 and MMP10 were chosen for further validation. Both genes showed a downregulation in the GDF15 KD tumors in both cisplatin treated (control vs KD) and PBS treated (control vs KD)(Figure 19). When comparing the PBS vs cisplatin treated for each group only MMP1 was upregulated in the cisplatin treated groups compared to the PBS treated groups suggesting that it is a cisplatin induced gene, whereas MMP10 is not.

Following this initial validation, real-time PCR was conducted on all the mouse tumors (Figures 20 and 21). As in the original samples submitted for mRNA-seq, both MMP1 and MMP10 mRNA levels were down compared to control tumors in both the cisplatin treated and the PBS treated tumors. Both MMP1 and MMP10 levels were statistically different in the PBS treated tumors when comparing control vs GDF15 KD. There was no statistical significance observed in the cisplatin (control vs kd) for MMP1, but there was significance for MMP10.

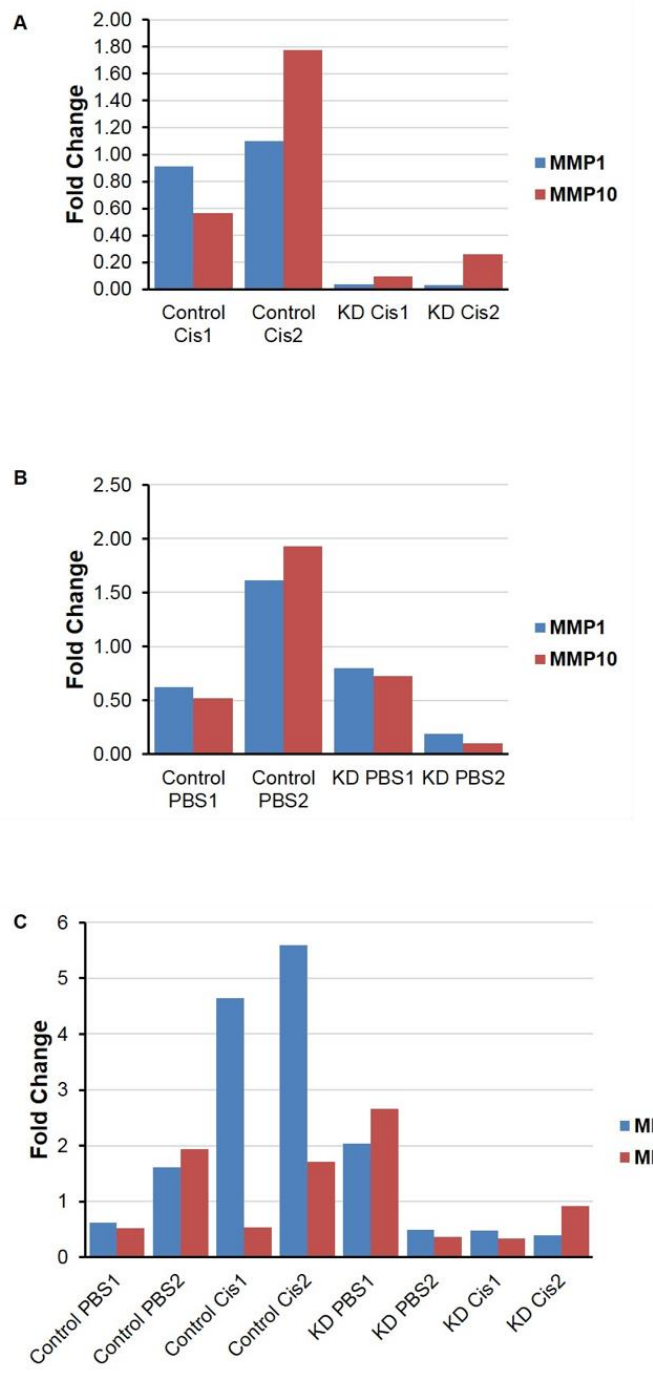

Figure 19: MMP1 and MMP10 expression in original mouse tumors submitted for RNA-seq.

(A) Cisplatin treated tumors comparing control vs. GDF15 KD, (B) PBS treated tumors comparing control vs. GDF15 KD, (C) Each group comparing PBS vs. Cisplatin.

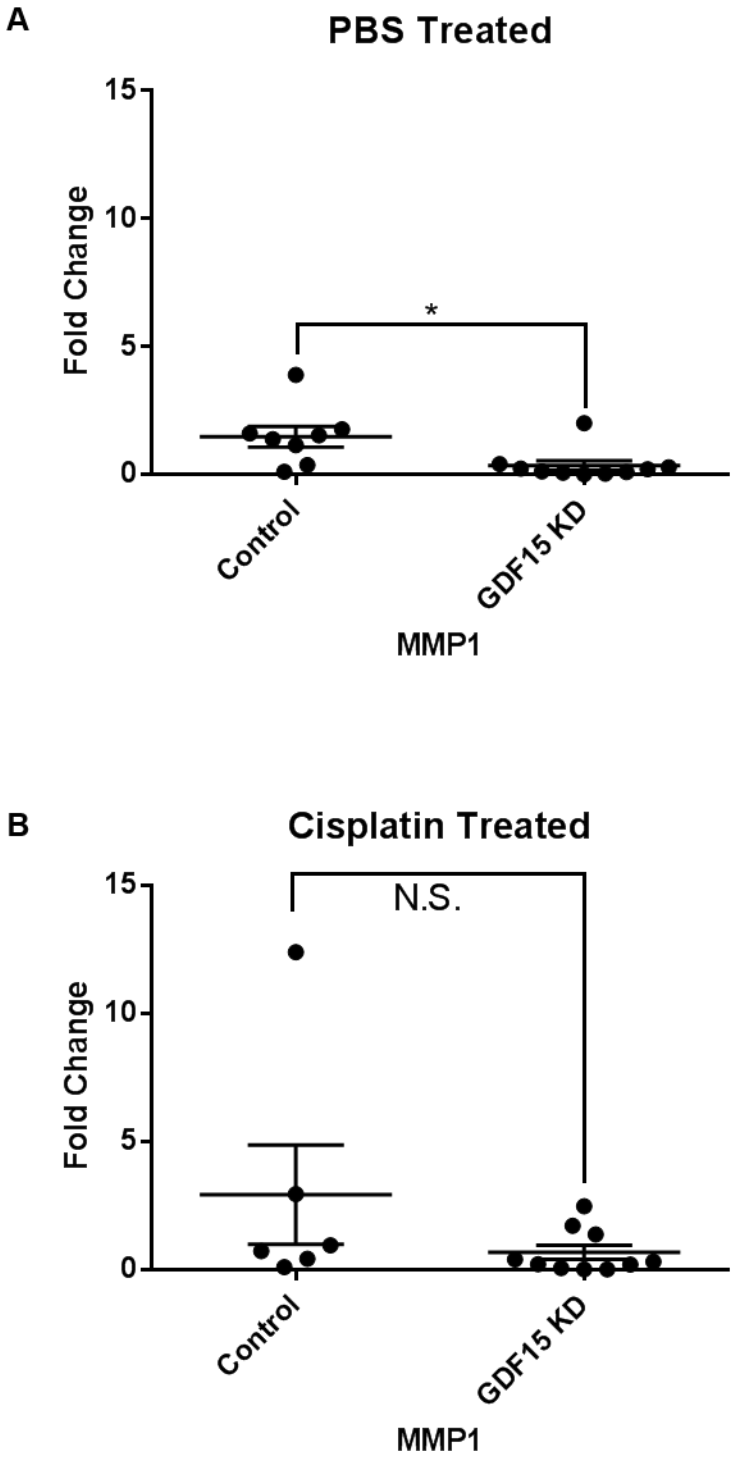

Figure 20: MMP1 expression in GDF15 KD mouse tumors.

PBS treated p-value = 0.02 and N. S. = not significant

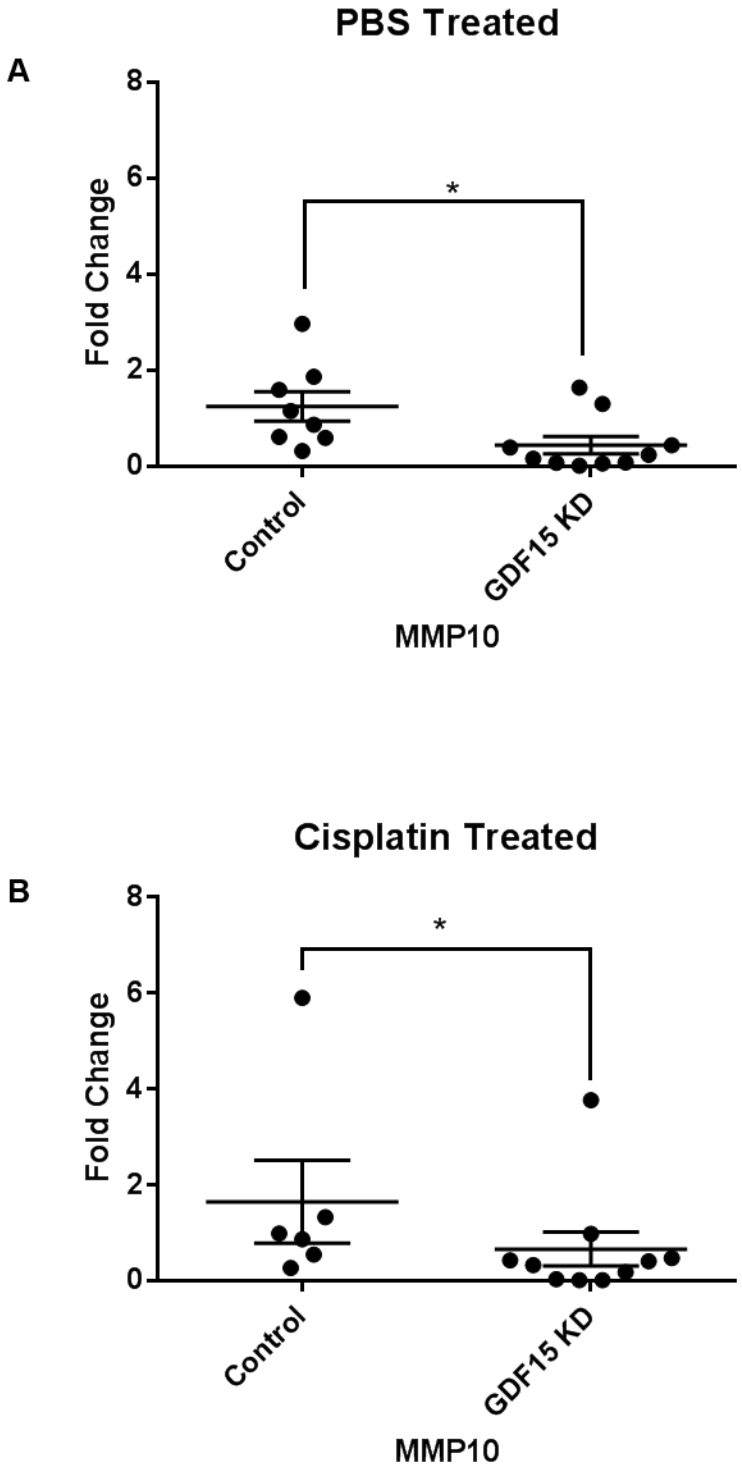

Figure 21: MMP10 expression in GDF15 KD tumors.

PBS treated p-value = 0.02, cisplatin treated p-value = 0.02

MMP1 and MMP10 in ovarian cancer cells

To further confirm our *in vivo* observation *in vitro*, GDF15 was knocked down in the A2780 and SMOV2 cell lines. Upon GDF15 KD, both MMP1 and MMP10 were upregulated in the GDF15 KD cells compared to the control cells (Figure 22). This is the opposite to our *in vivo* data in which MMP1 and MMP10 are downregulated in the GDF15 KD tumors suggesting additional factors are involved.

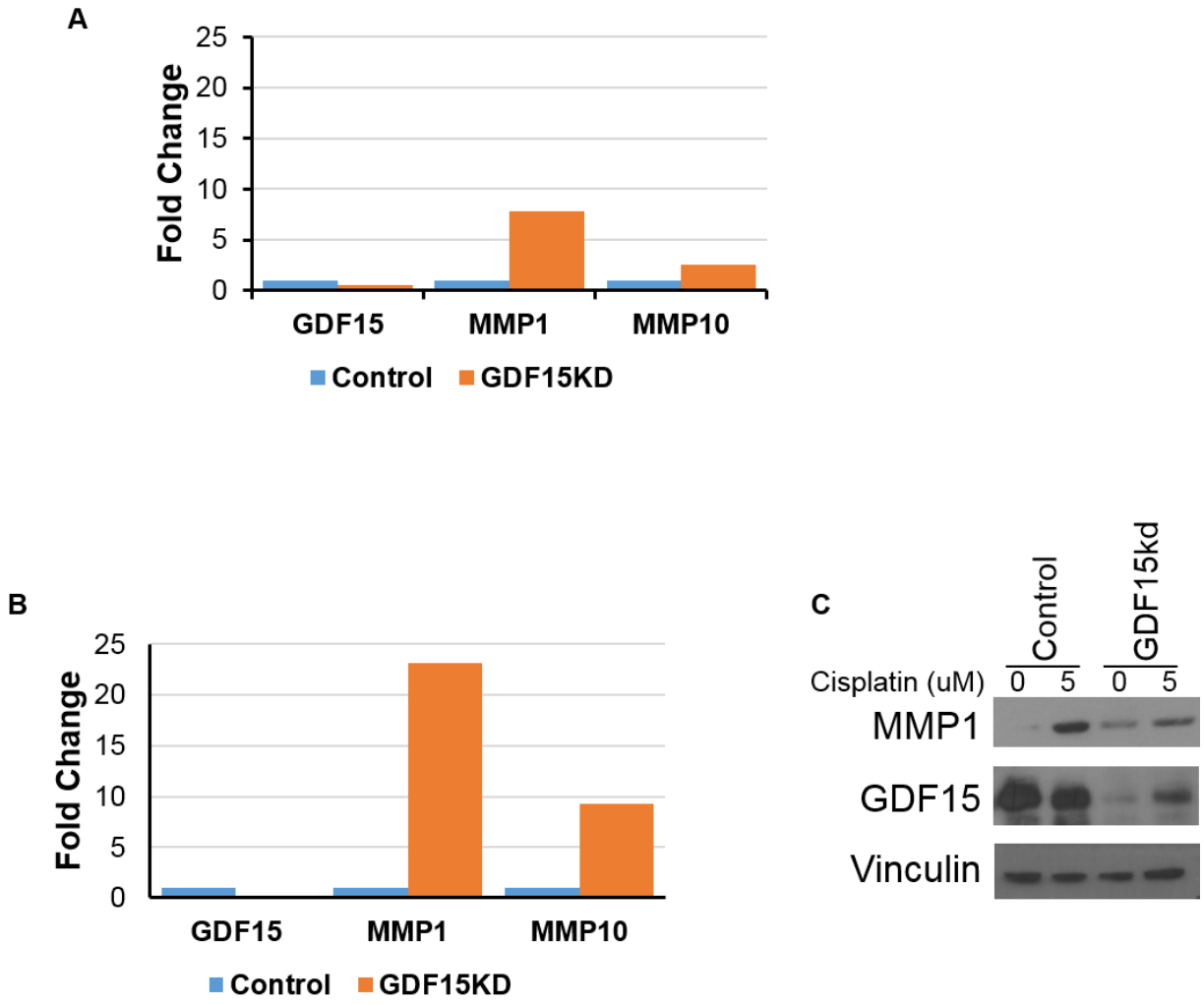

Figure 22: GDF15 KD *in vitro* revealed an upregulation of MMP1 and MMP10. A2780 cell line and (B-C) SMOV2

Correlation between GDF15 and MMPs in TCGA data set

TCGA RNA-seq data was downloaded from the cBio portal for both the ovarian serous and uterine endometrioid. RNA-seq samples were plotted for either MMP1 or MMP10 expression levels compared to GDF15 expression (Figure 23). Both MMP1 and MMP10 showed a statistically significant correlation with GDF15 expression following a Spearman correlation test. Our previous data shows that GDF15 induction is p53 dependent. 87% of ovarian serious tumors express mutant p53. In order to evaluate the correlation between GDF15 and MMP1 in p53 wild type tumors we examined TCGA RNA-seq data from the cBio portal was obtained for the endometrioid subtype of uterine cancer. RNA-seq data from the uterine endometriod p53 wild type samples was analyzed. From this analysis, a correlation between GDF15 and MMP1 expression was observed but not for MMP10 (Figure 23).

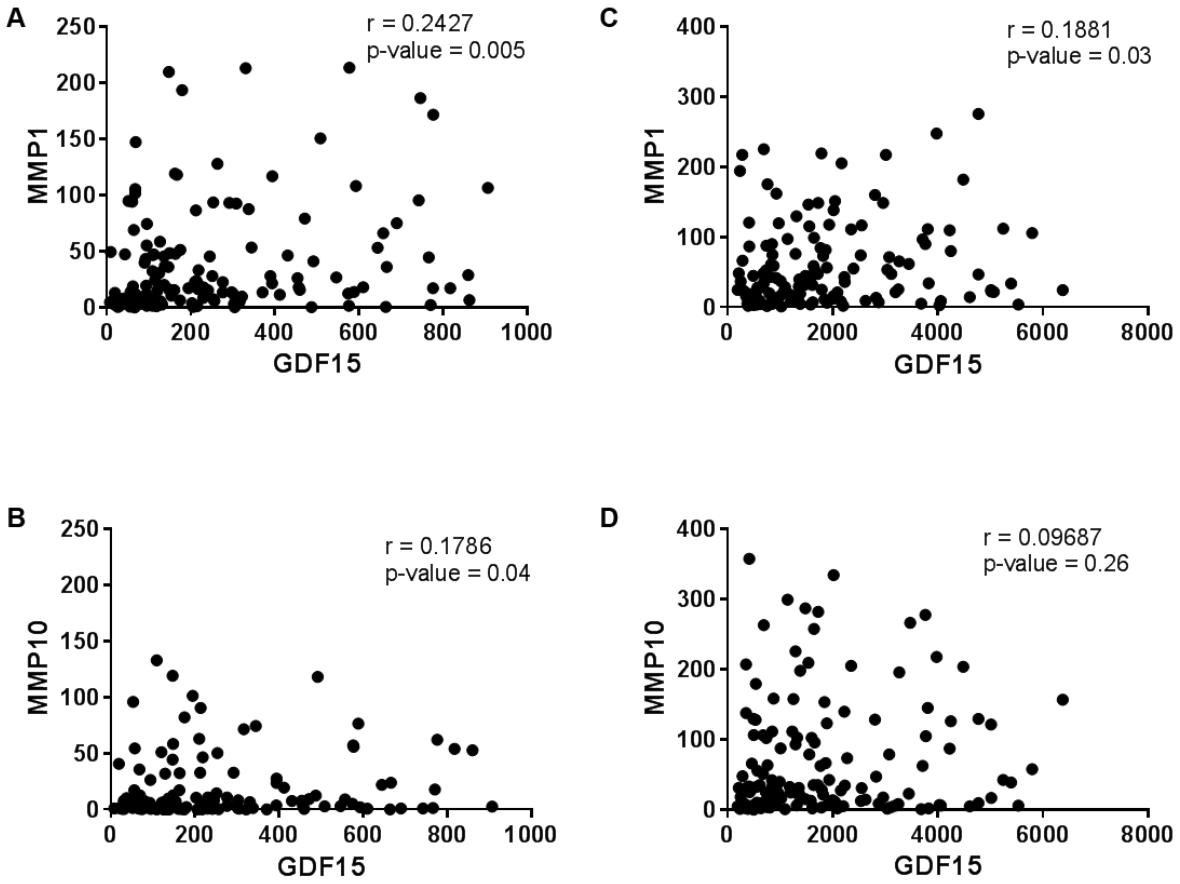

Figure 23: Correlation of MMP1 and MMP1o expression with GDF15 in patient samples.

RNA-seq expression data from TCGA patient samples (A-B) ovarian serous cysteadenocarcinoma-provisional data set and (C-D) uterine corpus endometrioid carcinoma [123].

MMP downstream targets in RPPA mouse tumor dataset

As previously described in this chapter, RPPA used to analyse changes in protein expression in mouse tumor samples (Figure 24). Among the proteins included in the RPPA analysis are FN1, a known target of MMP10 and COL6A1, which is an MMP target. A comparison between control vs GDF15 KD tumor samples in the cisplatin treated groups showed an upregulation of COL6A1 in the GDF15 KD tumors, whereas there was no difference in FN1.

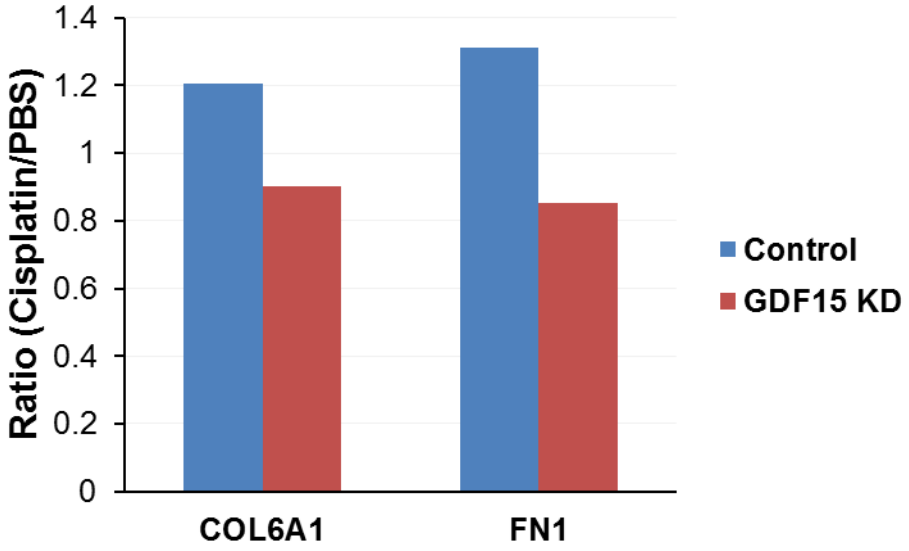

Figure 24: RPPA showing MMP downstream targets.GDF15 mouse tumor RNA-Seq data aligned to the mouse genome

To evaluate the paracrine effects of human xenograft tumor GDF15 on the mouse stromal tissue, the mRNA-seq data from the xenograft tumors was also aligned to the mouse genome. The following comparisons were performed for the mouse alignment: PBS vs Cisplatin treated, Control PBS vs. KD PBS, and Control Cisplatin vs KD Cisplatin (Table 3). Similar to the human alignment no gene was significantly different in the control PBS vs KD PBS treated comparison.

Table 3, shows the genes whose expression are statistically different in the cisplatin treated control vs GDF15 KD tumors. An ingenuity pathway analysis study showed the top five molecular and cellular functions were lipid metabolism (Clps and Pnlip), post-translational modifications and protein degradation in which Cela2a, Prss2, and Prss3 are involved. Genes from these categories were chosen for validation (Figure 25). All five genes were validated in the original RNA samples used for sequencing. However, when the entire mouse tumor set was analyzed using Real-time PCR, none were statistically different between groups (data not shown). Therefore, none of these genes were further studied. While having both human and mouse cells allowed us to look at autocrine and paracrine signaling, the tumor/stromal cell tissue ratio also became a limitation because depending on where the tissue piece that we extracted RNA was cut off some samples could have had more of one cell type than others.

Feature ID	Fold change	FDR p-value correction	Control Cis1 RPKM	Control Cis1 RPKM	KD Cis RPKM	KD Cis2 RPKM
2210010C04Rik	-3,778.01	0.02	8,174.34	2.66	2.27	1.94
Amy2a3	-4,413.37	0.02	11,873.80	4.07	2.73	2.54
Amy2a5	-10,256.79	0.04	19,885.06	1.57	2.92	1.05
Amy2b	-3,199.89	0.04	5,006.90	0.82	0.8	1.97
Cel	-3,504.62	0.02	6,679.79	3.45	2.66	1.18
Cela1	-2,929.57	0.02	42,529.72	17.85	16.35	13.55
Cela2a	-3,291.24	0.02	20,565.85	11.13	6.07	6.39
Cela3b	-3,276.22	0.02	22,085.74	10.74	7.15	6.48
Clps	-3,320.29	0.02	23,220.18	11.78	7.87	6.42
Cpa1	-4,936.16	0.02	10,004.21	2.66	2.47	1.54
Cpa2	-1,678.97	0.04	1,542.65	0.97	0.84	0.78
Cpb1	-2,904.53	0.04	3,032.00	0.95	1.15	0.76
Ctrb1	-4,358.00	0.02	105,193.64	37.1	24.4	24.58
Ctrc	-4,180.35	0.02	8,501.17	4.54	1.64	2.18
Ctrl	-4,375.22	0.02	12,005.10	2.92	3.45	2.1
Gp2	-3,973.37	0.04	1,638.11	0.39	0	0.52
Klk1	-3,967.31	0.04	3,121.97	1.18	0.62	0.71
Pnlip	-5,082.17	0.02	14,575.99	7.68	3.77	2.1
Pnliprp1	-3,166.69	0.04	6,382.31	1.44	2.45	1.54
Pnliprp2	-1,697.18	0.04	1,529.05	1.16	1.27	0.4
Prss2	-3,523.17	0.02	39,327.39	20.03	8.41	13.25
Reg2	-4,929.20	0.02	14,585.65	8.99	2.35	3.3
Reg3b	-2,677.32	0.02	11,220.69	6.14	4.99	3.56
Rnase1	-2,333.82	0.04	4,175.88	1.6	1.12	2.14
Spink3	-3,268.87	0.04	1,288.40	0.61	0	0.49
Sycn	-8,095.57	0.04	9,964.26	0.93	0.65	1.49
Try4	-4,657.18	0.02	41,125.00	15.85	7.88	9.59
Try5	-4,588.09	0.02	34,351.97	15.22	8.4	6.91
Zg16	-3,116.16	0.03	9,679.24	3.67	4.54	1.96

Table 3: RNA-seq of mouse cisplatin treated control vs. GDF15 KD tumor extracts

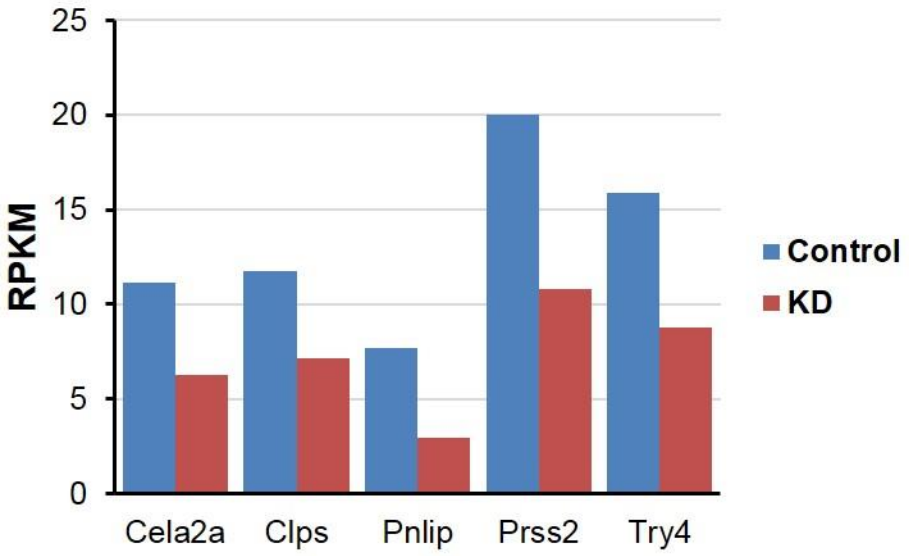

Figure 25: Mouse genes chosen for further validation.

Conclusion

The purpose of this chapter was to study how GDF15 affects the tumor microenvironment. As a secreted protein, GDF15 should have both autocrine and paracrine signaling. Using the tumors collected from the GDF15 KD mouse experiment from chapter 3, the tumor-stroma ratio was determined. The data showed GDF15 KD tumors had a lower stromal cell percentage than the control tumors. This analysis also revealed an increase in stromal tissue in the control tumors following cisplatin treatment, while the stromal component in the GDF15 KD tumors remained the same. These findings suggesting that GDF15 affects stromal composition in response to cisplatin.

RNA was also extracted from these tumors for sequencing. From the human alignment, MMP1 and MMP10 expression levels were further validated. In the mouse tumors both MMP1 and MMP10 RNA expression were downregulated in the GDF15 KD tumors compared to the control tumors. RNA-seq data from the TCGA ovarian serous and uterine corpus endometrioid patient samples further supported this observation. Interestingly, *in vitro* we observed the opposite as the *in vivo* data and the TCGA data,

an increase in both MMP1 and MMP10 RNA levels following GDF15 KD compared to the control. RPPA analysis of the mouse tumors further suggested a decrease in on metalloprotease activity by having an increase in COL6A1 and FN1, both of which are known to be degraded by MMPs. In the stromal cells we found no statistical difference in gene expression between control vs GDF15 KD tumors.

Chapter 5: Discussion

Induction of GDF15 by chemotherapeutic agents

Our microarray study in which the A2780 ovarian cancer cell line was treated with either 5 or 10 μM cisplatin at different time points (0, 30 minutes, 1, 2, and 16 hours) identified genes induced by cisplatin. We validated four cisplatin-induced genes (CDKN1A (p21), FDXR, GDF15, and TP53I3). These four genes are also known to be p53 regulated genes. This comes as no surprise as one of the mechanisms of cisplatin is to cause inter and intrastrand crosslinks with DNA resulting in the activation of the DNA damage response. As a result, p53 will then mediate transcription of genes leading to either cell cycle arrest and or apoptosis. We further focused on GDF15 because as a secreted protein it could be used as a biomarker.

GDF15 is rapidly induced under many situations including therapeutic agents and non-steroidal anti-inflammatory drugs. Our study showed that GDF15 is consistently induced by platinum agents (cisplatin and carboplatin) but not paclitaxel. Both cisplatin and carboplatin are DNA damaging agents whereas paclitaxel binds to microtubules and increases their stability [125]. The lack of GDF15 induction by paclitaxel is likely a result of the mechanisms that paclitaxel has on the cells and whether or not these pathways regulate GDF15.

GDF15 dependence on p53

While GDF15 expression has been shown to be both p53 dependent and independent, our study revealed that induction of GDF15 by both cisplatin and carboplatin is p53 dependent. We further showed that when you overexpress GDF15 in a p53 null cell line, GDF15 is expressed, whereas when you knockdown p53 in a p53

wild type cell line, GDF15 is no longer induced following cisplatin treatment. This observation further supports a study by Cheng *et al.* in which two p53 null cell lines were transfected with either wild type p53 or p53 mutants. Only the cells transfected with p53 wild type expressed GDF15 [126].

The dependence of GDF15 on p53 is of particular importance in our model. The most common type of ovarian cancer is of the high grade serous subtype. TCGA data of the ovarian high grade serous study analyzed through the cBioPortal has shown that 87% of these tumors contain p53 mutations [17, 18]. Therefore, our findings have further implications in p53 wild type tumors ovarian cancers.

GDF15 in tumorigenesis and chemoresistance

The overall purpose of my project was to identify a biomarker and/or therapeutic target to cisplatin chemoresponse. The reasoning was that if we could find a marker that could identify patients that would respond vs those who would not respond to cisplatin treatment, that the marker could help in determining if the current standard treatment (platinum-taxane) or an alternative would be a better option. In addition to showing in chapter 3 that GDF15 is induced following cisplatin treatment both *in vitro* and *in vivo*, we conducted an experiment *in vivo* looking at the effect of GDF15 knockdown in chemoresistance. Our experiment consisted of two groups (control vs GDF15 KD), which were further divided into PBS-treated and cisplatin-treated. This allowed us to look at GDF15 from two angles: (1) the role it plays in tumorigenesis and (2) the role it plays in ovarian cancer chemoresponse.

GDF15 KD did not affect cell proliferation in either the PBS treated or the cisplatin treated tumors, but it did affect tumor growth. GDF15 KD tumors were bigger when compared to the control tumors. Mutations that arrest the cell cycle do not block cell

growth just as cells can divide without growing. In essence, the cell cycle and cell growth are independently regulated [127]. However, control of tumor growth requires a balance between apoptosis and cell proliferation. A study by Mattern *et al.* revealed that apoptosis increased while proliferation decreased in large tumors compared to smaller tumors in lung cancer [128]. When we looked at PBS vs cisplatin treated tumors our data shows that GDF15 KD cisplatin tumors are smaller compared to control tumors once they have been normalized to their own PBS tumors to account for tumor size differences. In addition, RPPA data of these tumors revealed an increase in BIM and decrease in full length PARP1 in the GDF15 KD tumors, which is expected to which promote apoptosis. Essentially, GDF15 KD tumors are larger in size than the control tumors when the mice were not treated with cisplatin. However, GDF15 KD tumors have a higher increase in apoptosis than control tumor when the mice were treated with cisplatin. This may explain why we are not seeing an effect on cell proliferation.

Our mouse data further support observations from a study conducted by Meier *et al.* In their study, Meier *et al.* knocked down GDF15 in the A2780cis cell line. They tested the hypothesis that carboplatin induced genes in the sensitive cell line (A2780) should show higher basal expression levels in the cell line with acquired resistance (A2780cis). Their study showed that GDF15 levels increased by 14 fold in the plasma of carboplatin treated A2780 tumor bearing mice compared to a 2 fold increase in the A2780cis tumor bearing mice. In the Meier study, the cells were injected into nude mice subcutaneously and treated with carboplatin through tail vein injection. They also reported the same effects we saw with regards to cell proliferation, tumor growth, and response to a platinum agent, in their case carboplatin [129].

It is important to note several differences between the two studies. Our experiment was conducted in an orthotopic mouse model of ovarian cancer whereas they used a subcutaneous model. We treated the mice intraperitoneally with cisplatin whereas they treated through tail vain injection. Most importantly is the cell line they used. The A2780cis cell line has a p53 mutation (K351N). This mutation is found in the tetramerization domain. Mutations in this domain affect the affinity and conformation of its interaction with DNA, thus affecting the binding between p53 and DNA [130]. This K351 mutation also impairs the p53 nuclear export induced by cisplatin [131]. The data in this dissertation shows that at least in ovarian cancer GDF15 induction by platinum agents is p53 dependent. Figure 26 shows that when you treat the A2780cp20 cell line (which also has a p53 mutation (V173F mutation in the DNA binding domain) [132]) with 25 μM cisplatin, there is no induction of GDF15. Overall, both studies show the effect that GDF15 has in ovarian cancer tumorigenesis regardless of p53 status. Our study provides evidence that in p53 wild type ovarian cancers there is potential use of GDF15 as a biomarker. Although when deciding where the cutoff for GDF15 levels is determined, the fact that some tumors could be intrinsically resistant should be considered. Nevertheless, additional experiments are required that would need to be done before we can argue a role for GDF15 as a predictive chemotherapeutic response biomarker would be to conduct co-culture experiments with ovarian cancer cells and fibroblasts to check GDF15 levels. In addition, conducting IC_{50} experiments of the co-cultured ovarian cancer cells overexpressing or knocking down GDF15 are also needed..

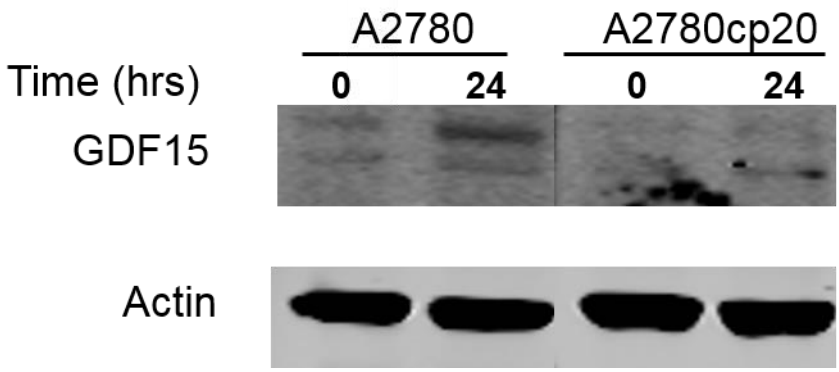

Figure 26: No induction of GDF15 in cisplatin resistant derivative of A2780 cell line. GDF15 expression in A2780 cell line and its derivative A2780cp20 before and after treatment with 25 µM cisplatin.

GDF15 secreted from tumors cells modulates the tumor microenvironment

The tumor microenvironment is known to play an important role in carcinogenesis. As a result there is a great interest in understanding the factors involved. As a secreted protein GDF15 has both autocrine and paracrine signaling. In chapter 4, we asked whether the crosstalk between GDF15 being secreted by the tumors and the stroma had any effect on the tumor microenvironment. Our GDF15 KD mouse study revealed that GDF15 plays a role in the tumor composition. GDF15 KD tumors had a lower percentage of the stromal component compared to control tumors. We know that this is a direct effect of GDF15 being secreted from the tumors as in our initial mouse study where we treated the mice containing tumors derived from either the A2780 or RMG1 cell lines to cisplatin we also included a control in which no tumor cells were injected with cancer cells but were treated with cisplatin and no GDF15 was detected in the sera of these mice. Further supporting this observation was the study conducted by

Staff *et al*. where IHC of ovarian tumor samples showed GDF15 expression in the tumor cells but not in the stromal component [121].

A study by Özdemir *et al.* is a clear example of the importance of the microenvironment. In the study, it was revealed that myoblast depletion in a pancreatic mouse model results in more invasive and undifferentiated tumors [133]. Further, proof that tumor derived factors can have a profound effect on cancer associated stroma is a recent study by Rhim *et al*., in which they showed that sonic hedgehog deficient pancreatic mouse tumors had a decrease in tumor stroma but also an increase in tumor vasculature, proliferation, and undifferentiated histology [134]. Not much is known about GDF15 and the microenviroment. Ectopic expression of GDF15 in fibroblasts has been shown to promote prostate cancer cell migration, invasion and tumor growth [135]. In myeloma, GDF15 increases survival of stroma-dependent cells and leads to resistance to therapeutic agents (melphalan, bortezemib, and lenalidomide) [136]. GDF15 has also been shown to be secreted from tumor –associated macrophages [137]. Other than the above mentioned examples not much else is known about GDF15 in the microenvironment. Our study is the first to show that GDF15 affects the tumor-stroma ratio. Given that GDF15 expression increases with tumor stage and this leads to poor survival, it would be important to further explore the role that GDF15 secreted from the tumor cells plays in tumorigenesis. In our study, orthotopic nude mouse models were used. In order to get an understanding of what is occurring with GDF15 and the microenviroment a syngeneic mouse model would be a better option to further explore as well as identifying the GDF15 receptor.

The tumor microenvironment has also been shown to be affected by therapeutic agents. Stromal cells are exposed to the same stresses as tumor cells such as hypoxic

and acidic conditions. They are also exposed to therapeutic agents. This environment can contribute to resistance by limiting drug bioavailability or creating selective pressure resulting in epigenetic changes or genetic mutations that in turn lead to acquired resistance [138]. In addition, our study showed cisplatin treatment affects the tumor/stromal ratio in the control tumors but not in the GDF15KD tumors, suggesting that GDF15 affects stromal response to cisplatin. This effect could very well be through the type of stromal cell that GDF15 is recruiting to the tumor. Nakasone *et al*. showed in a mammary tumor mouse model that myeloid cells were recruited to the tumor site following doxorubicin treatment. The tumor cells were recruiting the myeloid cells through secretion of CCL2/CCL12. Inhibition of the myeloid cells resulted in a better response.

GDF15 regulation of MMPs

In chapter 4, we aimed to identify downstream targets of GDF15. We reasoned that since GDF15 is a secreted protein it should have both autocrine and paracrine signaling. Since our GDF15 KD mouse model was an orthotopic model in which we essentially had human tumor cells mixed with mouse stromal tissue, we would be able to identify both autocrine and paracrine GDF15 regulated proteins.

From the RNA-seq data of the mouse tumors, only genes with an FDR p-value of 0.05 were further analyzed. Interestingly, only comparisons looking at the cisplatin treated tumors for both the mice and human sequence alignments had statistically significant FDR p-values. From the mouse alignment data comparing the cisplatin treated tumors Control vs GDF15 KD an analysis in IPA showed genes involved in lipid metabolism (Clps and Pnlip), post-translational modifications and protein degradation (Cela2a, Prss2, and Prss3) as being among the top functional molecular and cellular

functions. Lipid metabolism has been shown to promote cancer progression by promoting cancer cell survival as well as modulating cellular responses to anticancer drugs [139]. Post-translational modifications also play an important role in cancer because these chemical modifications dictate the activation state for the majority of physiological events. These modifications are targeted in therapeutics. An example is the EGFR inhibitors gefitinib (Iressa) and erlotinib (Tarceva) [140]. Protein degradation is important for homeostasis. Protein degradation is involved in the regulation of cell cycle, antigen processing, transcription and signal transduction. Aberrations in proteins involved in protein degradation can lead to stabilization of oncoproteins as well as destabilization of tumor suppressor genes [141]. Unfortunately, we were unable to validate those genes. A possibility could be that when we cut tissue for RNA extraction we do not really know what the tumor–stroma ratio is. Perhaps microdissection of stromal tissue would have been a better option to further validate these genes. While it would be important to look further into this, the Ingenuity Pathway Analysis suggests that GDF15 is promoting tumorigenesis.

From the RNA-seq data that was aligned to the human genome, the cisplatin Control vs GDF15 KD analysis only yielded four genes (GFY, MMP1, MMP10, and CRABP1) with statistically significant FDR p-values. Not much is known about GFY. It is a transmembrane protein that plays a role in olfactory sensory signaling [142]. CRABP1 is known to modulate retinoic acid function with a high affinity to the retinoic acid receptor. It is seen as a potential prognostic marker for the ovarian serous and clear cell carcinoma subtypes and its reduced expression in these ovarian cancer subtypes result in poor overall survival [143]. This supports our in vivo data. The GDF15 KD mice

have higher expression of CRABP1 than Control mice. While CRABP1 is certainly worth pursuing, we focused on MMP1 and MMP10.

GDF15 has previously been shown to be involved in migration and invasion. It has also been shown to regulate the expression of some matrix metalloproteinases (MMPs). Griner *et al*., showed a positive correlation between MMP2, MMP9 and GDF15 expression in ovarian cancer. The same study also shows that the pan-MMP inhibitor GM6001 suppressed invasiveness of GDF15 stable clones [144]. Our data further support this observation. In the mice, we observed a downregulation of both MMP1 and MMP10 in the GDF15 KD tumors compared to the Control in both the PBS treated and the cisplatin treated. We further show a positive correlation between MMP1, MMP10, and GDF15 in TCGA RNA-seq data from both the ovarian serous and the uterine endometrioid patient data set. However, knockdown of GDF15 in the A2780 and SMOV2 ovarian cancer cell lines resulted in an increase in both MMP1 and MMP10 expression (Figure 27). We attribute this to the crosstalk between the tumor and stromal cells. Based on our *in vivo* data from chapter 4, GDF15 clearly plays an important role in the tumor microenvironment. To date there has been no association between MMP10 and GDF15. However, a recent study did associate GDF15 and MMP1. Liu *et al.* showed that in coronary artery ectasia, GDF15 inhibits MMP1 expression in the sera of patients with coronary artery ectasia [145]. This observation is the opposite from what we have observed in the mice but GDF15 is very context and cell type dependent.

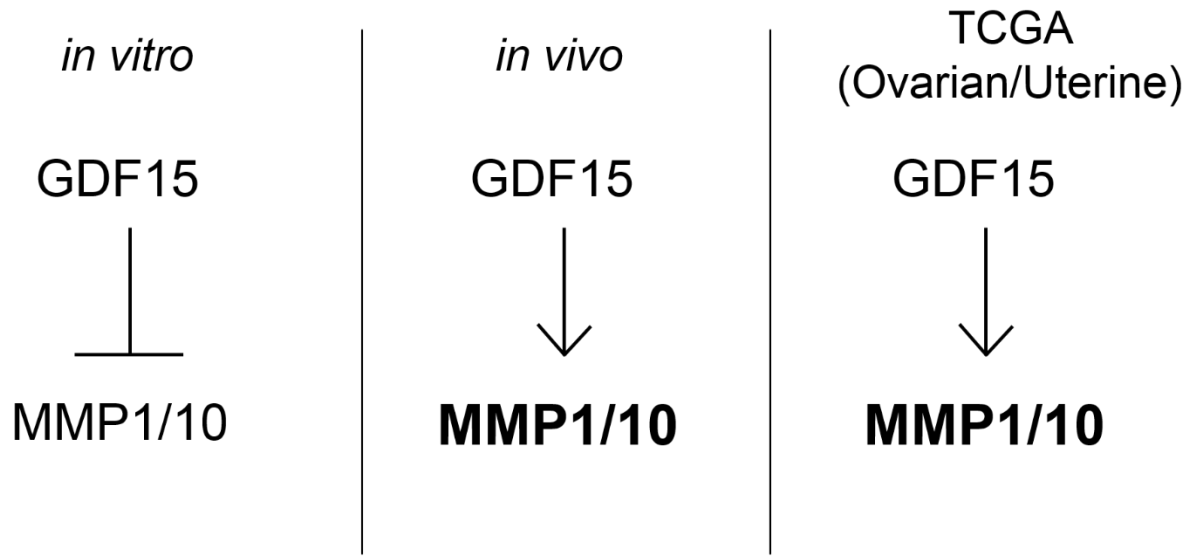

Figure 27: *in vivo* and *in vitro* inconsistencies.

Future Directions

The tumor microenvironment is comprised of a number of different types of cells (endothelial cells, fibroblasts, immune cells, etc.). Future studies should determine what cell type GDF15 is recruiting. IHC could be conducted on the tumor samples from the GDF15 KD mouse experiment using markers (such as: α smooth muscle for cancer associated fibroblasts or CD31 for endothelial cells) to determine specific stromal cell compositions. Identifying how GDF15 is recruiting stromal cells would also be important to understand. A way to do this might be to identify its receptor.

In this chapter I discuss how the regulation of MMP1 and MMP10 *in vitro* is the opposite of what was observed in the mouse tumors and in the TCGA data sets for both the ovarian and the uterine endometrioid patient samples. This could be attributed to the crosstalk between the tumor cells and the tumor microenvironment. Co-culture experiments between the cancer cells and endothelial cells/fibroblasts could be done to further study this discrepancy. Further analysis as to which pathways could be regulated by GDF15 might give an idea of how MMP1 and MMP10 are regulated. The GEO

dataset (GSE40595) consisting of gene expression profiling in the GeneChip Human Genome U133 Plus 2.0 microarray for Affymetrix of microdissected samples of normal ovarian stroma and ovarian cancer stroma was analyzed using the GEO2R program from the NCBI [146]. Genes with 2 fold change or higher where then analyzed using the Ingenuity Pathway Analysis software program. This analysis revealed the MAPK (p38, JNK, ERK), Jak/STAT, and TGF-β to be upregulated in the ovarian cancer stroma compared to the normal ovarian stroma. As a future direction it would be ideal to treat fibroblasts, as they are most abundant in tumor stroma [147], and conduct gene expression profiling to compare with the analysis just mentioned. In the literature there is evidence of GDF15 activating MAPK (p38,ERK) and PI3K/AKT. While this would allow us to study the pathways that GDF15 is activating in the stroma it still would not clarify why *in vivo* GDF15 promotes MMP1 and MMP10 upregulation yet in vitro inhibits their expression. Ovarian cancer cell lines in which GDF15 has been knocked down or overexpressed could be treated with inhibitors to pathways known to regulate MMP1 and MMP10.

Significance

GDF15 is generally expressed at low levels in tissue. It is rapidly induced in situations such as cancer, acute injury, cellular stress, inflammation, as well as by chemotherapeutic agents. GDF15 induction has been shown to be both p53 dependent and independent. However, our data would suggest that the induction of GDF15 is dependent on p53. This study showed for the first time that GDF15 affects the tumor composition. GDF15 KD tumors had less stroma compared to control tumors in addition GDF15 KD tumors were smaller following cisplatin treatment compared to control tumors. These observations suggests that GDF15 paracrine signaling plays an

important role in ovarian cancer tumorigenesis and chemoresponse. In addition, our study showed that GDF15 induction by platinum agents is p53 dependent, increasing interest on GDF15 as a marker of chemoresponse of the current treatment which consists of a chemotherapeutic combination of carboplatin and paclitaxel. Given that p53 wild type ovarian tumors are more platinum resistant than the high grade serous subtype that has a high p53 mutation rate dentifying the GDF15 receptor would be useful for future therapeutic development.

Concluding Remarks

To summarize, in this study we show that GDF15 is induced by platinum compounds in a p53 dependent manner. We further show that GDF15 affects the tumor-stroma ratio as well as the tumor response to cisplatin. (Figure 28).

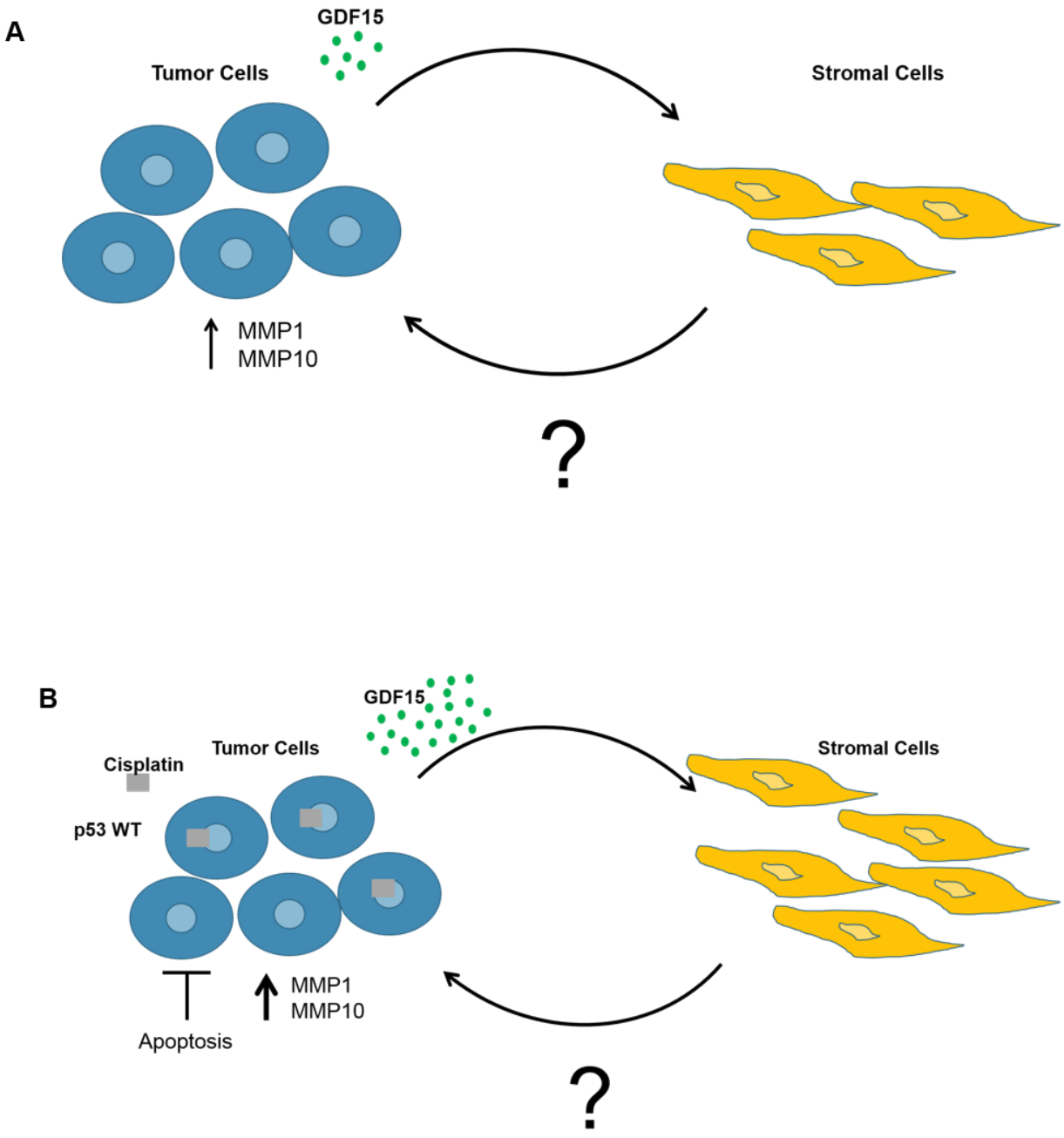

Figure 28: Model of GDF15 in ovarian cancer.

(A)GDF15 secretion from tumor cells regulates stromal cell signaling. By a yet unidentified pathway MMP1 and MMP10 are upregulated in tumor cells (B) following cisplatin treatment GDF15 is induced but only in p53 wild type (WT). This leads to an increase in stromal cells as well as an increase in MMPs and inhibition of apoptosis.

References

[1] Ovarian Cancer. In: American Cancer Society; 2016.

[2] Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Freur EJ, Cronin KA, (eds.). SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2012/, based on November 2014 SEER data submission, posted to the SEER web site, April 2015.

[3] Diseases and Conditions: Ovarian cancer. In: Mayo Foundation for Medical Education and Research; 2015.

[4] Gubbels JAA, Claussen N, Kapur AK, Connor JP, Patankar MS. The detection, treament, and biology of epithelial ovarian cancer. Journal of Ovarian Research l2010;3.

[5] Bast RC, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer l2009;9: 415-428.

[6] Romero I, Bast RC. Minireview: Human Ovarian Cancer: Biology, Current Management, and Paths to Personalizing Therapy. Endocrinology l2012;153: 1593-1602.

[7] McCluggage G. Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis. Pathology l2011;43: 420-432.

[8] Nakayama K, Nakayama N, Kurman RJ, Cope L, Pohl G, Samuels Y, Velculescu VE, Wang T-L, Shih I-M. Sequence mutations and amplification of PIK3CA and AKT2 genes in purified ovarian serous neoplasms. Cancer Biology & Therapy l2006;5: 779-785.

[9] Auner V, Kriegshäuser G, Tong D, Horvat R, Reinthaller A, Mustea A, Zeillinger R. KRAS mutation analysis in ovarian samples using a high sensitivity biochip assay. BMC Cancer l2009;9: 111-111.

[10] Jones S, Wang T-L, Shih I-M, Mao T-L, Nakayama K, Roden R, Glas R, Slamon D, Diaz LA, Vogelstein B, Kinzler KW, Velculescu VE, Papadopoulos N. Frequent Mutations of Chromatin Remodeling Gene ARID1A in Ovarian Clear Cell Carcinoma. Science l2010;330: 228-231.

[11] Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJM, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih I-M, Mes-Masson A-M, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG. ARID1A Mutations in Endometriosis-Associated Ovarian Carcinomas. New England Journal of Medicine l2010;363: 1532-1543.

[12] Kuo K-T, Mao T-L, Jones S, Veras E, Ayhan A, Wang T-L, Glas R, Slamon D, Velculescu VE, Kuman RJ, Shih I-M. Frequent Activating Mutations of PIK3CA in Ovarian Clear Cell Carcinoma. The American Journal of Pathology l2009;174: 1597-1601.

[13] Hashiguchi Y, Tsuda H, Inoue T, Berkowitz RS, Mok SC. PTEN expression in clear cell adenocarcinoma of the ovary. Gynecologic Oncology l2006;101: 71-75.

[14] Tan DSP, Miller RE, Kaye SB. New perspectives on molecular targeted therapy in ovarian clear cell carcinoma. Br J Cancer l2013;108: 1553-1559.

[15] Gilks CB, Prat J. Ovarian carcinoma pathology and genetics: recent advances. Human Pathology l2009;40: 1213-1223.

[16] Integrated genomic analyses of ovarian carcinoma. Nature l2011;474: 609-615.

[17] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. Cancer Discovery l2012;2: 401-404.

[18] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Science Signaling l2013;6: pl1-pl1.

[19] Gao J, Zheng Z, Rawal B, Schell MJ, Bepler G, Haura EB. Mirk/Dyrk1B, a novel therapeutic target, mediates cell survival in non-small cell lung cancer cells. Cancer biology & therapy l2009;8: 1671-1679.

[20] Kurman RJ. Origin and molecular pathogenesis of ovarian high-grade serous carcinoma. Annals of Oncology l2013;24: x16-x21.

[21] Vergote I, Tropé CG, Amant F, Kristensen GB, Ehlen T, Johnson N, Verheijen RHM, van der Burg MEL, Lacave AJ, Panici PB, Kenter GG, Casado A, Mendiola C, Coens C, Verleye L, Stuart GCE, Pecorelli S, Reed NS. Neoadjuvant Chemotherapy or Primary Surgery in Stage IIIC or IV Ovarian Cancer. New England Journal of Medicine l2010;363: 943-953.

[22] Kehoe S, Hook J, Nankivell M, Jayson GC, Kitchener H, Lopes T, Luesley D, Perren T, Bannoo S, Mascarenhas M, Dobbs S, Essapen S, Twigg J, Herod J, McCluggage G, Parmar M, Swart A-M. Primary chemotherapy versus primary surgery

for newly diagnosed advanced ovarian cancer (CHORUS): an open-label, randomised, controlled, non-inferiority trial. The Lancet.

[23] Sopik V, Rosen B, Giannakeas V, Narod SA. Why have ovarian cancer mortality rates declined? Part III. Prospects for the future. Gynecologic Oncology l2015;doi:10.1016/j.ygyno.2015.06.019.

[24] Treatment types. In: American Cancer Society; 2015.

[25] Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer l2013;13: 714-726.

[26] Goldstein LJ, Galski H, Fojo A, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I. Expression of Multidrug Resistance Gene in Human Cancers. Journal of the National Cancer Institute l1989;81: 116-124.

[27] Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J. DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Research l2006;34: D668-D672.

[28] Xu Y, Villalona-Calero MA. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. Annals of Oncology l2002;13: 1841-1851.

[29] Gagnon J-F, Bernard O, Villeneuve L, Têtu B, Guillemette C. Irinotecan Inactivation Is Modulated by Epigenetic Silencing of UGT1A1 in Colon Cancer. Clinical Cancer Research l2006;12: 1850-1858.

[30] Bell DW, Gore I, Okimoto RA, Godin-Heymann N, Sordella R, Mulloy R, Sharma SV, Brannigan BW, Mohapatra G, Settleman J, Haber DA. Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nat Genet l2005;37: 1315-1316.

[31] Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR Mutation and Resistance of Non–Small-Cell Lung Cancer to Gefitinib. New England Journal of Medicine l2005;352: 786-792.

[32] Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H. Acquired Resistance of Lung Adenocarcinomas to Gefitinib or Erlotinib Is Associated with a Second Mutation in the EGFR Kinase Domain. PLoS Med l2005;2: e73.

[33] Carter P, Presta L, Gorman CM, Ridgway JBB, Henner D, Wong WLT, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185^{HER2} antibody for human cancer therapy. Proc Natl Acad Sci U S A l1992;89: 4285-4289.

[34] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene. Science l1987;235: 177-182.

[35] Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF. Studies of the HER-2/neu Proto-Oncogene in Human Breast and Ovarian Cancer. Science l1989;244: 707-712.

[36] Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. Journal of Clinical Oncology l1996;14: 737-44.

[37] Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G, Slamon DJ. Multinational Study of the

Efficacy and Safety of Humanized Anti-HER2 Monoclonal Antibody in Women Who Have HER2-Overexpressing Metastatic Breast Cancer That Has Progressed After Chemotherapy for Metastatic Disease. Journal of Clinical Oncology l1999;17: 2639.

[38] Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. New England Journal of Medicine l2001;344: 783-792.

[39] Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M. Efficacy and Safety of Trastuzumab as a Single Agent in First-Line Treatment of HER2-Overexpressing Metastatic Breast Cancer. Journal of Clinical Oncology l2002;20: 719-726.

[40] Nahta R, Esteva FJ. Trastuzumab: triumphs and tribulations. Oncogene l2007;26: 3637-3643.

[41] Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, van de Vijver MJ, Bernards R. A Functional Genetic Approach Identifies the PI3K Pathway as a Major Determinant of Trastuzumab Resistance in Breast Cancer. Cancer Cell l2007;12: 395-402.

[42] Razis E, Bobos M, Kotoula V, Eleftheraki AG, Kalofonos HP, Pavlakis K, Papakostas P, Aravantinos G, Rigakos G, Efstratiou I, Petraki K, Bafaloukos D, Kostopoulos I, Pectasides D, Kalogeras KT, Skarlos D, Fountzilas G. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy

in metastatic breast cancer. Breast Cancer Research and Treatment l2011;128: 447-456.

[43] Browne BC, Crown J, Venkatesan N, Duffy MJ, Clynes M, Slamon D, O'Donovan N. Inhibition of IGF1R activity enhances response to trastuzumab in HER-2-positive breast cancer cells. Annals of Oncology l2011;22: 68-73.

[44] Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer l2008;8: 193-204.

[45] Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, Sarkar S. Drug Resistance in Cancer: An Overview. Cancers l2014;6: 1769-1792.

[46] Longley DB, Johnston PG. Molecular mechanisms of drug resistance. Journal of Pathology l2005;205: 275-292.

[47] Walsh CS. Two decades beyond BRCA1/2: Homologous recombination, hereditary cancer risk and a targer for ovarian cancer therapy. Gynecologic Oncology l2015;137: 343-350.

[48] Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, Villegas E, Jacquemont C, Farrugia DJ, Couch FJ, Urban N, Taniguchi T. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. Nature l2008;451: 1116-1120.

[49] Edwards SL, Brough R, Lord CJ, Natrajan R, Vatcheva R, Levine DA, Boyd J, Reis-Filho JS, Ashworth A. Resistance to therapy caused by intragenic deletion in BRCA2. Nature l2008;451: 1111-1115.

[50] Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicologic Pathology l2007;35: 495-516.

[51] Wong RSY. Apoptosis in cancer: from pathogenesis to treatment. Journal of Experimental & Clinical Cancer Research : CR l2011;30: 87-87.

[52] Raffo AJ, Perlman H, Chen M-W, Day ML, Streitman JS, Buttyan R. Overexpression of bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo*. Cancer Research l1995;55: 4438-4445.

[53] Minn AJ, Rudin CM, Boise LH, Thompson CB. Expression of bcl-xL can confer a multidrug resistance phenotype. Blood l1995;86: 1903-1910.

[54] Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. The Journal of Clinical Investigation 117: 326-336.

[55] Uramoto H, Iwata T, Onitsuka T, Shimokawa H, Hanagiri T, Oyama T. Epithelial-mesenchymal transition in EGFR-TKI acquired resistant lung adenocarcinoma. Anticancer Research l2010;30: 2513-2517.

[56] Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. Journal of Cell Science l2013;125: 5591-5596.

[57] Gilbert LA, Hemann MT. DNA Damage-Mediated Induction of a Chemoresistant Niche. Cell l2010;143: 355-366.

[58] Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, Davis A, Mongare MM, Gould J, Frederick DT, Cooper ZA, Chapman PB, Solit DB, Ribas A, Lo RS, Flaherty KT, Ogino S, Wargo JA, Golub TR. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature l2012;487: 500-504.

[59] Dasari S, Bernard Tchounwou P. Cisplatin in cancer therapy: Molecular mechanisms of action. European Journal of Pharmacology l2014;740: 364-378.

[60] Rosenberg B, Van Camp L, Krigas T. Inhibition of Cell Division in Escherichia coli by Electrolysis Products from a Platinum Electrode. Nature l1965;205: 698-699.

[61] Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, Castedo M, Kroemer G. Molecular mechanisms of cisplatin resistance. Oncogene l2012;31: 1869-1883.

[62] Prestayko AW, D'Aoust JCD, Issell BF, Crooke ST. Cisplatin (cis-diamminedichloroplatinum II). Cancer Treatment Reviews l1979;6: 17-39.

[63] Köberle B, Tomicic MT, Usanova S, Kaina B. Cisplatin resistance: Preclinical findings and clinical implications. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer l2010;1806: 172-182.

[64] Eastman A. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacology & Therapeutics l1987;34: 155-166.

[65] Siddik Z. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene l2003;22: 7265 - 7279.

[66] Ray PD, Huang B-W, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling l2012;24: 981-990.

[67] Basu A, Krishnamurthy S. Cellular responses to cisplatin-induced dna damage. Journal of Nucleic Accids l2010;2010: doi:10.4061/2010/201367.

[68] Donaldson KL, Goolsby GL, Wahl AF. Cytotoxicity of the anticancer agents cisplatin and taxol during cell proliferation and the cell cycle. International Journal of Cancer l1994;57: 847-855.

[69] Sorenson CM, Eastman A. Mechanism of *cis*-diamminedichloroplatinum(II)-induced cytotoxicity: role of G_2 arrest and DNA double-strand breaks. Cancer Research l1988;48: 4484-4488.

[70] Sorenson CM, Barry MA, Eastman A. Analysis of events associated with cell cycle arrest at G_2 phase and cell death induced by cisplatin. Journal of the National Cancer Institute l1990;82: 749-755.

[71] Wang X, Martindale JL, Holbrook NJ. Requirement for ERK Activation in Cisplatin-induced Apoptosis. Journal of Biological Chemistry l2000;275: 39435-39443.

[72] Cuadrado A, Lafarga V, Cheung PCF, Dolado I, Llanos S, Cohen P, Nebreda AR. A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. The EMBO Journal l2007;26: 2115-2126.

[73] Jones Emma V, Dickman Mark J, Whitmarsh Alan J. Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. Biochemical Journal l2007;405: 617-623.

[74] Tanida S, Mizoshita T, Ozeki K, Tsukamoto H, Kamiya T, Katoaoka H, Sakamuro D, Joh T. Mechanisms of cisplatin-induced apoptosis and of cisplatin sensitivity: Potential of BIN1 to act as a portent predictor of cisplatin sensitivity in gastric cancer treatment. International Journal of Surgical Oncology l2012;2012: Article ID 862879, 8 pages.

[75] Shen DW, Pouliot LM, Hall MD, Gottesman MM. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacological Reviews l2012;64: 706-721.

[76] Teicher BA, Herman TS, Holden SA, Wang Y, Pfeffer MR, Crawford JW, Frei III E. Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. Science l1990;247: 1457-1461.

[77] Kelland LR, Mistry P, Abel G, Loh SY, O'Neill CF, Murrer BA, Harrap KR. Mechanism-related Circumvention of Acquired cis-Diamminedichloroplatinum(II) Resistance Using Two Pairs of Human Ovarian Carcinoma Cell Lines by Ammine/Amine Platinum(IV) Dicarboxylates. Cancer Research l1992;52: 3857-3864.

[78] Howell SB, Safaei R, Larson CA, Sailor MJ. Copper Transporters and the Cellular Pharmacology of the Platinum-Containing Cancer Drugs. Molecular Pharmacology l2010;77: 887-894.

[79] Katano K, Kondo A, Safaei R, Holzer A, Samimi G, Mishima M, Kuo Y-M, Rochdi M, Howell SB. Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of cooper[1,2]. Cancer Research l2002;62: 6559-6565.

[80] Komatsu M, Sumizawa T, Mutoh M, Chen Z-S, Terada K, Furukawa T, Yang X-L, Gao H, Miura N, Sugiyama T, Akiyama S-i. Copper-transporting P-Type Adenosine Triphosphatase (ATP7B) Is Associated with Cisplatin Resistance. Cancer Research l2000;60: 1312-1316.

[81] Nakayama K, Kanzaki A, Ogawa K, Miyazaki K, Neamati N, Takebayashi Y. Copper-trasporting p-type adenosine triphosphatase (ATP7B) as a cisplatin based chemoresistance marker in ovarian carcinoma: comparative analysis with expression of MDR1, MRP1, MRP2, LRP, and BCRP. International Journal of Cancer l2002;101: 488-495.

[82] Jennerwein MM, Eastman A, Khokhar AR. The role of DNA repair in resistance of L1210 cells to isomeric 1,2-diaminocyclohexaneplatinum complexes and ultraviolet irradiation. Mutation Research l1991;254: 89-96.

[83] O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, Friend S, Fornace AJ, Kohn KW. Characterization of the p53 Tumor Suppressor Pathway in Cell Lines of the National Cancer Institute Anticancer Drug Screen and Correlations with the Growth-Inhibitory Potency of 123 Anticancer Agents. Cancer Research l1997;57: 4285-4300.

[84] Peng H-Q, Hogg D, Malkin D, Bailey D, Gallie BL, Bulbul M, Jewett M, Buchanan J, Goss PE. Mutations of the p53 Gene Do Not Occur in Testis Cancer. Cancer Research l1993;53: 3574-3578.

[85] Wong K-K, Izaguirre DI, Kwan SY, King ER, Deavers MT, Sood AK, Mok SC, Gershenson DM. Poor survival with wild-type *TP53* ovarian cancer? Gynecologic Oncology l2013;130: 565-569.

[86] Martinez-Rivera M, Siddik ZH. Resistance and gain of resistance phenotypes in cancers harboring wild-type p53. Biochemical Pharmacology l2012;83: 1049-1062.

[87] Brozovic A, Fritz G, Christmann M, Zisowsky J, Jaehde U, Osmak M, Kaina B. Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance. International Journal of Cancer l2004;112: 974-985.

[88] Deng X, Ewton DZ, Friedman E. Mirk/Dyrk1B Maintains the Viability of Quiescent Pancreatic Cancer Cells by Reducing Levels of Reactive Oxygen Species. Cancer Research l2009;69: 3317-3324.

[89] Wang X, Baek SJ, Eling TE. The diverse roles of nonsteroidal anti-inflammatory drug activated gene (NAG-1/GDF15) in cancer. Biochemical Pharmacology l2013;85: 597-606.

[90] Unsicker K, Spittau B, Krieglstein K. The multiple facets of the TGF-β family cytokine growth/differentiation factor-15/macrophage inhibitory cytokine-1. Cytokine & Growth Factor Reviews l2013.

[91] Mimeault M, Batra SK. Divergent molecular mechanisms underlying the pleiotropic functions of macrophage inhibitory cytokine-1 in cancer. Journal of Cellular Physiology l2010;224: 626-635.

[92] Eling TE, Baek SJ, Shim M, Lee CH. NSAID Activated Gene (NAG-1), a Modulator of Tumorigenesis. Journal of Biochemistry and Molecular Biology l2006;39: 649-655.

[93] Yokoyama-Kobayashi M, Saeki M, Sekine S, Kato S. Human cDNA Encoding a Novel TGF-β Superfamily Protein Highly Expressed in Placenta. Journal of Biochemistry l1997;122: 622-626.

[94] Paralkar VM, Vail AL, Grasser WA, Brown TA, Xu H, Vukicevic S, Ke HZ, Qi H, Owen TA, Thompson DD. Cloning and Characterization of a Novel Member of the Transforming Growth Factor-β/Bone Morphogenetic Protein Family. Journal of Biological Chemistry l1998;273: 13760-13767.

[95] Lawton LN, Bonaldo MdF, Jelenc PC, Qiu L, Baumes SA, Marcelino RA, de Jesus GM, Wellington S, Knowles JA, Warburton D, Brown S, Soares MB. Identification of a novel member of the TGF-beta superfamily highly expressed in human placenta. Gene l1997;203: 17-26.

[96] Hromas R, Hufford M, Sutton J, Xu D, Li Y, Lu L. PLAB, a novel placental bone morphogenetic protein. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression l1997;1354: 40-44.

[97] Min KW, Liggett JL, Silva G, Wu WW, Wang R, Shen RF, Eling TE, Baek SJ. NAG-1/GDF15 accumulates in the nucleus and modulates transcriptional regulation of the Smad pathway. Oncogene l2016;35: 377-388.

[98] Wakefield LM, Hill CS. Beyond TGF[beta]: roles of other TGF[beta] superfamily members in cancer. Nat Rev Cancer l2013;13: 328-341.

[99] Attisano L, Cárcamo J, Ventura F, Weis FMB, Massagué J, Wrana JL. Identification of human activin and TGFβ type I receptors that from heteromeric kinase complexes with type II receptors. Cell l1993;75: 671-680.

[100] Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, Brattain M, Willson JKV. Inactivation of the Type II TGF-β receptor in colon cancer cells with microsatellite instability. Science l1995;268: 1336-1338.

[101] Tan M, Wang Y, Guan K, Sun Y. PTGF-β, a type β transforming growth factor (TGF-β) superfamily member, is a p53 target gene that inhibits tumor cell growth via TGF-β signaling pathway. Proceedings of the National Academy of Sciences of the United States of America l2000;97: 109-114.

[102] Wiklund FE, Bennet AM, Magnusson PK, Eriksson UK, Lindmark F, Wu L, Yaghoutyfam N, Marquis CP, Stattin P, Pedersen NL, Adami HO, Gronberg H, Breit SN, Brown DA. Macrophage inhibitory cytokine-1 (MIC-1/GDF15): a new marker of all-cause mortality. Aging Cell l2010;9: 1057-64.

[103] Daniels LB, Clopton P, Laughlin GA, Maisel AS, Barret-Connor E. Growth-Differentiation Factor-15 is a robust, independent predictor of 11-year mortality risk in community -dwelling older adults: The Rancho Bernardo Study. Circulation l2011;123: 2101-2110.

[104] Brown DA, Breit SN, Buring J, Fairlie WD, Bauskin AR, Liu T, Ridker PM. Concentration in plasma of macrophage inhibitory cytokine-1 and risk of cardiovascular events in women: a nested case-control study. The Lancet l2002;359: 2159-2163.

[105] Kempf T, von Haehling S, Peter T, Allhoff T, Cicoira M, Doehner W, Ponikowski P, Filippatos GS, Rozentryt P, Drexler H, Anker SD, Wollert KC. Prognostic Utility of Growth Differentiation Factor-15 in Patients With Chronic Heart Failure. Journal of the American College of Cardiology l2007;50: 1054-1060.

[106] Corre J, Hebraud B, Bourin P. Concise review: growth differentiation factor 15 in pathology: a clinical role? Stem Cells Transl Med l2013;2: 946-52.

[107] Wang X, Kingsley PJ, Marnett LJ, Eling TE. The role of NAG-1/GDF15 in the inhibition of intestinal polyps in APC/Min mice by sulindac. Cancer Prev Res (Phila) l2011;4: 150-60.

[108] Koopmann J, Buckhaults P, Brown DA, Zahurak ML, Sato N, Fukushima N, Sokoll LJ, Chan DW, Yeo CJ, Hruban RH, Breit SN, Kinzler KW, Vogelstein B, Goggins M. Serum Macrophage Inhibitory Cytokine 1 as a Marker of Pancreatic and Other Periampullary Cancers. Clinical Cancer Research l2004;10: 2386-2392.

[109] Shnaper S, Desbaillets I, Brown DA, Murat A, Migliavacca E, Schluep M, Ostermann S, Hamou MF, Stupp R, Breit SN, de Tribolet N, Hegi ME. Elevated levels

of MIC-1/GDF15 in the cerebrospinal fluid of patients are associated with glioblastoma and worse outcome. Int J Cancer l2009;125: 2624-30.

[110] Ma J, Tang X, Sun W-w, Liu Y, Tan Y-r, Ma H-l, Zhu D-w, Wang M, Wang L-z, Li J, Tu Y-y, Zhang C-p, Zhang Z-y, Zhong L-p. Mutant GDF15 presents a poor prognostic outcome for patients with oral squamous cell carcinoma. Oncotarget l2016;7: 2113-2122.

[111] Wang J, Li W, Wang Y, Li C, Ding M, Zhang H, Lai M. The H6D genetic variation of GDF15 is associated with genesis, progress and prognosis in colorectal cancer. Pathology - Research and Practice l2015;211: 845-850.

[112] Baek SJ, Kim K-S, Nixon JB, Wilson LC, Eling TE. Cyclooxygenase Inhibitors Regulate the Expression of a TGF-β Superfamily Member That Has Proapoptotic and Antitumorigenic Activities. Molecular Pharmacology l2001;59: 901-908.

[113] Baek SJ, Okazaki R, Lee S-H, Martinez J, Kim J-S, Yamaguchi K, Mishina Y, Martin DW, Shoieb A, McEntee MD, Eling TE. Nonsteroidal anti-inflammatory drug-activated gene-1 over expression in transgenic mice suppresses intestinal neoplasia. Gastroenterology l2006;131: 1553-1560.

[114] Boyle GM, Pedley J, Martyn AC, Banducci KJ, Strutton GM, Brown DA, Breit SN, Parsons PG. Macrophage Inhibitory Cytokine-1 Is Overexpressed in Malignant Melanoma and Is Associated with Tumorigenicity. J Invest Dermatol l2008;129: 383-391.

[115] Lee DH, Yang Y, Lee SJ, Kim K-Y, Koo TH, Shin SM, Song KS, Lee YH, Kim Y-J, Lee JJ, Choi I, Lee J-H. Macrophage Inhibitory Cytokine-1 Induces the Invasiveness of Gastric Cancer Cells by Up-Regulating the Urokinase-type Plasminogen Activator System. Cancer Research l2003;63: 4648-4655.

[116] Senapati S, Rachagani S, Chaudhary K, Johansson SL, Singh RK, Batra SK. Overexpression of macrophage inhibitory cytokine-1 induces metastasis of human prostate cancer cells through the FAK-RhoA signaling pathway. Oncogene l2010;29: 1293-1302.

[117] Roth P, Junker M, Tritschler I, Mittelbronn M, Dombrowski Y, Breit SN, Tabatabai G, Wick W, Weller M, Wischhusen Jr. GDF-15 Contributes to Proliferation and Immune Escape of Malignant Gliomas. Clinical Cancer Research l2010;16: 3851-3859.

[118] Whiteside MA, Chen D-T, Desmond RA, Abdulkadir SA, Johanning GL. A novel time-course cDNA microarray analysis method identifies genes associated with the development of cisplatin resistance. Oncogene l2004;23: 744-752.

[119] Huang C-Y, Beer TM, Higano CS, True LD, Vessella R, Lange PH, Garzotto M, Nelson PS. Molecular Alterations in Prostate Carcinomas that Associate with In vivo Exposure to Chemotherapy: Identification of a Cytoprotective Mechanism Involving Growth Differentiation Factor 15. Clinical Cancer Research l2007;13: 5825-5833.

[120] Zhao L, Lee BY, Brown DA, Molloy MP, Marx GM, Pavlakis N, Boyer MJ, Stockler MR, Kaplan W, Breit SN, Sutherland RL, Henshall SM, Horvath LG. Identification of Candidate Biomarkers of Therapeutic Response to Docetaxel by Proteomic Profiling. Cancer Research l2009;69: 7696-7703.

[121] Staff AC, Bock AJ, Becker C, Kempf T, Wollert KC, Davidson B. Growth differentiation factor-15 as a prognostic biomarker in ovarian cancer. Gynecol Oncol l2010;118: 237-43.

[122] Bock AJ, Stavnes HT, Kempf T, Trope CG, Berner A, Davidson B, Staff AC. Expression and clinical role of growth differentiation factor-15 in ovarian carcinoma effusions. Int J Gynecol Cancer l2010;20: 1448-55.

[123] Integrated genomic characterization of endometrial carcinoma. Nature l2013;497: 67-73.

[124] Brun YF, Varma R, Hector SM, Pendyala L, Tummala R, Greco WR. Simultaneous modeling of concentration-effect and time-course patterns in gene expression data from microarrays. Cancer Genomics & Proteomics l2008;5.

[125] Weaver BA. How Taxol/paclitaxel kills the cells. Molecular Biology of the Cell l2014;25: 2677-2681.

[126] Cheng J-C, Chang H-M, Leung PCK. Wild-Type p53 Attenuates Cancer Cell Motility by Inducing Growth Differentiation Factor-15 Expression. Endocrinology l2011;152: 2987-2995.

[127] Su TT, O'Farrell PH. Size control: Cell proliferation does not equal growth. Current Biology l1998;8: R687-R689.

[128] Mattern J, Volm M. Imbalance of cell proliferation and apoptosis during progression of lung carcinoma. Anticancer Research l2004;24: 4242-4246.

[129] Meier JC, Haendler B, Seidel H, Groth P, Adams R, Ziegelbauer K, Kreft B, Beckmann G, Sommer A, Kopitz C. Knockdown of platinum-induced growth differentiation factor 15 abrogates p27-mediated tumor growth delay in the chemoresistant ovarian cancer model A2780cis. Cancer Medicine l2015;4: 253-267.

[130] Muscolini M, Montagni E, Caristi S, Nomura T, Kamada R, Di Agostino S, Corazzari M, Piacentini M, Blandino G, Costanzo A, Sakaguchi K, Tuosto L.

Characterization of a new cancer-associated mutant of p53 with a missense mutation (K351N) in the tetramerization domain. Cell Cycle l2009;8: 3396-3405.

[131] Muscolini M, Montagni E, Palermo V, Di Agostino S, Gu W, Abdelmoula-Souissi S, Mazzoni C, Blandino G, Tuosto L. The Cancer-associated K351N Mutation Affects the Ubiquitination and the Translocation to Mitochondria of p53 Protein. The Journal of Biological Chemistry l2011;286: 39693-39702.

[132] Skilling JS, Squatrito RC, Connor JP, Niemann T, Buller RE. p53 Gene Mutation Analysis and Antisense-Mediated Growth Inhibition of Human Ovarian Carcinoma Cell Lines. Gynecologic Oncology l1996;60: 72-80.

[133] Özdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu C-C, Simpson T, Laklai H, Sugimoto H, Kahlert C, Novitskiy SV, Acosta AD, Sharma P, Heidari P, Mahmood U, Chin L, Moses H, Weaver V, Maitra A, Allison JP, LeBleu VS, Kalluri R. Depletion of Carcinoma-Associated Fibroblasts and Fibrosis Induces Immunosuppression and Accelerates Pancreas Cancer with Diminished Survival. Cancer cell l2014;25: 719-734.

[134] Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, Dekleva EN, Saunders T, Becerra CP, Tattersall IW, Westphalen CB, Kitajewski J, Fernandez-Barrena MG, Fernandez-Zapico ME, Iacobuzzio-Donahue C, Olive KP, Stanger BZ. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. Cancer cell l2014;25: 735-747.

[135] Bruzzese F, Hagglof C, Leone A, Sjoberg E, Roca MS, Kiflemariam S, Sjoblom T, Hammarsten P, Egevad L, Bergh A, Ostman A, Budillon A, Augsten M. Local and systemic protumorigenic effects of cancer-associated fibroblast-derived GDF15. Cancer Res l2014;74: 3408-17.

[136] Corre J, Labat E, Espagnolle N, Hebraud B, Avet-Loiseau H, Roussel M, Huynh A, Gadelorge M, Cordelier P, Klein B, Moreau P, Facon T, Fournie JJ, Attal M, Bourin P. Bioactivity and prognostic significance of growth differentiation factor GDF15 secreted by bone marrow mesenchymal stem cells in multiple myeloma. Cancer Res l2012;72: 1395-406.

[137] Urakawa N, Utsunomiya S, Nishio M, Shigeoka M, Takase N, Arai N, Kakeji Y, Koma Y-i, Yokozaki H. GDF15 derived from both tumor-associated macrophages and esophageal squamous cell carcinomas contributes to tumor progression via Akt and Erk pathways. Lab Invest l2015;95: 491-503.

[138] Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev Cancer l2009;9: 665-674.

[139] Omabe M, Ezeani M, Omabe KN. Lipid metabolism and cancer progression: The missing target in metastatic cancer treatment. Journal of Applied Biomedicine l2015;13: 47-59.

[140] Krueger KE, Srivastava S. Posttranslational Protein Modifications: Current Implications for Cancer Detection, Prevention, and Therapeutics. Molecular & Cellular Proteomics l2006;5: 1799-1810.

[141] Burger AM, Seth AK. The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications. European Journal of Cancer l2004;40: 2217-2229.

[142] Kaneko-Goto T, Sato Y, Katada S, Kinameri E, Yoshihara S-i, Nishiyori A, Kimura M, Fujita H, Touhara K, Reed RR, Yoshihara Y. Goofy Coordinates the Acuity of Olfactory Signaling. The Journal of Neuroscience l2013;33: 12987-12996.

[143] Miyake T, Ueda Y, Matsuzaki S, Miyatake T, Yoshino K, Fujita M, Nomura T, Enomoto T, Kimura T. CRABP1-reduced expression is associated with poorer

prognosis in serous and clear cell ovarian adenocarcinoma. Journal of Cancer Research and Clinical Oncology l2010;137: 715-722.

[144] Griner SE, Joshi JP, Nahta R. Growth differentiation factor 15 stimulates rapamycin-sensitive ovarian cancer cell growth and invasion. Biochem Pharmacol l2012.

[145] F. LR, Wu W, Chen LF, Zhu XQ, Zhang SY. Growth differentiation factor 15 inhibits elevated expression of matrix metalloproteinase 1 induced by pooled serum in patients with coronary artery ecstasia. Zhong Yi Xue Ke Xue Yuan Xue Bao l2015;37: 520-527.

[146] Yeung TL, Leung CS, Wong KK, Samimi G, Thompson MS, Liu J, Zaid TM, Ghosh S, Birrer MJ, Mok S. TGF-β modulates ovarian cancer invasion by upregulating CAF-derived versican in the tumor microenvironment. Cancer Research l2013;73: 5016-5028.

[147] Augsten M. Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. Frontiers in Oncology l2014;4.

Vita

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