

**Tumor-Derived Exosomes Mediate Platinum-Resistance in Ovarian Cancer
Through Regulation of Epithelial to Mesenchymal Transition**

By

Jennifer M. Crow

Submitted to the graduate degree program in Pathology and Laboratory Medicine and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Andrew K. Godwin, Ph.D.

Dr. Adam Krieg, Ph.D.

Dr. Nikki Cheng, Ph.D.

Dr. Fariba Behbod, Ph.D.

Dr. Lane Christenson, Ph.D.

Date Defended: 26 May 2016

The Dissertation Committee for Jennifer M. Crow
certifies that this is the approved version of the following dissertation:

**Tumor-Derived Exosomes Mediate Platinum-Resistance in Ovarian Cancer
Through Regulation of Epithelial to Mesenchymal Transition**

Chairperson: Andrew K Godwin, PhD

Date approved: 13 June 2016

ABSTRACT:

Nano-sized vesicles, termed exosomes, have been implicated in the transfer of oncogenic proteins and genetic material from one cell to another. We speculated this may be one mechanism by which an intrinsically platinum-resistant population of epithelial ovarian cancer (EOC) cells imparts its influence on surrounding tumor cells. To explore this possibility we have utilized the platinum-sensitive A2780 cell line and independent platinum-resistant derivatives, *e.g.*, CP70 and C30, as well as a non-related platinum-resistant cell line, OVCAR10. We find A2780 cells treated with exosomes derived from highly resistant cells demonstrate up to a ~2-fold increase ($p < 0.05$) in resistance to carboplatin as compared to treatment with isolated autologous exosomes. Importantly, this exosome-associated phenotype is stable and associated with increased epithelial to mesenchymal transition (EMT) characteristics. In addition, we identified previously unreported somatic mutations in the *Mothers Against Decapentaplegic Homolog 4* (*SMAD4*), only in cells (OVCAR10, C30, and CP70) that demonstrated robust acquired resistance after platinum therapy. Cells displaying mutations in *SMAD4* exhibited significant changes in EMT-related markers following treatment with carboplatin. Interestingly, exosomes derived from A2780 cells engineered to exogenously express specific *SMAD4* mutations resulted in ~1.7-fold ($p < 0.05$) increase in resistance as compared to exosomes isolated from exogenous wildtype *SMAD4* (*SMAD4*^{WT}) expressing A2780 cells, suggesting these mutations are contributing to the development of a resistant phenotype. Additionally, cells expressing mutations in *SMAD4* exhibit a loss of phosphorylation of SMAD2 but retain activated SMAD3, which is important for EMT. Importantly, inhibition of SMAD3 via the small

molecule inhibitor, SIS3, reversed the EMT phenotype and acted synergistically with carboplatin to enhance cell death. Lastly, we identified a clinically relevant inhibitor of SMAD3, Eribulin Mesylate (Halaven®), which also acted synergistically with carboplatin *in vitro*. Altogether, our findings provide the first evidence that ovarian tumor cells use exosomes as a vehicle to achieve tumor cell-cell crosstalk and this exchange advantageously impacts the recipient cells response to platinum. We continue to describe a novel mechanism of action by which an EMT phenotype is perpetuated via exchange of tumor-derived exosomes, ultimately leading to the development of a subpopulation of chemotherapy refractory cells. Importantly, we present a novel therapeutic strategy that targets resistant cells and ‘tricks’ them into responding once again to the most effective therapeutics to date, cisplatin and carboplatin.

ACKNOWLEDGEMENTS

There are several key persons who helped to make this work possible. First I would like to thank my mentor, Dr. Andrew K. Godwin who provided me with the intellectual freedom to pursue my interests, the support to make this work possible, and the constant challenges which drove me to become a better scientist each day.

I would also like to acknowledge my fellow lab mates, both past and present, each of whom has had a direct impact on helping to shape the course of this work. Most especially, I would like to thank Dr. Safinur Atay, for sharing her love of exosomes, Dr. Thuy Vy Do, for her guidance as I embarked on adventures in TGF- β /SMAD signaling, Dr. Harsh Pathak, for his constant words of encouragement and advice, and Mrs. Susan Ard, for being my 'lab mom'.

Most importantly I would like to thank the love and support of my husband, Dr. Joseph Crow and our children, Alice and Jack. My family has inspired me, fed me, and given me unconditional love (and willing agreed to numerous family trips to the lab on Saturday and Sunday afternoons).

DEDICATION

This work is dedicated to my grandfather, Dr. Aloysius C. Gerst, M.D., who once wrote me a letter advising me to pursue biology as my career. While he is no longer physically on this earth I know he is here and is happy that I FINALLY got around to taking his advice.

TABLE OF CONTENTS

CHAPTER 1- OVARIAN CANCER.....	1
EPIDEMIOLOGY.....	2
RISK FACTORS.....	2
SCREENING AND DETECTION.....	7
OVARIAN CANCER HISTOLOGY AND SUBTYPES.....	8
STAGING.....	15
TREATMENT.....	16
PLATINUM-BASED CHEMOTHERAPY.....	21
METHODS OF PLATINUM-RESISTANCE.....	25
CHAPTER 2- EXOSOMES.....	28
OVERVIEW.....	29
HISTORY.....	30
EXOSOME BIOGENESIS.....	31
EXOSOMES IN CANCER.....	35
ONCOGENIC TRANSFORMATION AND TUMOR FORMATION.....	36
ANGIOGENESIS.....	43
NICHE FORMATION, METASTASIS.....	44
IMMUNE EVASION.....	45
CHEMOTHERAPY EVASION.....	47
THERAPEUTIC USE.....	50
BIOMARKERS.....	52
CHAPTER 3 - EXOSOMES AS MEDIATORS OF PLATINUM RESISTANCE IN OVARIAN CANCER	57
INTRODUCTION.....	58
MATERIALS AND METHODS.....	60
RESULTS.....	67
DISCUSSION.....	76
CHAPTER 4 - TARGETING TGF-B/SMAD SIGNALING TO OVERCOME PLATINUM-RESISTANCE IN OVARIAN CANCER.....	100

INTRODUCTION	101
MATERIALS AND METHODS	103
RESULTS	106
DISCUSSION.....	112
CHAPTER 5 - CONCLUSIONS AND FUTURE DIRECTIONS	127
CONCLUSIONS.....	128
FUTURE DIRECTIONS	131

TABLES

Table 1.01	FIGO Staging for Ovarian Cancer	15
Table 2.01	Exosomal Content Associated with Tumorigenesis	41
Table 2.02	Exosomal miRNAs as Biomarkers.....	55
Table 3.01	EOC Cell Line Carboplatin IC ₅₀ Values	80
Table 3.02	Mutations Identified With the TruSeq Amplicon Cancer Panel	81
Table 4.01	Additional Mutations in Platinum-Resistant EOC Cell Lines	114

FIGURES

Figure 1.01 The Origins of Ovarian Cancer.....	13
Figure 1.02 Genetic Dysregulation in High Grade Serous Ovarian Cancer.....	14
Figure 1.03 Chemotherapeutic Advancements in Ovarian Cancer.	20
Figure 1.04 Platinum-Based Chemotherapeutics and Traditional Mechanisms of Drug Resistance.	24
Figure 2.01 Exosome Biogenesis in Cancer.	34
Figure 2.02 Exosome-Mediated Effects on Recipient Cells.....	37
Figure 2.03 Tumor-Derived Exosomes Contribute To Cancer Development and Progression.	49
Figure 3.01 Characterization of EOC Derived Exosomes.	82
Figure 3.02 A2780 Cell Uptake of A2780-Derived Exosomes.....	83
Figure 3.03 A2780 Cell Uptake of CP70-Derived Exosomes.	84
Figure 3.04 A2780 Cell Uptake of C30-Derived Exosomes.....	85
Figure 3.05 Exosome Transfer Corresponds with a Loss of Sensitivity to Platinum.....	86
Figure 3.06 Transfer of a Platinum-Resistant Phenotype Extends to Other EOC Cells	87
Figure 3.07 Extended Effects of Exosome Uptake.....	88
Figure 3.08 A2780 Cells Have Enhanced Exosome-Mediated Pt Export with Prior Exposure to CP70-Exosomes.	89
Figure 3.09 A2780 Cells Undergo EMT When Treated with Platinum-Resistant Exosomes.	90
Figure 3.10 Platinum-Resistant EOC Cells Have Altered miRNA21 Expression.....	91

Figure 3.11 A2780 Cells Exhibit a Mesenchymal Phenotype after Exposure to Platinum-Resistant Exosomes.	92
Figure 3.12 The TGF- β /SMAD Signaling Pathway. Schematic representation of TGF- β /SMAD Signaling.	93
Figure 3.13 SMAD4 Components Within Exosomal Samples.	94
Figure 3.14 Mutations in SMAD4 are Associated with Platinum Resistance.	95
Figure 3.15 Validation of Mutant Plasmids.	96
Figure 3.16 Mutations in SMAD4 Contribute to Platinum Resistance and EMT.	97
Figure 3.17 A2780 ^{MUT} Cells Undergo EMT in Response to Carboplatin.	98
Figure 3.18 Exosomes from Mutant SMAD4 Cell Lines Transfer Platinum Resistance.	99
Figure 4.01 SMAD4 Alterations Across All TCGA Cancers.	115
Figure 4.02 Dysregulation of TGF- β /SMAD Signaling Components in TCGA Samples.	116
Figure 4.03 Dysregulation of miR-21 in TCGA Samples.	117
Figure 4.04 Dysregulation of SMAD3 impacts Overall Survival.	118
Figure 4.05 SMAD4 is Present in EOC Cell Lines.	119
Figure 4.06 Loss of SMAD2 Activation Corresponds with a Loss of Sensitivity to Platinum.	120
Figure 4.07 Loss of SMAD2 Expression Corresponds with a Platinum-Resistant Phenotype.	121
Figure 4.08 Working Model.	122

Figure 4.09..Combination of SMAD4 inhibition with Carboplatin Enhances Cell Death	123
Figure 4.10 SIS3 Inhibits Phosphorylation of SMAD3 and Triggers EMT.....	124
Figure 4.11 Synergistic Effects of SIS3 and Carboplatin.....	125
Figure 4.12 Combination Therapy of Eribulin and Carboplatin is Synergistic.....	126

CHAPTER 1- OVARIAN CANCER

EPIDEMIOLOGY

Ovarian cancer is the 5th leading cause of death in women and the primary cause of mortality in gynecological malignancies due to a dearth of treatment options coupled with late Stage detection and diagnosis. The American Cancer Society (ACS) predicts that ~22,280 women will be diagnosed with ovarian cancer and ~14,240 women will die from the disease alone in 2016. Unfortunately, the incidence rate of mortality for ovarian cancer has not significantly changed in the past 30 years. This trend is largely due to the fact that ovarian cancer manifests with non-specific clinical symptoms and is often diagnosed at Stage III or IV when the 5 year survival rate is less than 25% (Luvero, Milani et al. 2014). Symptoms of ovarian cancer include frequent urination, bloating, early satiety, and fatigue, all of which are common to a variety of ailments including gastrointestinal disorders and changes in the female reproductive cycle. Given that a majority of ovarian cancer cases are diagnosed in women over the age of 45, (with the most common age being 65), ovarian cancer is frequently misdiagnosed as early menopause and, often, women will not be screened for ovarian cancer until months after initial complaints (Babst, Katzmann et al. 2002; Babst, Katzmann et al. 2002).

RISK FACTORS

The chance of an average healthy women developing ovarian cancer is relatively low (1 in 70) as compared to other cancers (*i.e.*, 1 in 8 in breast cancer) (Siegel, Ma et al.

2014). However, the presence of one or more risk factors can increase this rate. A lifetime risk of developing cancer, (ovarian cancer included), increases with tobacco use, poor diet, obesity, and advanced age (McLemore, Miaskowski et al. 2009). For ovarian cancer specifically, age is the most significant risk factor, with the highest incidence occurring in women between the ages of 60 and 64. In fact, women over the age of 65 have up to a 5 times greater chance of developing ovarian cancer as compared to women under the age of 65 (Howlader N 2015). Given this statistic, it naturally follows that women who experience a greater number of ovulations in their lifetime have an increased chance of developing ovarian cancer over women with reduced ovulations, (*i.e.*, by means such as oral contraceptive use, multiple births, breastfeeding, and surgical procedures which include removal one or both ovaries such as oophorectomy) (Fathalla 1971; Sundar, Neal et al. 2015). The follicular rupture generated at each ovulation is repaired by surface epithelial cells through several rounds of replication. Godwin and colleagues were the first to demonstrate, *in vitro*, that this incessant replication of epithelial cells resulted in genetic and phenotypic changes typically seen during tumorigenesis, including a loss of contact independent growth, increased ability to form tumors in mice, and increased chromosomal abnormalities (Godwin, Testa et al. 1992). Outside of the ovary, additional research has shown that persistent chronic inflammation, as seen in cases of endometriosis, and inflammation, caused by talc and asbestos, correlates with an elevated risk of developing ovarian cancer (Sundar, Neal et al. 2015). Notably, studies that evaluated the effects of non-steroidal anti-inflammatory drugs (NSAIDs) and ovarian cancer incidence reported an

inverse correlation between NSAID use and ovarian cancer diagnosis (2014; Baandrup 2015) suggesting a potential preventative strategy.

The most direct risk factors for ovarian cancer (outside of age) are based on family history and genetics (CSSOCR 2016). Women with one or more close blood relatives (mother, sister, aunt), whom have been diagnosed with ovarian cancer, have ~3 times greater risk of developing the disease (ASCO 2015). In addition to ovarian cancer, the diagnosis of breast or colorectal cancers in close family members or oneself also increases ovarian cancer risk (ASCO 2015; ACS 2016). Most of these inherited cases of ovarian cancer (termed hereditary ovarian cancer), are linked to hereditary breast-ovarian cancer syndrome or Lynch syndrome (Lynch, Casey et al. 2009). Hereditary breast ovarian cancer syndrome is characterized by mutations in the tumor suppressor genes known as Breast Cancer Susceptibility (*BRCA*) 1 or *BRCA2* (Rebbeck, Mitra et al. 2015; ACS 2016) *BRCA1/2* mutations were first associated with an increased risk of breast cancer (Casey, Plummer et al. 1993), but more recently have been linked to tumor occurrences in ovarian, fallopian tube cancer, peritoneal cancer, prostate cancer, and pancreatic cancer (Brose, Rebbeck et al. 2002; Levy-Lahad and Friedman 2007; Ferrone, Levine et al. 2009; Mavaddat, Peock et al. 2013). While mutations in both *BRCA1* and *BRCA2* have been shown to affect ovarian cancer risk, there is some disagreement regarding the individual effects of mutations in either *BRCA1* or *BRCA2* on risk profiles. The ACS reports that the lifetime ovarian cancer risk for women with a mutation in *BRCA1* is between 35%-70% and 10%-30% for women with mutations in *BRCA2* (ACS 2016). Alternately, the Consortium of Investigators of Modifiers of

BRCA1/2 (CIMBA) reported that out of 19,581 *BRCA1* mutation carriers and 11,900 *BRCA2* mutation carriers (spanning 55 centers in 33 countries), 5% (1,041) of *BRCA1* mutant carriers and 6% (682) of *BRCA2* mutant carriers were diagnosed with ovarian cancer suggesting that mutations in either of these genes may correspond near equally with risk status (Mavaddat, Barrowdale et al. 2012; Rebbeck, Mitra et al. 2015). One possibility for the variability observed in the ACS report is that it was a summary of many studies with a variety of inclusion/exclusion factors. In fact, the society notes that their reported rates vary and increase with age. Additionally, other studies have demonstrated that *BRCA1/2* mutations can vary with tumor type (Mavaddat, Barrowdale et al. 2012). For these reasons, the CIMBA studies may be more accurate in relating *BRCA1/2* mutations with overall risk of being diagnosed with ovarian cancer. It is important to note; however, that while presence of mutations in the *BRCA* genes increases risk, the actual percentage of women diagnosed with ovarian cancer who carry mutations in *BRCA1/2* is low (5-15%) and therefore, is not an inclusive marker for the disease (Swisher, Sakai et al. 2008; Fong, Yap et al. 2010)

Mutations in genes associated with autosomal dominant hereditary Lynch syndrome (formally known as hereditary nonpolyposis colorectal cancer), make up the remaining hereditary ovarian cancers (Lynch, Casey et al. 2009). Lynch syndrome is characterized by mutations in DNA mismatch repair genes such as MutL Homolog 1 (*MLH1*) and MutS Homolog 2 (*MSH2*) (Lynch and Lynch 1979). In the vast majority of these cases, a single mutated allele is inherited and the second allele is lost via somatic mutation, methylation, or both (Toss, Tomasello et al. 2015) Women with mutations in

this panel of genes have up to a 12 times increased risk of developing ovarian cancer depending upon ancestry (CSSOCR 2016). Importantly, the average age of diagnosis of ovarian cancer in women with a family history of Lynch syndrome, especially the *MSH2* mutation type, is at least 10 years earlier than the average population, with mean ages of diagnosis between 45 and 51 years of age (compared to 60-64 years for non-Lynch associated ovarian cancers), as reviewed in Lynch and Godwin *et al* (Lynch, Casey et al. 2009). Despite this discrepancy in the age of diagnosis, overall survival is not significantly different between Lynch and non-Lynch groups. Analysis of a small study involving 27 patients on the Dutch Lynch syndrome registry and 52 sporadic ovarian cancer cases reported cumulative 5- year survival rates of 64.2 and 58.1 respectively (Crijnen, Janssen-Heijnen et al. 2005).

The ability to detect and assess risk based upon these above factors, especially the discussed genetic factors, has empowered women considered to be at high risk to more confidently make life changing decisions which have the potential to significantly lower their chances of developing ovarian cancer. For example, women with a high risk of developing the disease may choose to undergo selective surgeries such as oophorectomy, hysterectomy, tubal ligation and bilateral Salpingo-oophorectomy (BSO) procedures which can reduce their chance of developing ovarian cancer by as much as 90% (in pre-menopausal women) and 50% (in post-menopausal women) (Domchek, Friebel et al. 2010). These more extreme preventative strategies are considered for women at high risk primarily because of a lack of ability to detect the disease at an early stage, when the overall survival is above 90%.

SCREENING AND DETECTION

Currently, there is no routine screening procedure available to directly detect ovarian cancer in the general population akin to mammograms for the detection of breast cancer and the pap smear for the detection of cervical cancer. Women who have one or more of the above risk factors and/or experience symptoms for more than two weeks should undergo further testing. Common diagnostic tests for ovarian cancer include pelvic exam, transvaginal ultrasound (TVUS), and evaluating serum levels of cancer antigen 125 (CA125) (Jacobs, Menon et al. 2016; Terry, Schock et al. 2016). A pelvic exam is a simple, in-clinic procedure, which involves a physician physically feeling for a mass. TVUS uses ultrasound waves to detect abnormal tissue masses and fluid filled cysts on the ovaries, uterus, fallopian tubes, and other areas of the pelvic and abdominal cavities. This technique can detect smaller abnormalities, which can be missed in the pelvic exam. CA125 is a membrane bound protein which is expressed on the surface of cells that undergo differentiation into a Müllerian-type epithelium and can be released as a soluble form into bodily fluids (Bischof, Tseng et al. 1986). Women with elevated (> 35 units/mL) of CA125 are considered to be at a higher risk of having ovarian cancer (Eagle and Ledermann 1997). However, CA125 is also elevated in other malignant and benign conditions such as breast cancer (Berruti, Tampellini et al. 1994) and endometriosis (Pittaway and Fayez 1986) which makes diagnosis based on CA125 levels impossible. In addition, in a recent longitudinal study evaluating potential biomarkers for ovarian cancer, the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, researchers found little significance for using CA125, or one of

4 other promising markers for the detection of early Stage (Stages I and II) ovarian cancer (Terry, Schock et al. 2016). Likewise, the UK collaborative Trial of Ovarian Cancer Screening (UKCTOCS) recently conducted one of the largest ever randomized control trials investigating the effects of early disease detection, via annual CA125 screening coupled with risk algorithm software to detect trends, transvaginal ultrasound, or no screening, on ovarian cancer mortality in post-menopausal women (ages 50-74yrs). Sadly, in the primary analysis of this study involving 202,638 women, there was little to no significant impact on detection of ovarian cancer between screening methods. While the authors suggest that, upon further scrutiny of the data, there may be more significant differences in detection of ovarian cancer; there was still little impact on overall survival of women diagnosed with ovarian cancer (Jacobs, Menon et al. 2016).

Despite the combination of risk factors and screening modalities, a formal diagnosis of ovarian cancer is not made until a biopsy is performed on tissue taken from the initial cytoreductive or debulking surgeries, at which point the tumor can be classified by Type and Stage. Both Type and Stage are important factors for predicting overall survival and determining the best course of treatment.

OVARIAN CANCER HISTOLOGY AND SUBTYPES

The term ovarian cancer is an umbrella statement, which actually covers a multitude of diseases sharing common occurrences in and/or on the ovary. One such reason for this disease heterogeneity is the lack of a specific cell of origin. Ovarian cancer is

traditionally believed to arise from three different populations of cells; germ cells, gonadal-stromal cells, or the surface epithelium of the ovary. Current evidence suggests that many ovarian cancers may not be of ovarian origin at all and may, in fact, arise from the neoplastic spread of cells out of areas such as the endometrium and/or fallopian tubes (Kurman and Shih le 2010). Regardless of source, approximately 90% of ovarian cancers are of epithelial origin and are collectively termed epithelial ovarian cancer (EOC) and these are the focus of our research. EOC tumors are classified into Types based upon clinical behavior and genetic abnormalities (Kurman and Shih le 2010). Type I tumors are characterized by a step wise evolution and are diagnosed at a range from benign to malignant whereas Type II tumors are fast growing and aggressive and are almost always diagnosed at a high Stage (Kurman and Shih le 2010). Ovarian tumors are further classified into 4 main subtypes, mucinous, clear cell, endometrioid, and serous (McCluggage 2011). These subtypes are similar to their counterparts in normal tissues in that they display a shared morphology as well as similar patterns of gene expression. For example, *TP53* alterations are common to serous tumors and tissue within normal fallopian tube. Likewise, studies from Marquez et al demonstrated that, using a 63,000 probes set to correlated genetic signatures between 50 ovarian cancers of different histological subtypes, and corresponding normal tissue controls demonstrated significant shared alterations between mucinous cancers with those in normal colonic mucosa, and both endometrioid and clear cell subtypes with normal endometrium (Marquez, Baggerly et al. 2005) That being said, Of the subtypes of ovarian cancer, mucinous makes up the smallest percent (~3%) followed by clear cell and endometrioid, both of which occur with an approximate

frequency of 7-10% (McCluggage 2011). The vast majority of EOCs are serous (~70%) which are further divided into either high grade serous (HGS) or low grade serous (LGS) with HGS being the most common (~70%), and the most deadly.

Almost half of all endometrioid and clear cell carcinomas are diagnosed as a Stage I (Anglesio, Carey et al. 2011), which is a positive statistic given that these types of tumors are difficult to treat. The 5-year survival rate for women diagnosed with either endometrioid or clear cell carcinoma at higher stages is poor (Seidman, Horkayne-Szakaly et al. 2004; Storey, Rush et al. 2008). Mucinous carcinoma is not only the least common type of tumor, it is now debated that many of these tumors are actually metastasis from other sites such as the gastrointestinal and biliary tracts (Lee and Young 2003). Like endometrioid and clear cell, the prognosis of women diagnosed with mucinous is largely dependent upon the Stage of the tumor. Tumors diagnosed at an early Stage have excellent prognosis but this quickly drops with diagnosis at more advanced Stages. LGS are relatively uncommon; however, these types of tumors tend to be slower growing and therefore more chemo-resistant (Gourley, Farley et al. 2014). LGS tumors typically harbor a mutation in either, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), or *ERBB2* (Vang, Shih le et al. 2009). Each of these genes encodes for upstream regulators of mitogen-activated protein kinase (*MAPK*) and mutations in these genes leads to constitutive activation of the pathway resulting in uncontrolled proliferation (Vang, Shih le et al. 2009). Like mucinous, clear cell, and endometrioid, LGS tumors are generally less responsive to traditional platinum and taxane-based chemotherapy;

however, they are rarely diagnosed at an early stage, and therefore, women diagnosed with LGS carcinoma have a poor prognosis. Similar to LGS, HGS ovarian cancer is most commonly diagnosed at a late Stage when a complete resection of the tumor is difficult. In fact, less than 5% of HGS cancers are diagnosed at a Stage 1 (when the tumor is confined to the ovaries). Recently, two novel hypotheses for the pathogenesis of HGS ovarian cancer have been proposed. In the first mechanism, ovarian cancer precursors develop in the fimbria from occult serous tubal intraepithelial carcinoma (STIC), prior to metastasis to the ovary. The second theory describes genetic alterations occurring within the normal ovarian surface epithelium or inclusion cysts which either proceed via a high grade pathway with no perceivable intermediate histology or a low grade pathway encompassing several, benign and non-invasive steps (**Figure 1.01** – courtesy of Dr. Andrew K. Godwin). HGS tumors lack mutations in *KRAS*, *BRAF*, and *ERBB2*, but almost always contain mutations in the tumor suppressor gene *TP53* (Vang, Shih le et al. 2009). In addition to mutations in *TP53*, data from the TCGA and other published studies have shown that HGS is characterized by numerous and inconsistent somatic mutations, DNA copy number alterations, and up-regulation and down-regulation of a multitude of genes. To provide an example of this, I utilized data available through The Cancer Genome Atlas (TCGA) to demonstrate genetic aberrations within 34 common cell cycle control genes from 316 HGS ovarian cases with complete mutation, copy number alteration, and mRNA data (2011) (**Figure 1.02**). While some alterations were fairly consistent across patient samples (such as up-regulation or amplification of *MYC* in ~30% of cases, down-regulation of *RBL2* in ~25% of cases, and up-regulation or amplification of *CCNE1* in ~20% of cases) the

remaining 31 queried genes had between 3-29% alteration rates of which there was little discernable pattern. As a comparison, *TP53* is shown to be altered in >95% of cases. Examples such as this demonstrate just how difficult high-grade EOC is to treat with single molecularly-targeted therapies (Singer, Kurman et al. 2002; Salani, Kurman et al. 2008). To add to this hurdle, while HGS tumors are initially responsive to platinum-chemotherapy, recurrent tumors become resistant quickly and treatment options are limited for women with platinum-resistant disease.

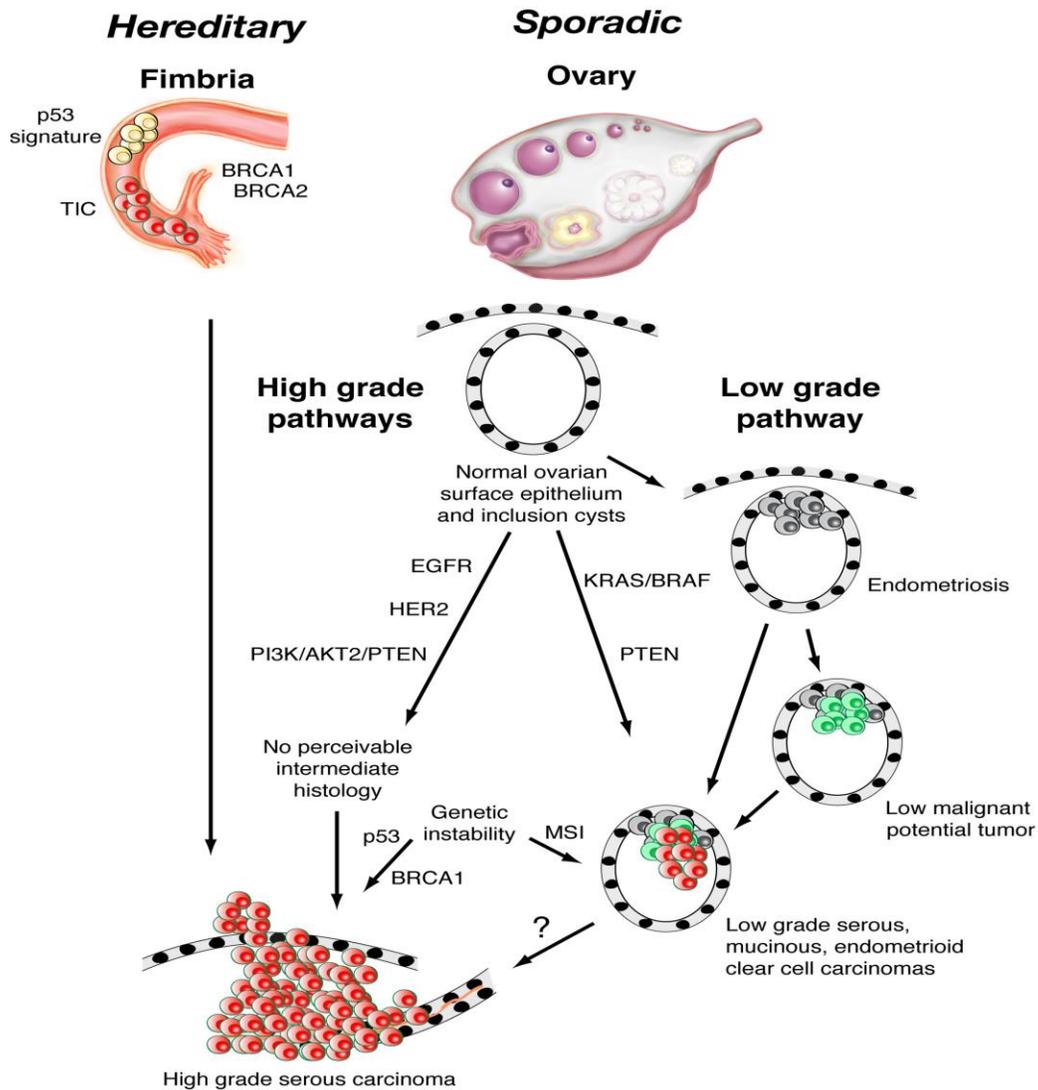


Figure 1.01 The Origins of Ovarian Cancer. Schematic representation of the prevailing theories behind ovarian cancer development. Hereditary EOC is largely associated with genetic abnormalities found within the fimbria which lead to development of tubal intraepithelial carcinoma capable of metastasizing to the ovary. The sporadic route involves a multi-step process and can involve the loss of common tumor suppressor genes leading to genetic instability. This path is characterized by stages of benign and no-invasive pathologies. Figure kindly reprinted with permission from Dr. Andrew K. Godwin ([Lynch, Casey et al. 2009](#))

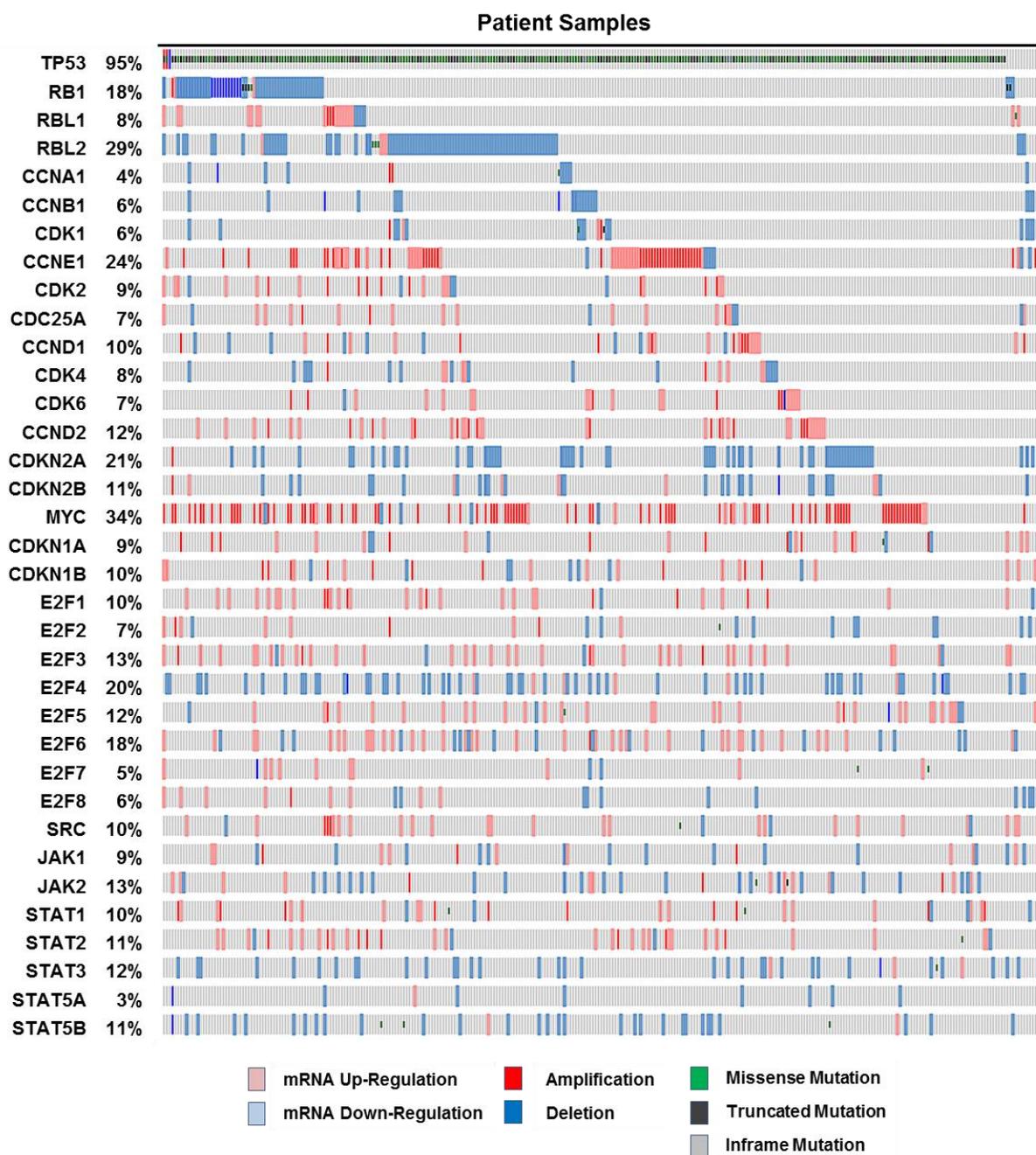


Figure 1.02 Genetic Dysregulation in High Grade Serous Ovarian Cancer. Data from the TCAG showing mutation, copy number alteration, and mRNA dysregulation of 34 cell cycle control genes and TP53 alteration status (as a comparison) within 316 cases of high grade serous ovarian cancer demonstrates the overall heterogeneity of the disease.

STAGING

The International Federation of Gynecology and Obstetrics (FIGO) staging system (Table 1.1) (Prat 2015) was recently updated as of January 1st 2014 to more fully encompass the importance of correctly identifying the extent of cancer spread. It is used by clinicians, in conjunction with histology and identification of origin (when possible) to select the most appropriate treatment.

Table 1.01 FIGO Staging for Ovarian Cancer

Stage I: Tumor Confined to Ovaries

IA	Tumor limited to 1 ovary, capsule intact, no tumor on surface, negative washings	
IB	Tumor involves both ovaries + IA	
IC	Tumor limited to 1 or both ovaries	
	IC1	Surgical Spill
	IC2	Capsule rupture before surgery or tumor on ovarian surface
	IC3	Malignant cells in the ascites or peritoneal washings

Stage II: Tumor involves 1 or both ovaries with pelvic extension (below the pelvic brim) or primary peritoneal cancer

IIA	Extension and /or implant on uterus and/or fallopian tubes
IIB	Extension to other pelvic intraperitoneal tissues

Stage III: Tumor involves 1 or both ovaries with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes

IIIA	(Positive retroperitoneal lymph nodes and/or microscopic metastasis beyond the pelvis)	
	IIIA1	Positive retroperitoneal lymph nodes only
	IIIA2	Microscopic, extrapelvic (Above the brim) peritoneal involvement +/- positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen
IIIB	Macroscopic, extrapelvic, peritoneal metastasis <= 2 cm +/- positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen	
IIIC	Macroscopic, extrapelvic, peritoneal metastasis > 2 cm +/- positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen	

Stage IV: Distant metastasis excluding peritoneal metastasis

IVA	Pleural effusion with positive cytology
IVB	Hepatic and/or splenic parenchymal metastasis, metastasis to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

TREATMENT

The primary treatment for high grade serous EOC is surgical de-bulking followed by platinum-based chemotherapy. The primary goals of surgical intervention are; i) to establish a diagnosis, ii) stage the tumor, and iii) remove as much of the tumor burden as possible (Coleman, Monk et al. 2013). Optimal surgical cytoreduction defined as the removal of all visible tumors and is based upon studies demonstrating the OS benefit of removal of all tumors <1.0 cm in diameter (Bristow, Tomacruz et al. 2002; du Bois, Reuss et al. 2009). Standard of care is surgery followed by chemotherapy; however, the concept of neoadjuvant chemotherapy has been discussed. In an analysis of the European Organization for Research and Treatment of Cancer (EORTC) 55971 trial in which 670 Stage III or IV ovarian cancer patients were randomly assigned to neoadjuvant chemotherapy or surgery without neoadjuvant treatment there was little significant difference in overall survival. However, analysis of the trial results observed trends which suggest that women with stage III disease and tumors <45 mm may benefit more from primary surgery whereas women with stage IV disease and tumor >45 mm may benefit from neoadjuvant chemotherapy (van Meurs, Tajik et al. 2013). The lack of clinical trials to further provide statistically significant support for neoadjuvant chemotherapy is lacking, and therefore, primary surgery remains the standard of care. Chemotherapy follows next, typically with a platinum-based agent.

In the 1970's the clinical introduction and use of platinum-based chemotherapeutics, specifically cisplatin [*cis*-diamminedichloroplatinum(II)], significantly improved overall

survival (OS) by ~6 months in 29% of patients with ovarian cancer, leading the way to its adoption as the backbone of most chemotherapeutic regimens (Rossof, Talley et al. 1979; Thigpen, Shingleton et al. 1979). In the mid-1980s, a cisplatin analog, Carboplatin [cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II)], with improved toxicity profile and equivalent therapeutic efficacy replaced cisplatin as the standard of care (Evans, Raju et al. 1983; Joss, Kaplan et al. 1984; Alberts, Green et al. 1992). The last major advance occurred in early 1990's with the introduction of the mitotic inhibitor paclitaxel, which further improved OS by 3-15 months (depending on the study) when used in combination with platinum (McGuire, Hoskins et al. 1996; Piccart, Bertelsen et al. 2000; Kyrgiou, Salanti et al. 2006) (**Figure 1.03**).

The development of more molecularly targeted therapeutics has become increasingly popular in the quest to provide new arsenal for the treatment of ovarian cancer. Recently, genetic and functional evidence has suggested that tumors with mutations in *BRCA1* or *BRCA2* may be sensitive to poly (adenosine diphosphate [ADP]) ribose polymerase inhibitor (PARPi) treatment (Swisher, Sakai et al. 2008; Wang and Figg 2008). PARPi therapies (*i.e.* olaparib and rucaparib) (Audeh, Carmichael et al. 2010; Drew, Mulligan et al. 2011) are emerging as an effective class of targeted therapies which are demonstrating promising potential in treating ovarian tumors harboring mutations in *BRCA1*, *BRCA2*, and potentially other components of homologous recombination (HR)- DNA repair (Turner, Tutt et al. 2004; 2011). Olaparib, (trade name, Lynparza (AstraZeneca) was FDA approved for treatment of advanced ovarian cancer in 2014 concurrent with the approval of the BRCAAnalysis CDx (Myriad Genetics) for the

qualitative detection of *BRCA1* and *BRCA2* mutations (Gunderson and Moore 2015; Kim, Ison et al. 2015). That being said, it has been documented that PARP secondary mutations may develop over the course of treatment resulting in expression of functional BRCA1, which allows for renewed DNA repair and development of resistance to both PARPi and platinum agents (Swisher, Sakai et al. 2008).

The anti-Vascular Endothelial Growth Factor (VEGF) therapeutic, bevacizumab (Avastin[®], Roche), is an example of a target-specific drug, which can be added to a front-line chemotherapy regimen. In a large Phase III trial, 1,528 women were randomized into either carboplatin alone or bevacizumab with carboplatin arms. For women in the bevacizumab arm, progression free survival (PFS) increased by only ~1.5 months. However, in patients at high risk of recurrence, the addition of bevacizumab increased PFS and extended OS by ~8.2 months (Perren, Swart et al. 2011). Results from a recent Phase III trial, entitled “Avastin Use in Platinum-Resistant Epithelial Ovarian Cancer (AURELIA)”, indicated that the addition of Avastin in combination with chemotherapy significantly increased PFS over chemotherapy alone (8.1 vs. 3.9 months, $P < 0.001$). However there was no significant increase in OS and it must be noted that, while significant, the overall increase in PFS of ~4 months is small (Husain, Wang et al. 2016).

While continued drug development attempts have been prolific, the fact remains that the most effective therapeutic for treatment of EOC is platinum-based chemotherapy. Unfortunately the development of resistance to this class of therapeutics is inevitable in

the vast majority of recurrent cases leaving a void in second-line treatment options. The current status quo for patients with platinum-resistant disease is a balance between prescribing additional chemotherapy or inclusion in experimental clinical trials, all while attempting to maintain an acceptable level of quality of life (Ledermann and Kristeleit 2010). Frequently, monotherapy of liposomal doxorubicin, topotecan, etoposide, gemcitabine or increased intervals of paclitaxel are prescribed (Gordon, Fleagle et al. 2001; Markman, Blessing et al. 2006; Fung-Kee-Fung, Oliver et al. 2007). However, these are far from a cure and the hurdle of overcoming platinum-resistance remains one of the lasting key milestones in advancing the overall survival of women with ovarian cancer.

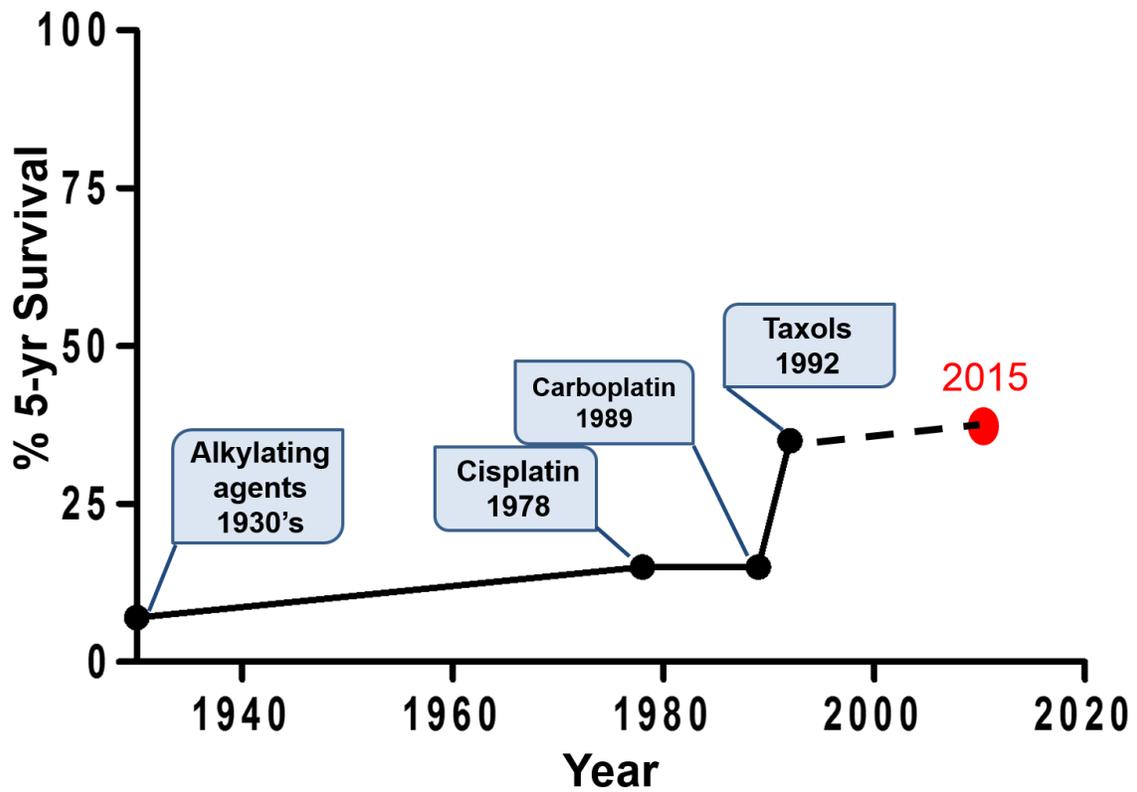


Figure 1.03. Chemotherapeutic Advancements in Ovarian Cancer. This graph depicts the 5-year survival rate of women with stage III or IV disease and corresponding therapeutic advancements. The 5-year survival rate has not significantly increased since the addition of taxols, (specifically paclitaxel) to platinum-based chemotherapy in 1992.

PLATINUM-BASED CHEMOTHERAPY

Cisplatin is currently used to treat a variety of solid tumors and has been called the 'penicillin of cancer drugs' (Kelland 2007). It and other platinum-based chemotherapeutics remain the gold standard for treatment of EOC. Cisplatin, also known as Peyrone's Chloride, was first synthesized by Michael Peyrone in 1845 (Kelland 2007) and is a simple inorganic structure of $\text{Cl}_2\text{H}_6\text{N}_2\text{Pt}$ (**Figure 1.04**). The use of cisplatin as a chemotherapeutic agent was brought to light in the early 1960's by the biophysicist, Barnett Rosenberg (Muggia, Bonetti et al. 2015). Rosenberg observed that electrodes composed of platinum halted cell cycle progression in *Escherichia coli* and triggered the cells to form long (300-fold greater) filamentous morphologies (Rosenberg, Vancamp et al. 1965). The *in vivo* use of cisplatin in animal models quickly followed (Rosenberg, VanCamp et al. 1969) and cisplatin was first used to treat cancer patients in 1971 (Rossof, Talley et al. 1979). While cisplatin was highly effective in treating a variety of cancers, severe side effects, notably nephrotoxicity, lead to the development of next-generation platinum-based compounds.

Carboplatin was the first derivative of cisplatin to be approved for clinical use in the treatment of a wide variety of cancers. Carboplatin forms the same DNA adducts as its parent compound however the rate of adduct formation is ~10-fold slower and therefore 20-40-fold higher concentrations of carboplatin are required to achieve the same therapeutic effect when compared to the parent compound (Knox, Friedlos et al. 1986). This change in the rate of aquation was accomplished through the replacement of

cisplatin's chloride ions with 1,1 cyclobutanedicarboxylate, which is a slower leaving group and less reactive with water (**Figure 1.04**). Oxaliplatin, a third generation compound, was approved for use in the treatment of cancers, such as colorectal cancer, in the early 2000's (Kelland 2007). While similar to carboplatin, oxaliplatin has a hydrophobic dach ligand which points into the major groove of DNA (**Figure 1.04**). It is hypothesized that this inhibits the binding of DNA repair proteins thus preventing DNA repair and more efficiently activates apoptotic pathways (Hector, Bolanowska-Higdon et al. 2001).

Platinum compounds enter the cell through passive diffusion across the plasma membrane, active transporters such as copper transporter proteins, or organic cation transporters (**Figure 1.04**) (Wheate, Walker et al. 2010). Once inside the cell the reduced Cl concentration (4-20 mM intracellular vs 100 mM extracellular) causes the leaving groups to aquate thus exposing the reactive Pt. Functionally, Pt acts by covalently binding DNA through N7 on purines and subsequently forming DNA adducts. This twists or kinks the DNA 30°-60° towards the major groove, disrupts the cell cycle, activates DNA repair pathways, and triggers apoptosis. Platinum compounds are especially effective in cells with deficiencies in DNA repair. For example, cells with mutations in components of the homologous end joining (HR) DNA repair pathways (*i.e.*, *BRCA* mutants) are forced to utilize the more error-prone non-homologous end joining (NHEJ) to repair platinum-induced DNA damage. This causes a rapid accumulation of genetic errors and subsequently increased cell death.

Platinum-based compounds can be exported from the cell through membrane transporters, packaged and secreted in extracellular vesicles, or degraded by the tripeptide L-glutathione. Changes in drug uptake/efflux, responses to adduct formation, and/or elevated glutathione levels have been shown to influence how tumor cells respond to platinum-based chemotherapy.

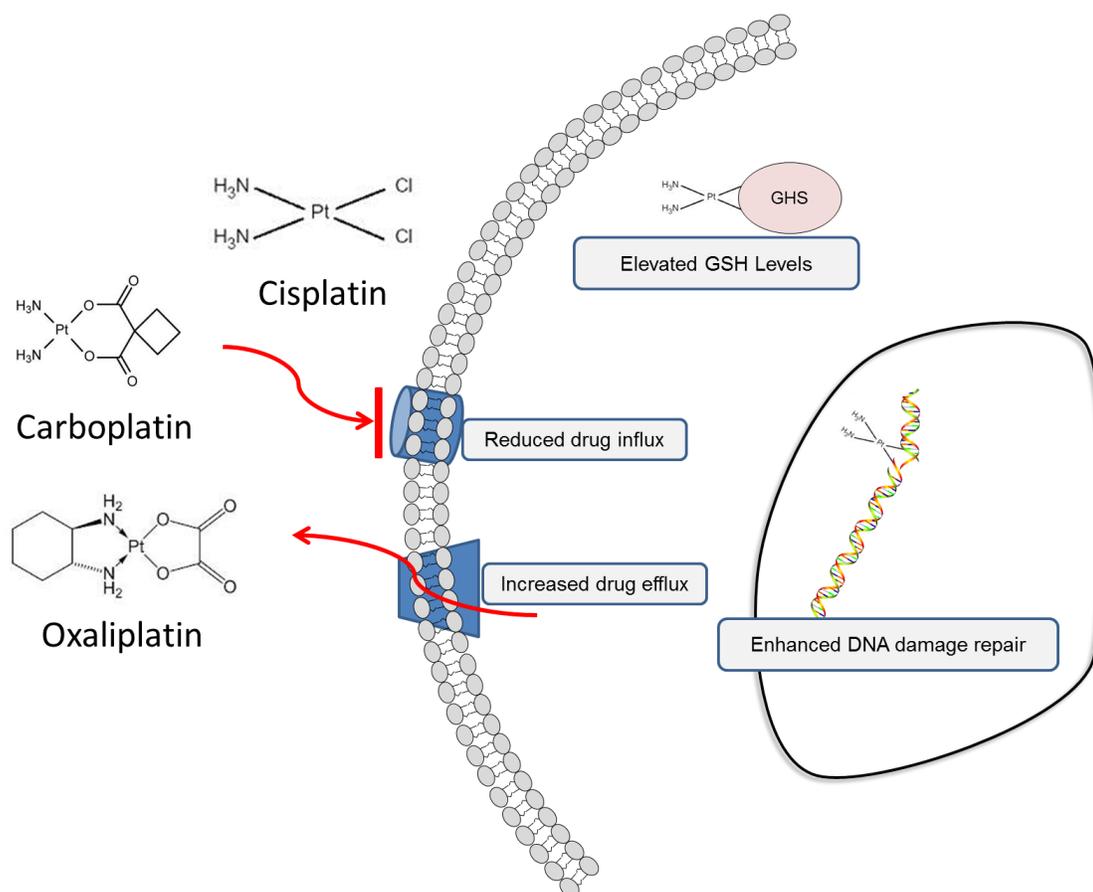


Figure 1.04 Platinum-Based Chemotherapeutics and Traditional Mechanisms of Drug Resistance. Molecular structures of the three most common platinum-based chemotherapeutics are shown (cisplatin, carboplatin, and oxaliplatin). Drugs enter the cell by passive diffusion and/or active drug transport. Once inside the compounds aquate and travel to the nucleus where they bind DNA and cause DNA damage. Traditional mechanisms of resistance to platinum chemotherapy includes reduced drug influx, decreased drug efflux, elevated drug degradation by intracellular glutathione and enhanced DNA damage repair systems.

METHODS OF PLATINUM-RESISTANCE

While over 90% of EOC patients will respond to initial treatment with platinum or platinum and taxane combinations, over 50% of these women will relapse with resistant or refractory disease. The loss of sensitivity to platinum-chemotherapy has been extensively studied in EOC, in fact, a PubMed search using the terms 'ovarian cancer' and 'platinum-resistance' reveals over 1,600 results dating back to the mid 1970's. Through this somewhat overwhelming amount of research there have emerged several classical, well documented mechanisms of platinum-resistance including, but not limited to; increased drug efflux/reduced influx, increased glutathione synthesis, increased DNA damage repair, and increased ability to undergo epithelial to mesenchymal transition (EMT) (Hamilton, Lai et al. 1989; Perez, Hamilton et al. 1990; Stewart 2007; Latifi, Abubaker et al. 2011; Galluzzi, Senovilla et al. 2012; Marchini, Fruscio et al. 2013; Yew, Crow et al. 2013).

For obvious reasons, extracellular platinum is ineffective and in some instances, the development of platinum-resistance is associated with changes in how the drug enters and leaves the cell. Ishida and colleagues were the first to identify the role of copper transporter-1 (CTR-1) in the uptake of cisplatin (Ishida, Lee et al. 2002). Importantly, they observed that yeast cells with deficiencies in the *CTR-1* gene and *mCTR-1*^{-/-} mice were more resistant to cisplatin than their WT counterparts (Ishida, Lee et al. 2002). In the same year Katano and colleagues further elucidated the relationships between Cu and cisplatin import/export in ovarian cancer cells (Katano, Kondo et al. 2002). The

group provided strong evidence that cisplatin utilized the same transporters as Cu for both intake and efflux and cells with resistance to cisplatin also exhibited similar increases in resistance to Cu as compared to the sensitive, parental cell lines (Katano, Kondo et al. 2002).

Once inside the cell, platinum-compounds can be bound by thiol-containing species, such as glutathione, which are rich in sulfur-containing amino acids (Mistry, Kelland et al. 1991). Platinum binds readily to sulfur and the resulting compounds bind with glutathione S-transferase for cellular export via ATP-dependent pumps (*i.e.*, MRP1 and MRP2) (Mistry, Kelland et al. 1991; Ishikawa 1992). Increases in intracellular glutathione (Godwin, Meister et al. 1992) as well as increases in the numbers or activity of export pumps (Ohishi, Oda et al. 2002) have been correlated with platinum-resistance.

Platinum-chemotherapeutics, which are able to bind to DNA from adducts, lead to DNA damage. These lesions are repaired by major DNA-repair pathways including; nucleotide-excision repair (NER), mismatch repair (MMR), DNA strand cross-link repair, homologous recombination, and non-homologous end joining (Helleday, Petermann et al. 2008). In ovarian cancer, Ferry and colleagues have demonstrated that EOC cell lines derived from clonal evolution after exposure to cisplatin have elevated excision repair cross-complementation group 1 (ERCC1) which is essential to NER response to platinum-induced DNA damage (Ferry, Hamilton et al. 2000). To complicate things, a loss of pathway components can also lead to resistance. For example, it has also been

shown that cells with deficiencies in MMR have elevated resistance profiles (Fink, Nebel et al. 1996).

Lastly, several reports have also suggested that treatment with platinum may induce physiological changes in recipient cells which are representative of EMT and cells with an increased ability to undergo EMT are more resistant to therapy (Latifi, Abubaker et al. 2011). While the exact mechanism behind this phenomenon is still unclear, there is a push to develop anti-EMT therapeutics in an effort to reduce and/or treat the development of platinum resistant disease (Chen, Wang et al. 2014; Smolle, Taucher et al. 2014), which will be discussed in detail later.

Despite decades of research elucidating various mechanisms of resistance, few therapeutic strategies have shown to have a significant clinical benefit. There is an urgent need to develop a better standard of care for women in late stage high grade EOC. A current avenue of study focuses on genetic changes that define the recurrent and resistant tumor burden. Importantly, recent attention on extracellular vesicles has suggested that transfer of genetic and proteomic content via these vesicles may play an important role in the development of platinum resistance in EOC.

CHAPTER 2- EXOSOMES

OVERVIEW

The recent discovery of exosome-mediated cellular communication has greatly transformed our understanding of the way contact independent cell-to-cell communication occurs in physiological and pathological processes. These nano-sized vesicles of endocytic origin are secreted by most cell types and represent a “natural” packaged delivery system that efficiently transports a wide range of informative molecules such as nucleic acids, proteins and retrotransposons, often representative of the cell of origin. Although exosomes were originally described in 1980s, recent discoveries have sparked renewed interest in their role as mediators of intercellular communications, as well as their potential value as biomarkers. Exosomes are able to travel systemically throughout the body to potentially target a variety of recipient cells. Upon surface contact and/or uptake, exosomes exert molecular and physiological changes via the delivery of their content and/or activation of signaling pathways. Recent studies have highlighted the importance of exosomal communication in “normal” biology and pathological states such as cancer. Examples of these activities include angiogenesis, wound healing, inflammation, cell migration and mediating phenotype alterations. In many types of neoplasia current evidence demonstrates that tumor cells display an enhanced exosomal output in contrast to their normal counterparts which is not only important for communication between tumor cells but also between tumor cells and their surrounding microenvironment. This dynamic interplay contributes to the development and progression of diseases such as cancer, and enhances processes such as tumor metastasis, anti-tumor immuno-responses, and drug resistance.

HISTORY

The term 'exosomes' was first coined by Trams and colleagues in the late 1970s as extracellular vesicles with 5'-nucleotidase activity that were reflective of the activity of the parent cells (Trams, Lauter et al. 1981). While this study mainly described membrane fragments, not exosomes as we know them today, the authors proposed that these secreted vesicles may serve a physiologic functional role. It wasn't until six years later that Rose Johnstone and colleagues observed vesicles released from the plasma membrane of sheep reticulocytes during their maturation into erythrocytes which they termed as exosomes (Johnstone, Adam et al. 1987). Most importantly, Johnstone *et al.*, reported that exosome biogenesis was most likely linked to the formation of multivesicular bodies (MVB) as previously described by two independent groups (Pan *et al.*, and Harding *et al.*,) while studying transferrin receptor sorting (Harding, Heuser et al. 1983; Pan, Teng et al. 1985). Additionally, Johnstone demonstrated that exosomes isolated from the *in vitro* culture of sheep reticulocytes contained several proteins (acetylcholinesterase, glucose transporters, nucleoside transporters, and the transferrin receptor), which maintained biological activity while in exosomes and were capable of mimicking a portion of the parental cell's functions (Johnstone, Adam et al. 1987). In 1989 Johnstone further expanded the understanding of exosome biogenesis by providing evidence that the sorting of specific proteins into exosomes is an active and orderly process which may be conserved across species (Johnstone, Bianchini et al. 1989). Since these initial discoveries four-decades ago we have finally begun to understand the biological significance of this specific class of secreted vesicles. What

were once believed to be “nothing more than the cell’s disposal system” have emerged as intricate and organized intracellular communicators which are essential in many aspects of physiology such as development, immune surveillance, and, importantly, the development of disease states such as cancer.

EXOSOME BIOGENESIS

Extracellular vesicle production and release occurs in all cells types under normal physiologic as well as in diseased states (Robbins and Morelli 2014). This conserved evolutionary process, inherent to both prokaryotic and eukaryotic cells (Johnstone 2006; Keller, Sanderson et al. 2006; They, Ostrowski et al. 2009), leads to the formation of membrane-derived vesicles, which based upon vesicular size, intracellular contents and biogenesis, are categorized into various classes of cell-derived extracellular vesicles (EVs). Following their respective biogenesis, three main classes of cell-derived EVs are now recognized: (1) microvesicles/microparticles, (2) apoptotic bodies, and (3) exosomes. Distinct from microvesicles and apoptotic bodies, exosomes are defined by their biogenesis, size (display a diameter within 30-150 nm), and their specific protein content. While not all exosomes contain universal markers (such as β -actin, GAPDH, or tubulin in eukaryotic cell lysates), our current understanding of exosome biogenesis and biology has identified several key proteins which are typically (but not always) found on and within exosomes including tetraspanins such as CD9, CD63, and CD81 (They, Ostrowski et al. 2009), members of the Endosomal Sorting Complexes Required for

Transport (ESCRT) such as ALIX, and TSG101, and heat shock proteins such as Hsp70 and Hsp90 (Thery, Zitvogel et al. 2002; Thery, Amigorena et al. 2006).

Exosome formation begins in the early endosome by a mechanism of inward budding of the limiting membrane which results in the formation of intraluminal vesicles (ILV) (**Figure 2.01**) (Harding, Heuser et al. 1983). The ESCRT complex proteins (ESCRT-0, I, II, & III, TSG101, and accessory proteins ALIX and VPS4) are important to this process and carry out activities such as ubiquitin-mediated cargo trafficking, vesicle formation, and scission (Katzmann, Babst et al. 2001; Babst, Katzmann et al. 2002; Babst, Katzmann et al. 2002; Bilodeau, Winistorfer et al. 2003; Katzmann, Stefan et al. 2003; Raiborg, Rusten et al. 2003; Baietti, Zhang et al. 2012). Exosome biogenesis, independent of the ESCRT machinery has also been described. Trajkovic and colleagues were the first to identify the importance of sphingolipid ceramide content in generating the inward curvature of ILV formation (Trajkovic, Hsu et al. 2008). Since then, evidence has emerged highlighting the potential roles of additional lipids and lipid-associated machinery; including cholesterol and phospholipase D2, in exosome biogenesis (Wubbolts, Leckie et al. 2003; Laulagnier, Grand et al. 2004; Strauss, Goebel et al. 2010). Of note, recent studies suggest that the membrane lipid composition is often altered in tumor cells, and select tumor-associated lipids may be enriched within exosomes and serve biological functions which act to enhance tumorigenesis (Nicolson 2015).

Mature ILV's are termed multivesicular bodies (MVB). MVBs either fuse with lysosome or, through mechanisms which are still unclear, travel to and fuse with the plasma

membrane of the producing cell. Several reports highlight the significance of Rab proteins (*i.e.*, Rab35, Rab27a, and Rab27b) in this process (Hsu, Morohashi et al. 2010; Ostrowski, Carmo et al. 2010) and, in addition to their roles in exosome biogenesis, ongoing evidence suggests that these proteins may be important in tumor development and metastasis (**Figure 2.01**) (Li, Hu et al. 2014; Ostenfeld, Jeppesen et al. 2014; Wang, Gilkes et al. 2014; Yang, Liu et al. 2015). For example, Bobrie and colleagues have demonstrated the importance of Rab27a in the ability of breast cancer cells to secrete exosomes capable of recruiting pro-tumor neutrophils into the tumor microenvironment (Bobrie, Krumeich et al. 2012). Likewise, Hendrix and colleagues have provided evidence that Rab27b is essential for lymph node metastasis in a breast cancer mouse model (Hendrix, Braems et al. 2010; Hendrix, Maynard et al. 2010). In addition to Rab GTPases, the p53 protein, which is highly dysregulated in a variety of malignancies (Levine, Reich et al. 1983; Cordani, Pacchiana et al. 2016), has been implicated in the regulation of exosome release (**Figure 2.01**). It was reported that DNA damage activation of p53 induced transcription of TSAP6 (which has been associated with exosome biogenesis) (Yu, Harris et al. 2006). In addition, p53 activation has also been correlated with other exosome biogenesis pathway components such as Caveolin-1 (Feng 2010). In fact, several studies have illustrated that enhanced exosome biogenesis and release may be triggered by stressors such as chemotherapeutics, which activate p53 by causing DNA damage; it is thought that exosome biogenesis and release may be a survival mechanism in these cells (Merendino, Bucchieri et al. 2010; Khan, Jutzy et al. 2011; King, Michael et al. 2012; Yang, Wu et al. 2013) (**Figure 2.01**).

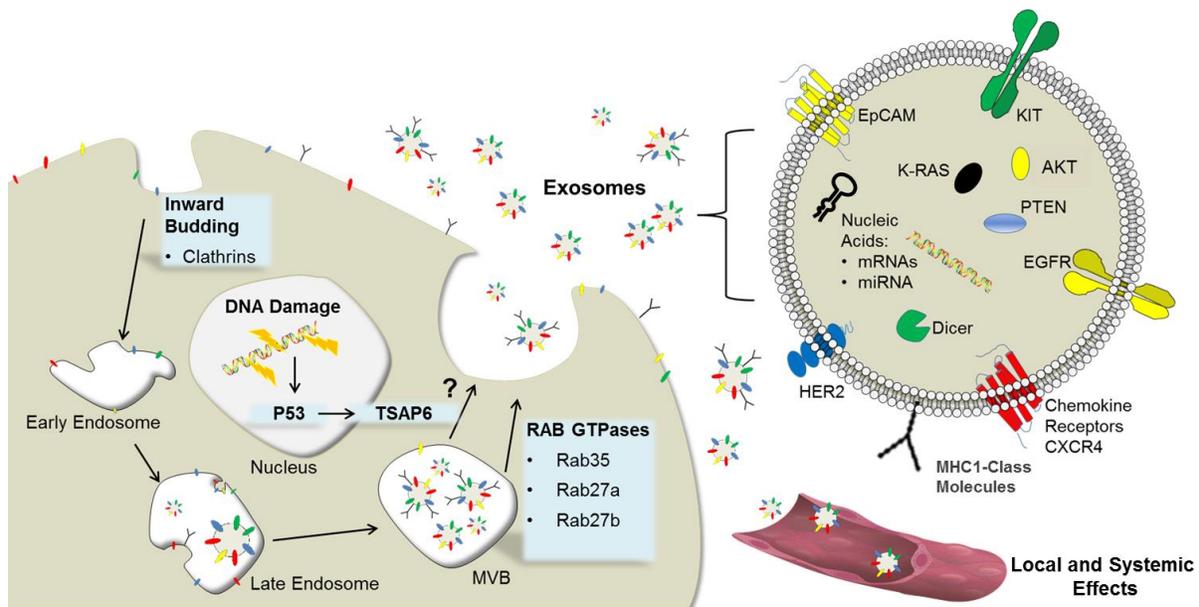


Figure 2.01. Exosome Biogenesis in Cancer. Exosomal biogenesis machinery, which has been reported to be necessary or dysregulated in malignancies, includes clathrins (responsible for inward budding and exosomal uptake), members of the RAB GTPase family (involved in MVB trafficking and fusion with the cell membrane), P53 (tumor suppressor gene – upregulates TSAP), TSAP (whose function in exosome biogenesis is unknown). Exosomes released from cancer cells carry oncogenic cargo including mRNAs, miRNAs, and biologically active proteins including oncogenic receptors; KIT, EGFR, CXCR3, and HER2, as well as intracellular proteins, K-RAS, PTEN, AKT and Dicer. Exosomes have a dense lipid membrane enabling them to travel through blood and lymph systems to interact with cells at very distal locations.

EXOSOMES IN CANCER

The first report of the ability of tumor cells to release microvesicles was initially described by Taylor et al., in early 1980's in his studies of ovarian cancer (Taylor and Doellgast 1979; Taylor, Homesley et al. 1980). Since this seminal work, exosomes have been identified as a previously unappreciated contributor in the development of many types of cancer including breast, gastrointestinal stromal tumors (GIST), non-small cell lung, glioblastoma multiforme (GBM), and leukemia (Baynes, Shih et al. 1991; Perez-Torres, Valle et al. 2008; Skog, Wurdinger et al. 2008; Nazarenko, Rana et al. 2010; Atay, Banskota et al. 2014). Neoplastic cells become cancerous upon acquisition of specific characteristics, or 'hallmarks,' which enable them to develop, survive, and metastasize (Hanahan and Weinberg 2011). The "Hallmarks of Cancer" were defined by Hanahan and Weinberg to include replicative immortality, resisting cell death, evading extracellular cues, increased angiogenesis, invasion and metastasis, and immune escape. The ability of tumors to accomplish such hallmarks requires intracellular communication between tumor cells and the complex network of cells and molecules that make up the surrounding tumor stroma including cancer-associated fibroblasts (CAFs), endothelial cells, tumor associated macrophages (TAMs), as well as the extracellular matrix. To date, exosomes derived from both cells of the tumor and stroma have been shown to play key roles in a variety of cancers and work is ongoing to demonstrate just how essential these vesicles are in the process of tumorigenesis.

ONCOGENIC TRANSFORMATION AND TUMOR FORMATION

Tumor cells distinguish themselves from their normal cellular counterparts by their abilities to replicate indefinitely, evade extracellular cues to stop proliferation, and survive genomic alterations, which would otherwise trigger apoptosis. Gain-of-function mutations in proto-oncogenes or loss of tumor suppressor activities contribute to the pathogenesis of most types of cancers and current research has emerged suggesting that exosomes may be involved in the propagation of these malignant changes (Skog, Wurdinger et al. 2008; Atay, Banskota et al. 2014). Since a portion of the parental cell's membrane proteins and cytosolic components are retained within exosomes, tumors are capable of using this endogenous vesicle trafficking system to horizontally transfer oncogenic mRNAs, micro-RNAs (miRNAs), and proteins as well as other tumor-promoting materials to neighboring cells within the microenvironment. Exosomes are believed to interact with recipient cells by either fusing with the recipient cell's plasma membrane, thereby transferring both membrane and cytosolic components and activating signaling pathways, through ligand-receptor binding interactions, or through uptake via endocytosis (**Figure 2.02**). In many instances, exosome contact triggers phenotypic transformation of recipient cells which, in cancer, can enhance overall tumorigenicity (**Figure 2.02**) (Webber, Steadman et al. 2010; Yang, Chen et al. 2011).

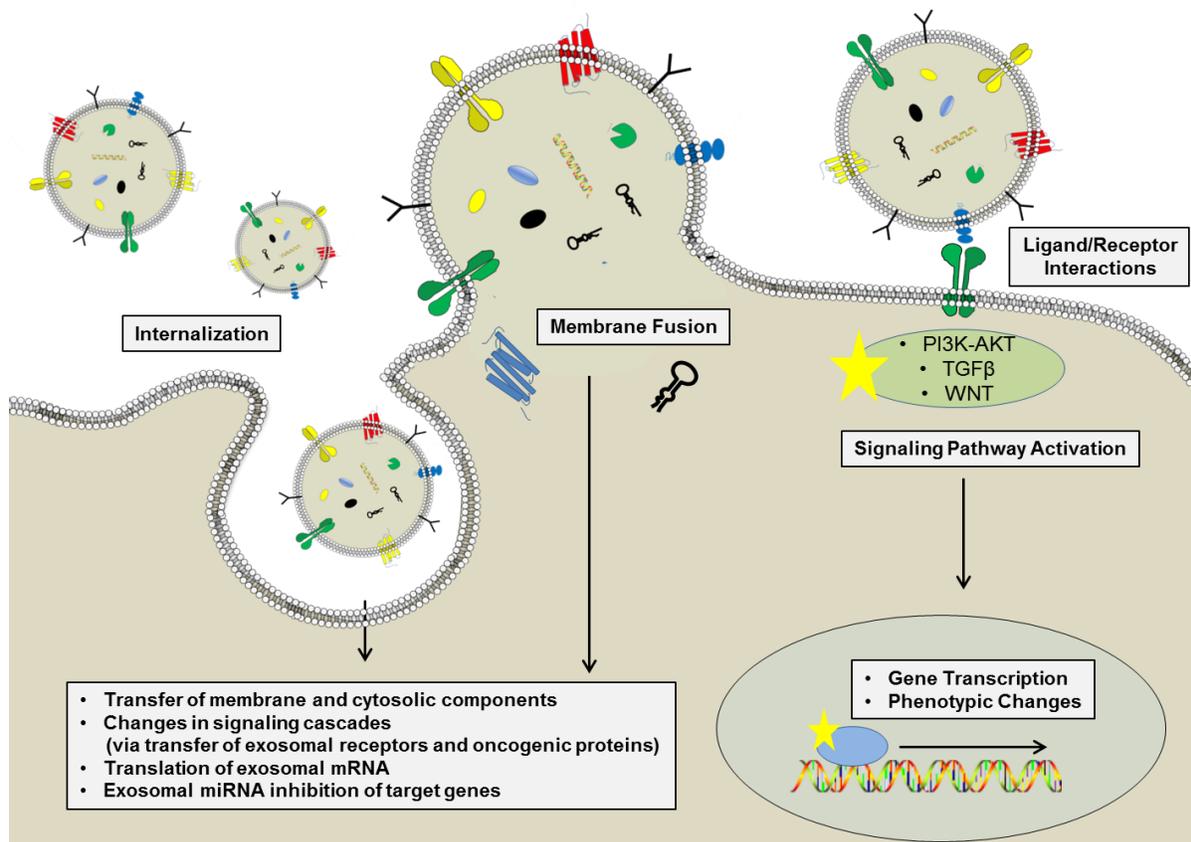


Figure 2.02 Exosome-Mediated Effects on Recipient Cells. Exosomes interact with recipient cells in one of three identified ways. They may be internalized via pinocytosis or endocytosis, make contact and fuse with the plasma membrane of the recipient cell, or interact with receptors on the cell surface. The first two of these interactions result in the transfer of exosomal material including oncogenic proteins, mRNAs and miRNAs. The last interaction may result in activation of signaling cascades such as PI3K-AKT, TGFβ, and WNT, which leads to changes in gene expression and phenotype. Importantly, tumor cells use these mechanisms to exchange information and increase tumorigenesis.

In 2007, Valadi and colleagues were the first to report that cell-cell communication via exosomes mediated the exchange of mRNA and miRNAs. More importantly, they demonstrated that certain mRNAs within exosomes were translated into protein within the recipient cells, thus highlighting the functional significance of exosome-mediated RNA transfer in cell-cell communication (Valadi, Ekstrom et al. 2007). The first direct evidence of this form of communication within a neoplastic setting was identified in GBM. In this study, Skog and colleagues demonstrated how GBM cell-derived exosomes contained mRNAs, which could be transferred to and translated within recipient cells (Skog, Wurdinger et al. 2008). In addition, the uptake of these exosomes induced proliferation in a human glioma cell line as well as tubule formation in non-malignant endothelial cells (Skog, Wurdinger et al. 2008). Subsequent studies demonstrated that exosomes derived from colorectal cancer cells contained mRNAs involved in cell cycle regulation which, upon transfer to untransformed endothelial cells, promoted uncontrolled proliferation (Hong, Cho et al. 2009). Tumor-derived exosomes have also been shown to be involved in the recruitment of transformed mesenchymal stem cells into the tumor microenvironment (Webber, Steadman et al. 2010; Cho, Park et al. 2011). Likewise, tumor cells exposed to exosomes derived from cells within the microenvironment, such as CAFs or TAMs, demonstrated enhanced proliferation and invasion capacities (Yang, Chen et al. 2011; Luga and Wrana 2013). Subsequent to these studies, numerous miRNAs, mRNAs, and proteins have since been identified and shown to induce tumorigenesis in a wide variety of malignancies (**Table 2.01** and **Figure 2.02**) and ongoing work suggests that the transfer of oncogenic material, especially short non-coding RNAs, such as miRNAs, may be an essential step in cancer

development (Haug, Hald et al. 2015; Rodriguez, Silva et al. 2015; Warnecke-Eberz, Chon et al. 2015; Rooj, Mineo et al. 2016).

Perhaps one of the most interesting discoveries of the past few years was the identification of miRNA processing machinery within tumor-derived exosomes (Melo, Sugimoto et al. 2014). Melo and colleagues identified Dicer, Ago2, and TRB (all members of the RISC-loading complex) within exosomes. The discovery of this machinery was significant because of the integral role it plays in pre-miRNA processing into mature miRNA. This study provided intriguing evidence that exosomes derived from both breast cancer cell lines and patient sera were capable of processing pre-miRNA into mature miRNA independent of the cellular environment. In addition, uptake of these exosomes triggered “normal” epithelial cells to form tumors in a Dicer-dependent manner.

The transfer of proteins, especially oncogenic proteins, from tumor cells to cells in the microenvironment has been observed in the evolution of multiple types of cancers. Our group was the first to show that circulating exosomes derived from GIST patient serum or conditioned media of GIST cell lines contain the constitutively active, oncogenic KIT. KIT, also known as CD117 is the normal cellular homologue of the viral oncoprotein v-Kit (v-Kit, Hardy Zuckerman 4 feline sarcoma viral oncogene homologue) and is a member of the receptor tyrosine kinase subclass III superfamily that includes receptors for platelet derived growth factor (PDGF), macrophage-colony stimulating factor (M-CSF), and FLT3 (Tarn and Godwin 2006).

Atay and colleagues reported that uptake of these so called “oncosomes” by normal progenitor smooth muscle cells triggered the activation of oncogenic signaling pathways downstream of KIT and lead to a tumor-promoting phenotype within these recipient cells (Atay, Banskota et al. 2014). We reported that GIST cells not only constitutively released low levels of MMP1, but that challenging myometrial smooth muscle cells with GIST patient-derived exosomes (but not exosomes from healthy donors) significantly increased MMP1 production, which in turn enhanced GIST cell invasion. We assessed direct/indirect exosome-mediated MMP1 induction using siRNA and inhibitory drug strategies to reduce MMP1 production by myometrial cells and were able to mimic, *in vitro*, the exosome–MMP1 expression feedback loop. In particular, *in vivo*-derived exosomes appeared to be potent exogenous source of MMP induction in stromal cells, which in turn acted as a pro-invasion factor for GIST cells (Atay and Godwin 2014).

Likewise, oncogenic KRAS has also been reported as a component of exosomes derived from mutant KRAS-expressing colon cancer cells and uptake of these exosomes triggered enhanced invasiveness in recipient cells (Higginbotham, Demory Beckler et al. 2011). In other studies, exosomes derived from mutant KRAS cell lines were shown to increase the production of tumor-promoting proteins such as EGFR, SRC family kinases and integrins (Higginbotham, Demory Beckler et al. 2011; Demory Beckler, Higginbotham et al. 2013). Rodriguez and colleagues reported that breast cancer cells over expressing oncogenic CXCR4 exhibited an increase in stem cell markers, as well as proliferative, migratory and invasiveness properties. Importantly,

exosomes derived from these cells were able to transfer a portion of these characteristics to recipient non-tumorigenic (T47D) cells (Rodriguez, Silva et al. 2015).

While the presence of genomic DNA in exosomes is still under debate recent work has identified dsDNA of tumor suppressor genes *TP53* and *KRAS* in exosomes derived from the pancreatic cell lines and serum of patients with pancreatic adenocarcinoma. Importantly, deep sequencing of the exosomal DNA revealed mutations identical to the parental cell lines (Kahlert, Melo et al. 2014). Through the transfer of mRNA, miRNAs, DNA, or proteins, exosomes have shown to be effective vehicles for oncogenic transformation, (**Figure 2.03**) and, as we will discuss, tumor progression.

Table 2.01 Exosomal Content Associated with Tumorigenesis

Function	Malignancy	Exosomal Content	Reference
Neoplastic Transformation	Prostate cancer Breast cancer	miR-125b, miR-130b, miR-155 miR-140	(Abd Elmageed, Yang et al. 2014) (Gernapudi, Yao et al. 2015)
		Dicer, Ago2, TRB	(Melo, Sugimoto et al. 2014)
		CXCR4	(Rodriguez, Silva et al. 2015)
	GIST	KIT	(Atay, Banskota et al. 2014)

Angiogenesis	Chronic Myeloid Leukemia (CML)	miR-210	(Umezu, Ohyashiki et al. 2013) (Zhuang, Wu et al. 2012)
	Colorectal Cancer Glioblastoma Multiforme (GBM) Lung Cancer Melanoma Pancreatic Cancer	miR-9	(Umezu, Tadokoro et al. 2014)
Invasion/ Migration	Multiple Myeloma Glioblastoma	miR-135b EGFRvIII	(Al-Nedawi, Meehan et al. 2008)
	Breast cancer Chronic Myeloid Leukemia (CML)	miR-223 miR-92a	(Yang, Chen et al. 2011) (Tadokoro, Umezu et al. 2013)
	Gastric cancer Breast cancer	miR-221, miR-222 miR-10b	(Wang, Zhao et al. 2014) (Singh, Pochampally et al. 2014)
	Colon cancer	miR-223 KRAS	(Yang, Chen et al. 2011) (Higginbotham, Demory Beckler et al. 2011)
	Breast cancer	miR-122	(Fong, Zhou et al. 2015)
Metastasis	Bladder cancer	miR-105 miR-23b	(Zhou, Fong et al. 2014)
	Hepatocellular carcinoma (HCC) Lung Cancer Nasopharyngeal carcinoma	miR-584, miR-517c, miR-378 miR-21, miR-29a miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p, miR-1908	(Ostenfeld, Jeppesen et al. 2014) (Kogure, Lin et al. 2011) (Fabbri, Paone et al. 2012) (Ye, Li et al. 2014)
Immune Inhibition	Glioblastoma	miR-451, miR-21	(van der Vos, Abels et al. 2016)
	Prostate cancer	FasL	(Abusamra, Zhong et al. 2005)
Therapeutic Escape	Breast cancer	miR-127, miR-197, miR-222, miR-223 miR-222 HER2, EPCAM	(Lim, Bliss et al. 2011) (Chen, Liu et al. 2014) (Battke, Ruiss et al. 2011)

ANGIOGENESIS

The high energy demands of growing tumors and exposure to hypoxic conditions requires increased angiogenesis (Shweiki, Itin et al. 1992; Paduch 2016). Exosomes have been implicated in the production of new blood vessels (neo-angiogenesis), which supply developing tumors with nutrients and oxygen (Janowska-Wieczorek, Wysoczynski et al. 2005; Al-Nedawi, Meehan et al. 2008). Likewise, exosome secretion has been implicated as a mechanism by which neoplastic cells survive the oxygen depleted hypoxic core of developing tumors (King, Michael et al. 2012; Kucharzewska, Christianson et al. 2013; Ramteke, Ting et al. 2015). Janowska-Wieczorek *et al* were the first to demonstrate that uptake of platelet-derived microvesicles and exosomes resulted in increased expression of angiogenic factors, as well as, invasive capacities in recipient human lung cancer cell lines (Janowska-Wieczorek, Wysoczynski et al. 2005). Likewise, Al-Nedawi *et al* reported that transfer of the mutated oncogenic epidermal growth factor receptor (EGFRvIII) from EGFRvIII⁺ to EGFRvIII⁻ glioma cells elicited enhanced production of the angiogenic factor VEGF and anchorage independent growth via activation of MAPK and AKT pathways (Al-Nedawi, Meehan et al. 2008). Hypoxic conditions in GBM promote secretion of tumor-derived exosomes which trigger protease-activated receptor 2 (PAR-2) signaling in endothelial cells thus enhancing angiogenesis (Svensson, Kucharzewska et al. 2011) and exosomes derived from multiple myeloma cells under hypoxic conditions are enriched with miR-135b which enhances angiogenesis in endothelial cells by suppressing targeting-factor inducing hypoxia factor 1 (HIF-1) (Umezū, Tadokoro et al. 2014). Additionally, hypoxic

conditions may also trigger exosome secretion, which acts to enhance metastasis to new sites as reported in breast and squamous cell carcinomas (Park, Tan et al. 2010; Wang, Gilkes et al. 2014).

NICHE FORMATION, METASTASIS

Upon the establishment of the primary tumor, severe conditions established during cancer progression frequently result in the movement of tumor cells to secondary metastatic sites. Exosomes have been implicated in preparing such sites or 'niches' for suitable and sustainable secondary tumor establishment, a task, which involves multilayered communication. Peinado and colleagues reported that melanoma-derived exosomes injected into mice functioned to induced vascular leakiness at pre-metastatic sites and triggered changes gene expression (such as increases in extracellular matrix remodeling factors) in lung tissue towards a more tumor-supporting environment (Peinado, Aleckovic et al. 2012). Importantly, the exosome-mediated cross talk between tumor cells and the surrounding stroma has recently been shown to be bi-directional. For example, Zhang and colleagues reported how organized exosome-mediated conversations between tumor cells and cells of the microenvironments of specific organs dictated future sites of metastasis (Zhang, Zhang et al. 2015). This group demonstrated that astrocyte-derived exosomes were capable of transferring tumor suppressor gene, *PTEN*-targeting miRNAs to metastatic tumor cells, thus promoting the advancement of metastatic sites within the brain. In addition, exosome-inflicted loss of *PTEN* within these tumor cells increased secretion of myeloid cell

recruiting chemokines, which further enhanced tumor development (Zhang, Zhang et al. 2015). Interestingly exosomal PTEN-targeting miRNAs were not found in exosomes derived from other organs, and therefore provided a novel insight alluding to the increased prevalence in certain malignancies towards metastasis of disease to the central nervous system. Additionally, it has been suggested that exosomes can contribute to the accumulation of tumor cells in sentinel lymph nodes (Hood, San et al. 2011). Hood and colleagues demonstrated that exosomes derived from melanoma cell lines preferentially localized to 'sentinel' lymph nodes *in vivo*. Uptake of these tumor-derived exosomes primed the lymph nodes to become more tumor-supportive and mice with pre-treatment of tumor-derived exosomes had increased tumor cell accumulation within the sentinel lymph nodes.

IMMUNE EVASION

Solid tumor malignancies are frequently staged according their tumor size, sites of metastasis and involvement of their nearby (regional) lymph nodes, which can be thought of as the 'base camps' of the body's immune system. In order for continued proliferation within immunocompetent hosts, tumor cells have adapted complex mechanisms (*i.e.*, the secretion of immunosuppressive factors, antigen presentation, and the ability to target regulatory T-cell function), which allow them to evade and/or suppress the body's innate anti-tumor machinery (Vinay, Ryan et al. 2015). The role of exosomes in immunology is perhaps the most extensively studied exosomal function to date. Clayton and Tabi were the first to report on the ability of exosomes to inhibit

immunological responses in cancer (Clayton and Tabi 2005). In this formative study, the researchers demonstrate that the natural killer group 2D (NKG2D) is downregulated in leukocytes following exposure to tumor-derived exosomes, and their effector cytotoxic functions are impaired as a result. Around the same time, Abusamra and colleagues identified the ability of exosomes derived from a prostate cell line to mediate apoptosis of CD8+ T-cells by the transfer of the Fas-L ligand (Abusamra, Zhong et al. 2005). Additionally, tumor-derived exosomes have been shown to impair anti-tumor immunity by inhibition of lymphocyte responses to IL-2 (Clayton, Mitchell et al. 2007), enhancement of pro-inflammatory cytokines (Deng, Cheng et al. 2012), binding tumor-reactive antibodies (Battke, Ruiss et al. 2011), and education of macrophages and mast cells to become tumor-supportive (Yang, Chen et al. 2011). In breast cancer, Yang and colleagues found that uptake of exosome-bound miRNA-223 from IL-4 activated macrophages increased the invasive potential of breast cancer cells *in vitro* (Yang, Chen et al. 2011). Additionally, van der Vos and colleagues demonstrated that microvesicles from human GBM cells contained miR-451/miR-21 and were taken up by cells in the microenvironment (microglia, monocyte/macrophages) (van der Vos, Abels et al. 2016). Upon exosome exposure, these recipient cells exhibited an increase in proliferation and changes in released cytokine profiles, which ultimately contributed to an immunosuppressive phenotype and tumor growth. Lastly, Battke and colleagues demonstrated the seemingly simple, yet profoundly important role of tumor-derived exosomes in binding to tumor-reactive immunotherapies (*i.e.*, HER2 targeting Herceptin[®]), thus sequestering the therapeutic compounds and protecting the parental tumor from attack (Battke, Ruiss et al. 2011).

CHEMOTHERAPY EVASION

Despite a tumor's ability to grow and metastasize, the ultimate cause of cancer-related mortality is the development of resistance to therapeutic interventions. It should be of no surprise that exosomes have been linked to this process, for example, by excretion of chemotherapeutic drugs and transfer of resistance traits (mRNAs and protein) between individual tumor cells (Andre Mdo, Pedro et al. 2016). In ovarian cancer, Safaei and colleagues demonstrated that platinum-resistant ovarian cancer cells exhibited increased intracellular pH levels, which corresponded with enhanced exosome-mediated efflux of the chemotherapeutic agent cisplatin (Safaei, Larson et al. 2005). In addition to physically exporting drugs, exosome transfer of factors which contribute to resistant phenotypes from drug-resistant to drug-sensitive tumor cells has been identified in prostate, lung, breast, neuroblastoma, and ovarian cancers (Corcoran, Rani et al. 2012; Chen, Liu et al. 2014; Lv, Zhu et al. 2014; Xiao, Yu et al. 2014; Challagundla, Wise et al. 2015). The most notable mechanism of such action is through transfer of miRNAs. One such example of this is in breast cancer, where exosomes derived from tamoxifen resistant-MCF-7 cells were found to contain and transfer miR-221/222 to tamoxifen-sensitive-MCF-7 cells. This transfer resulted in decreased target gene expression of P27 and ER α , which subsequently decreased tamoxifen sensitivity in recipient cells (Wei, Lai et al. 2014). In Chapter 3 my studies demonstrate that uptake of exosomes derived from platinum-resistant ovarian cancer cell lines by platinum sensitive cells corresponds with increased epithelial to mesenchymal transition (EMT) and a loss of sensitivity to carboplatin. Exosome-mediated EMT has been

observed in multiple types of cancer including melanoma and bladder cancers (Vella 2014) and the addition of pharmacological EMT inhibitors is currently being evaluated for treatment of malignancies such as prostate and lung cancers (Fischer, Durrans et al. 2015; Zheng, Carstens et al. 2015).

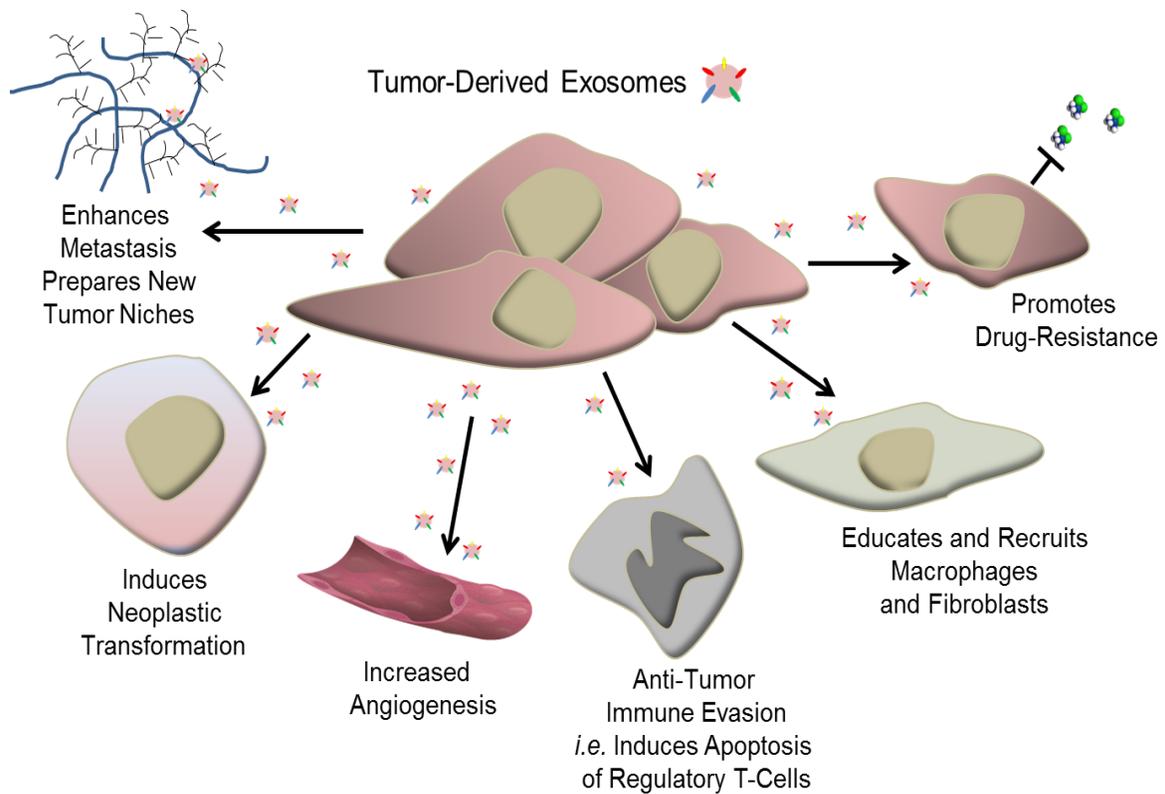


Figure 2.03. Tumor-Derived Exosomes Contribute To Cancer Development and Progression. Exosomes derived from cancer cells act on cells in the local and distal micro-environment to enhance tumorigenesis. Examples of this are; preparing extracellular matrix for establishment of new tumors, inducing neoplastic transformation of epithelial and other healthy cells, increasing angiogenesis, contributing towards anti-tumor immune evasion, recruiting and educating fibroblasts and other cells in the microenvironment to become tumor-supporting, and transferring factors which enhance drug resistance in neighboring tumor cells.

THERAPEUTIC USE

Given the overwhelming importance of exosomes in cancer development and progression it is only natural that researchers have contemplated mechanisms in which to target and/or utilize exosomes in therapeutic applications. The pivotal work for the latter came in 1998 when Zitvogel and colleagues demonstrated how exosomes derived from tumor-peptide pulsed dendritic cells (DEX) could be used as a tumor-vaccine. Researchers identified MHC-I, MHC-II, and T-cell co-stimulatory molecules on DEX and effectively used these exosomes to suppress growth of established murine tumors. This line of research was continued with the introduction of tumor-derived exosomes (TEX) as an alternative exosomal therapeutic option (Wolfers, Lozier et al. 2001). In this study, Wolfers and colleagues demonstrated that TEX were more effective than irradiated tumor cells, apoptotic bodies, and tumor lysates at eliciting a T-cell mediated antitumor immune response and effective elimination of autologous tumors. Clinical trials utilizing DEX (including studies with second generation DEX2) have been introduced in non-small cell lung cancer, melanoma, and primary as well as recurrent gliomas (Morse, Garst et al. 2005). While results have been modest, there is sufficient evidence to warrant additional clinical trials in patients with glioma and NSCLC (Tan, De La Pena et al. 2010).

In addition to their potential as a tumor-vaccine, exosomes have recently been evaluated for their uses as therapeutic delivery vehicles. Strategies to load therapeutic material into exosomes include modifying parental cells to exogenously express small

non-coding RNAs, mRNAs, or proteins which are then sorted into exosomes (Lee, Kim et al. 2011; Wahlgren, De et al. 2012; Koppers-Lalic, Hogenboom et al. 2013; Ohno, Takanashi et al. 2013; Shtam, Kovalev et al. 2013; Cooper, Wiklander et al. 2014; Wang, Wang et al. 2015) and biomechanical loading of drug compounds directly onto isolated exosomes via various co-culture techniques. Sun and colleagues were the first to demonstrate that exosomes loaded with the anti-inflammatory agent, curcumin, increased the solubility, stability, and bioavailability of curcumin and were more effective than curcumin alone at reducing inflammation (Sun, Zhuang et al. 2010). Likewise, *in vivo* xenograft models have demonstrated that the efficiency of drug delivery is enhanced when pharmacological agents such as paclitaxel are pre-loaded into bovine-derived milk exosomes as compared to drug alone (Munagala, Aqil et al. 2016). In many cases, exosomes can be engineered to preferentially target specific subsets of cells, thus minimizing off-target effects and toxicity. The idea of generating exosomes with this sort of targeting ligand has been shown to be effective in cancers such as in breast and lung (Tian, Li et al. 2014; Munagala, Aqil et al. 2016). Importantly, in many cases, exosomes are derived from modified host-derived cells, thus the autologous exosomes are able to travel throughout the body with little to no immunological interference.

Lastly – devices such as the M-trap and Aethlon ADAPT™ (adaptive dialysis-like affinity platform technology) have been developed which exploit the pro-tumorigenic potential of exosomes *in vivo*. In the M-trap therapeutic strategy, exosomes isolated from the ascites fluid of ovarian cancer patients are embedded on a 3-D scaffold, which is then

placed within the peritoneal cavity. Researchers observed that the implanted device attracted and sequestered circulating cancer cells which resulted in a reduced number of metastatic sites overall. Importantly, the use of devices such as this is thought to generate more focal and therefore operable sites of metastasis (de la Fuente, Alonso-Alconada et al. 2015). The Aethlon ADAPT™ utilizes a technology previously designed to filter viral loads from patient plasma (Marleau, Chen et al. 2012). In this proposed therapeutic strategy, exosomes bearing specific markers or oncoproteins (*i.e.*, HER2) are captured and removed from patient plasma which results in a reduction of exosome-mediated immune inhibition as well as decreased exosome-mediated inhibition of antibody-based therapies (*i.e.*, Herceptin®). Of course, while promising, this type of therapy is reliant upon identification of tumor-derived exosomal biomarkers, a field that is rapidly expanding.

BIOMARKERS

The unique phenotypes of tumor-derived exosomes make them not just an appealing choice for therapeutics, but also ideal targets for biomarker discovery given their relative abundance in bodily fluids and ease of accessibility. Notably, exosomes biogenesis and output has been shown to be enhanced in cancer cells (Baran, Baj-Krzyworzeka et al. 2010) and tumor-derived exosomes have been identified and isolated from several biological fluids including; blood, (Alegre, Zubiri et al. 2016; Herreros-Villanueva and Bujanda 2016; Taverna, Giallombardo et al. 2016) ascites (Runz, Keller et al. 2007; Peng, Yan et al. 2011; Carbotti, Orengo et al. 2013; Tokuhisa, Ichikawa et al. 2015),

saliva (Sharma, Gillespie et al. 2011; Lau, Kim et al. 2013; Yang, Wei et al. 2014; Sivadasan, Gupta et al. 2015), and urine (Mitchell, Welton et al. 2009; Berrondo, Flax et al. 2016; Hendriks, Dijkstra et al. 2016). The presence of tumor-specific cell surface proteins allows for tumor-specific exosome isolation using immunocapture techniques. Upon capture, analysis of exosomal cargo provides a wealth of information regarding the current state of disease. For example, exosome samples positive for EpCAM have been isolated from the ascites of patients and corresponded with overall survival and prognosis (Runz, Keller et al. 2007; Taylor and Gercel-Taylor 2008). Additionally, the presence of Glypican-1 on exosomes has been identified as a marker for pancreatic cancer at both early and late stage disease (Melo, Luecke et al. 2015). The most recent interest in the field has identified miRNAs as ‘disease signatures’ and multiple exosome-associated miRNAs are now being used as disease biomarkers (as listed in **Table 2.02**).

With the expansion of exosomal biomarkers there is a tremendous need for tools to rapidly capture and analyze circulating exosomes. Our lab was the first to truly develop an integrated microfluidic platform capable of isolating and interrogating specific subpopulations of exosomes from small volumes of plasma (He, Crow et al. 2014). This novel microfluidic strategy or “lab-on-a-chip” was able to rapidly and quantitatively isolate and analyze exosomes, as well as intravesicular markers directly from human blood. This microfluidic platform was shown able to integrate immunomagnetic isolation and enrichment, chemical lysis, and immune-sandwich chemifluorescence probing in one sequential process. He and colleagues developed assays to capture non-small cell

lung cancer (NSCLC) and ovarian cancer-associated exosomes and showed that exosomal protein markers could accurately distinguish cancer cases from healthy individuals. Importantly, the complete analysis, including two-stage immunomagnetic capture, was completed in ~2 hrs (0.5 hrs. off-chip incubation and ~1.5 hrs. on-chip assay) with as low as 30 μ L of plasma samples. The future relevance of this type of non-invasive biomolecular profiling cannot be understated in both diagnosis and the early detection of cancers where tumor biopsies are invasive, often difficult to obtain, and overly expensive.

Table 2.02 Exosomal miRNAs as Biomarkers

Malignancy	Exosomal miRNA biomarker	Biological Fluid Isolated	Diagnostic Value	Reference
Breast cancer	miR-101, miR-372, miR-373	Serum	Increased expression in breast cancer patients and miR-373 expression increased in receptor-negative breast cancer	(Eichelser, Stuckrath et al. 2014)
Cervical cancer	miR-21, miR-146a	Cervicovaginal lavage	Increased expression in cervical cancer patients exosomes compared to healthy controls	(Liu, Sun et al. 2014)
Colon cancer	miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a	Serum	Increased expression in exosomes of colon cancer patients	(Ogata-Kawata, Izumiya et al. 2014)
Colon cancer	miR-17-92a cluster	Serum	Increased expression in colon cancer patients and higher levels were predictive of poorer prognosis	(Matsumura, Sugimachi et al. 2015)
Esophageal squamous cell cancer	miR-21	Serum	Increased expression in exosomes of cancer patients compared to those with benign disease	(Tanaka, Kamohara et al. 2013)
Hepatocellular carcinoma	miR-718	Serum	Exosomal miR-718 expression decreased in HCC recurrent patients status post liver transplant versus healthy controls	(Sugimachi, Matsumura et al. 2015)
Hepatocellular carcinoma	miR-21	Serum	HCC patients with increased levels compared to healthy controls and hepatitis patients	(Wang, Hou et al. 2014)
Lung cancer	miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, miR-214	Plasma	12 miRNAs detectable in exosomes and up-regulation of total exosomes and miRNA levels in lung cancer patients	(Rabinowits, Gercel-Taylor et al. 2009)

Ovarian cancer	miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214	Serum	Elevation of 8 miRNAs in ovarian cancer patients exosomes compared to those with benign tumors and healthy controls	(Taylor and Gercel-Taylor 2008)
Pancreatic cancer	miR-1246, miR-3976, miR-4306, miR-4644	Serum	In comparison to healthy controls up-regulation of expression in pancreatic cancer patients	(Madhavan , Yue et al. 2015)
Prostate cancer	miR-375, miR-1290	Plasma	Increased expression in castration-resistant prostate cancer patients and levels are associated significantly with poor overall survival	(Huang, Yuan et al. 2015)

**CHAPTER 3 - EXOSOMES AS MEDIATORS OF PLATINUM RESISTANCE
IN OVARIAN CANCER**

INTRODUCTION

Current interest in the EOC field is focused on the importance of intercellular cross talk mediated by soluble and insoluble factors between the EOC tumor and stromal cells during development, progression, and evolution of drug-resistance (Sung, Jan et al. 2015; Wang, Niu et al. 2015; Coffman, Choi et al. 2016). The EOC tumor microenvironment includes recruited host cells (*i.e.* endothelial cells, fibroblasts, and macrophages) that communicate with tumor cells and often are reeducated to supply functions, which enhance metastasis, vascularization, and immuno-evasion. For instance, EOC tumors have been shown to produce significant levels of interleukin (IL)-6, which triggers recruitment of monocytes from the peripheral blood, and via activation of STAT3, converts these cells into tumor supportive M2 tumor associated macrophages (Dijkgraaf, Heusinkveld et al. 2013). Previous work from our lab demonstrated fibroblasts from normal human ovaries secrete high levels of hepatocyte growth factor, (HGF) which binds to and activates c-MET mediated signaling on EOC tumor cells. This interaction leads to changes in biological processes including increases in tumor proliferation and metastasis (Kwon, Smith et al. 2015). The mechanisms of cellular communication in these types of experiments include secretion of soluble factors, such as cytokines, mitogens, and growth factors; however, recently exosomes have been shown to be released by tumor cells and are emerging as a novel vehicle of cell-cell communication within the development and progression of EOC (Stoek, Keller et al. 2006; Keller, Konig et al. 2009).

Exosomes contribute to EOC development by inducing immune evasion, assisting in the establishment of secondary tumor niches, and serve as intracellular communicators for cross talk between tumor cells and the surrounding stroma (Cho, Park et al. 2011; Peng, Yan et al. 2011). More recently, exosomes have been shown to have a functional role in the development of chemotherapy resistance in breast, non-small cell lung, and prostate cancer; however, their role in platinum-resistance in EOC is unknown (Corcoran, Rani et al. 2012; Chen, Liu et al. 2014; Xiao, Yu et al. 2014). While emphasis in the EOC field is focused on tumor-stroma communication during neoplastic advancement, we report here, the first evidence of the importance of the intricate exchange of exosome-mediated crosstalk within EOC tumors leading to chemotherapeutic resistance by way of activation of EMT. Based on these novel findings, we propose the release of exosomes is a mechanism by which neoplastic EOC cells 'educate' each other, thereby exasperating the development of platinum-resistant disease.

MATERIALS AND METHODS

Cells and Culture Conditions. We utilized human ovarian cancer cells A2780, C30, CP70, C200, OVCAR5, A1847, and OVCAR10 (Behrens, Hamilton et al. 1987; Hamilton, Lai et al. 1989; Perez, Perez et al. 1992). Ovarian cancer cell lines A2780, A1847, and OVCAR5 were authenticated by using multiplex short tandem repeat (STR) testing and compared to historical reference DNA preserved in the lab for these cell lines. Testing was performed by the Clinical Molecular Oncology Lab at KUMC, a CLIA/CAP-accredited molecular diagnostics laboratory using the Promega PowerPlex 16 System used for human identity testing run on an Applied Biosystems instrument.

All cell lines were cultured in RPMI-1640 (Gibco, Thermo Fisher) media supplemented with 10% (v/v) exosome-depleted FBS, 2 mM L-glutamine, 0.2 units/mL human insulin, and 100 units/mL penicillin-streptomycin at 37°C with 5% CO₂. Exosome-depleted FBS was obtained by centrifuging FBS for 18 hours at 100,000 x g followed by filtration through a 0.22 µm filter.

Exosome Isolation. Cell lines were cultured to 70-80% confluency and conditioned media was collected after 24-48 hours, spun for 10 minutes at 2000 x g, and pooled together. Exosomes were isolated by differential centrifugation as previously reported (They, Zitvogel et al. 2002). Briefly media was spun for 45 minutes at 10,000 x g to pellet large vesicles and twice at 100,000x g to pellet and wash exosomes. Exosome pellets were resuspended in 50-100 µL of cold PBS and stored at -80°C.

Nanoparticle Tracking Analysis. Purified exosomes were resuspended in 100 μ L of 0.22 μ m filtered PBS and analyzed using a NanoSight LM10 instrument (NanoSight, Salisbury, United Kingdom). Analysis was performed by applying a monochromatic 404 nm laser to diluted exosomal preparation and measuring the Brownian movements of each particle. The Nanoparticle Tracking Analysis software version 2.3 was used to analyze 60 second videos of data collection to give mean, median, and mode of vesicle size and concentration.

Electron Microscopy. Exosomes were purified as above and fixed using 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C overnight. The pellet was rinsed in 0.15 M sodium cacodylate buffer (pH 7.4), followed by a post fixation in 1% osmium tetroxide containing 0.1% potassium ferricyanide buffered in 0.1 M cacodylate buffer for 1 hour. Exosomes were dehydrated through a series of ethanol washes followed by a propylene oxide bath for 10 minutes. Prepared exosome pellets were embedded in half propylene oxide/half embed 812 resin and cured in a 60°C oven overnight. 80 nm sections were cut using a Leica UC7 ultramicrotome and were picked up on copper thin bar 300-mesh grids and contrasted with 4% uranyl acetate and Sato's lead stain. Samples were examined using a transmission electron microscope JEOL JEM-1400 TEM at 80 KV. Images were captured using a digital camera.

Exosome Uptake and Fluorescent Microscopy. Exosomes were labeled with PKH67 Green Fluorescent Cell Linker (Sigma Aldrich, St. Louis, MO) in a modified protocol as described previously (Atay, Banskota et al. 2014). A2780 cells were labeled with

PKH26 Red Fluorescent Cell Linker (Sigma-Aldrich) according to manufacturer's instructions. Adhered A2780 cells were exposed to exosomes at a concentration of 1 $\mu\text{g}/10,000$ cells plated in time points of 0.5, 1, 2, and 24 hours. Media was removed upon completion of all time points and cells were gently washed once with PBS and fixed with 4% paraformaldehyde. After washing with PBS, cover slides were attached using a mounting medium containing DAPI. 3-6 images were taken of each time point and overlaid using MetaMorph Software (Molecular Devices, SunnyVale, CA).

Cell Viability Assays. Cells were treated with carboplatin (SelleckChem, Houston, TX) diluted from a 20 mM stock in PBS or PBS alone as a vehicle control. Cell viability was assessed using Cell Titer Blue (ThermoFisher), as previously published (Sethi, Pathak et al. 2012). Fluorescence was read using the Tecan Plate reader by 560/590 excitation/emission spectra. All assays were conducted in technical triplicates and replicated at least 2 times. Caspase 3/7 activity was conducted using Caspase-Glo[®] 3/7 (Promega), according to manufacturer's instructions. Statistical significance between experimental and control groups was determined using Student's T-test. Values of < 0.05 were considered significant.

Platinum Detection and Mass Spectroscopy. To detect levels of platinum in conditioned media, cell lysates, and exosomes we utilized inductively coupled plasma mass spectrometry (ICP-MS). To prepare media for Pt detection, 1 mL of exosome-depleted media was added to 9 mL of 2% trace metal grade nitric acid and sonicated for 30 minutes on ice. Exosome pellets were prepared by re-suspending the pellet in 75 μL

of sub-micron filtered water upon which 215 μL of 70% trace-metal grade nitric acid was added. For cellular lysate preparation 430 μL of 70% trace-metal grade nitric acid was added to 150 μL cellular lysate. Both cell lysates and exosome preparations were incubated in a 60°C water bath overnight. Platinum content was measured using the Agilent 7500 ICP-MS.

SDS Page and Western Blot Analysis. Exosome samples and cell lysates (prepared in Rippa buffer) were separated by adding 40 μg protein on 7%, 10%, and 4-20% Mini-PROTEAN® TGX™ Precast Gels, (BioRad, Hercules, CA) and transferred to a supported nitrocellulose membrane (BioRad). The membranes were blocked with 5% non-fat milk for one hour at room temperature. Primary antibodies for exosome characterization were anti-tumor suppressor gene 1 (TSG101-clone C2), anti-asparagine-linked glycosylation homolog-2-interacting protein (ALIX-clone 3A9), and anti-glucose regulated protein 78 kDa (GRP78-clone G-10) (all from Santa Cruz Biotechnology). Anti- β -Actin clone AC-74 was purchased from Sigma-Aldrich. Primary antibodies for EMT and TGF- β pathway analysis were; Mothers Against Decapentaplegic Homolog 4 (SMAD4), SMAD2 (clone 86F7), programmer of cell death 4 (PDCD4-clone D29C6 XP®), Zinc Finger E-Box Binding Homeobox 1(ZEB1- clone D80D3) and N-Cadherin were from Cell Signaling. TGF β I (lot 2344723) and TGF β II (lot 2283846) were purchased from Millipore (Temecula CA). Membranes were incubated with primary antibodies overnight and washed thrice for 10 minutes before addition of HRP conjugated anti-rabbit or anti-mouse secondary antibody (BioRad) for 1 hour at room temperature. After secondary incubation membranes were washed and treated with

ECL Western Blotting Substrate (Fisher Scientific) according to manufacturer's instructions.

Real time PCR. Cells were harvested at 80% confluency and RNA was extracted using Trizol and Direct-soltm RNA mini-prep system (Zymo Research, Irvine CA). Reverse Transcriptase was performed using the GoScriptTM Reverse Transcriptase System (Promega, Madison, WI) according to manufacturer's instructions. Real time PCR was conducted using SoSoGreen real time PCR Master Mix (BioRad). Primers for EMT-related genes were taken from Yew *et al* (Yew, Crow et al. 2013). For miR-21 identification, TaqMan Probes (Invitrogen) and RT-PCR primers specific to miR-21 (Invitrogen) were used according to manufacturer's instructions. Primers for primary miR-21 were designed as previously published (Davis, Hilyard et al. 2008).

Next Generation Sequencing

DNA Extraction. EOC cells at 80% confluency were trypsonized, collected, and washed 2x with cold PBS. DNA isolation was accomplished using the DNEasy blood and tissue kit (Qiagen, Hilden Germany) according to manufacturer's instructions.

Library Preparation. The TruSeq Amplicon Cancer Panel (Illumina) was used according to manufacturer's instructions. Amplicon libraries were generated by hybridizing pairs of oligonucleotides specific to targeted regions to each DNA sample. Unbound oligonucleotides were removed, and DNA polymerase and ligase were used to connect bound oligonucleotides by extension and ligation. Primers containing index sequences

for multiplexing and adapter sequences for cluster generation were used for PCR amplification. Libraries were quantified using the KAPA Library Quantification Kit (KAPA Biosystems, Inc.) specific to Illumina platforms and optimized for the Roche LightCycler 480 ii. Sample libraries were normalized and equal volumes pooled in the final multiplexed sequencing libraries.

Sequencing and Data Analysis. Pooled libraries were sequenced on a MiSeq System (Illumina) using a 2 x 150 paired-end format using the Custom Amplicon workflow. Base calls were generated on-instrument with the Real Time Analysis (RTA) software (Illumina). Reads were aligned to the Homo Sapiens – UCSC (h19) genome assembly and the Somatic Variant Caller (Illumina) was used for identification of variants. The Illumina Variant Studio software was used to annotate all detected variants. All alternate variant calls were required to have Q scores of at least Q30 and occur at a frequency of $\geq 15\%$.

Mutant Plasmid Generation and Transfection. The *SMAD4* plasmid (pcDNA FLAG-SMAD4M; Joan Massague, Addgene) was altered using the Quick Change II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) according to manufacturer's instructions. Primers were designed using the Quick Change Primer Design software (Agilent). All sequence validation was done using Sanger sequencing by GeneWiz, INC. Transfection of plasmids into A2780 cells was accomplished using Lipofectamine® 2000 (Invitrogen) according to manufacturer's instructions. Transfected cells were maintained in media containing 500 $\mu\text{g}/\text{mL}$ G418 (Corning).

Statistical Analysis. Statistical analysis was performed using the two tailed Student's t-test on both excel and Graph Pad Prism Programs and one-way ANOVA analysis on Graph Pad Prism.

RESULTS

Exosomes from carboplatin-resistant cells modify the platinum sensitivity profile of recipient cells.

Exosomes carry a range of nucleic acids and proteins and can have a significant impact on the phenotype of recipient cells as reported in melanoma, breast, non-small cell lung, and gastrointestinal stromal tumors (GIST) amongst others (Peinado, Aleckovic et al. 2012; Atay, Banskota et al. 2014; Xiao, Yu et al. 2014; Rodriguez, Silva et al. 2015; Gorczynski, Erin et al. 2016). For this phenotypic effect to occur, exosomes need to fuse with target cell membranes, either directly with the plasma membrane or with the endosomal membrane after endocytic uptake. To examine if exosomes isolated from platinum-resistant clones are taken up by the platinum-sensitive parental population we utilized the classic A2780 cell line and two independently-derived carboplatin resistant clones, C30 (IC₅₀ 325 µM) and CP70 (IC₅₀ 120 µM) (Louie, Behrens et al. 1985; Behrens, Hamilton et al. 1987; Godwin, Meister et al. 1992) (**Table 3.01**).

We first isolated exosomes from conditioned media of A2780, CP70, and C30 cells by ultracentrifugation (Johnstone, Bianchini et al. 1989; Raposo, Tenza et al. 1997). Isolated vesicles displayed between ~80-150 nm in size as determined by Nanoparticle Tracking Analysis (NTA) and scanning electron microscopy and contained common exosomal markers such as ALIX, TSG101, CD60, and the absence of either β-actin or GRP78 suggesting a true exosome population (**Figure 3.01**). To visualize uptake of exosomes by recipient cells, the exosomes were labeled with PKH67 green and the

cells with PKH red fluorescent membrane dyes. Recipient A2780 cells were then exposed to exosomes (1 $\mu\text{g}/10,000$ cells) derived from A2780 (autologous), C30, or CP70 cells for up to 24 hours. Regardless of the source, exosome uptake was rapid and uniform across all experimental groups (**Figures 3.02-3.04**). We have previously shown that the phenotypes of normal primary myometrial cells are dramatically altered towards the characteristics of donor tumor cells following uptake of tumor-derived exosomes from GIST cells and patient samples (Atay, Banskota et al. 2014). To investigate if a platinum-resistant phenotype could be transferred by this mechanism we treated platinum-naive A2780 cells (carboplatin IC_{50} 11 μM) with exosomes (0.5 μg exo/5,000 cells) from A2780 (autologous), CP70, C30, or vehicle (PBS) for 24 hours prior to treatment with 20 μM carboplatin for 48 hours (**Figure 3.05**). We observed a near 2-fold ($P < 0.05$) and 1.5-fold increase in viability of A2780 cells exposed to CP70-derived and C30-derived exosomes, respectively, as compared to autologous exosomes or PBS (**Figure 3.05**). Parallel analysis of caspase 3/7 activity by way of a fluorescent reporter assay revealed an average 20% decrease in cleavage of caspases 3 and 7 indicating reduced apoptosis in cells treated with C30- or CP70-derived exosomes as compared to controls (**Figure 3.05**).

To confirm this phenomenon is not limited to a select lineage of cells, we investigated the effects of exosomes derived from the unrelated, platinum-resistant OVCAR10 cell line (carboplatin IC_{50} 200 μM) on A2780 cells and the effects of C30- and OVCAR10-derived exosomes on the A1847 (IC_{50} 67 μM) and OVCAR5 (IC_{50} 44 μM) ovarian cancer cell lines. OVCAR10 was previously derived from a high-dose carboplatin and

cisplatin refractory advanced ovarian tumor (Hamilton, Lai et al. 1989), while A1847 and OVCAR5 were derived from untreated advanced ovarian tumors (Eva, Robbins et al. 1982; Godwin, Meister et al. 1992). Exosomes were isolated and characterized as before. Similar to previous observations, A2780 cells treated with OVCAR10-derived exosomes exhibited up to a 1.7-fold ($P < 0.05$) increase in viability as well as 50% decreases in caspase 3/7 activity following carboplatin treatment (**Figure 3.06**). In addition, we observed ~1.7 and ~2.0-fold increases in viability at 50 μM carboplatin for both A1847 and OVCAR5, respectively when treated with exosomes derived from C30 or OVCAR10 as compared to cells treated with autologous exosomes (**Figure 3.06**). In A1847 and OVCAR5 cells each group demonstrated a $\geq 50\%$ change in caspase 3/7 activity at 50 μM carboplatin concentrations in cells treated with C30- or OVCAR10-derived exosomes as compared to controls (**Figure 3.06**). Taken together our data demonstrate the development of platinum resistant disease may, in part, be mediated by cell-cell communication via exosomes.

Exosomes induce a protracted phenotypic change.

Recent studies by our lab and others have shown the effects of tumor-derived exosomes on recipient neighboring and distal cells can be both transient as well as prolonged and, in some cases, even exert a permanent phenotypic shift (Ogorevc, Kraljic et al. 2013; Abd Elmageed, Yang et al. 2014; Atay, Banskota et al. 2014). Therefore, we next asked if the acquired platinum-sensitivity profile was durable. We repeated the studies as indicated above and utilized an additional highly platinum-resistant cell line C200 ($\text{IC}_{50} > 500 \mu\text{M}$), which was derived from C30 by continuous

exposure to 200 μM cisplatin (Godwin, Meister et al. 1992). A2780 cells were exposed to autologous, CP70-, C30-, or C200-derived exosomes (1.0 μg exosome/10,000 cells plated), for 24 hours after which the media was replaced and cells were cultured for up to 14 days in standard conditions. Two-weeks after exosome exposure the recipient A2780 cells exposed to C200 or CP70-derived exosomes maintained more a resistant (~1.2-fold increase in viability, $P < 0.05$) phenotype as compared to controls. This increase in resistance was consistent across 20, 40, and 80 μM concentrations of carboplatin (**Figure 3.07**).

A2780 cells treated with platinum-resistant exosomes have enhanced exosome-mediated export of Pt.

Several mechanisms have been previously described relating exosomes to the development of chemotherapeutic resistance. These include the transfer or sequestering of miRNAs, transfer of oncogenic proteins or oncogene mRNA, and enhanced export of chemotherapeutic drugs including cisplatin and carboplatin (Atay, Banskota et al. 2014; Chen, Liu et al. 2014; Federici, Petrucci et al. 2014). To address the latter, we determined the ability of A2780 cells pre-exposed to autologous, CP70, and C200-derived exosomes to export Pt by way of exosomes. 500,000 A2780 cells were treated with 250 μg exosomes, (autologous, CP70, or C200) for 24 hours. The media was replaced and each group was treated with 100 μM Carboplatin. After 24 hours the media was removed, and depleted of exosomes. Exosomes, exo-free media, and cells were processed to remove organic material with 70% trace-metal grade nitric acid (see materials and methods for further detail), and total Pt was detected using

inductively coupled plasma mass spectrometry. A2780 cells exposed to CP70-derived exosomes had a significant ($P<0.05$) ~20% decrease in the amount of residual Pt within the media and a significant ($P<0.05$) ~25% decrease in the amount of cellular Pt (**Figure 3.08**). Importantly, A2780 cells exposed to CP70 exosomes had a near 6-fold ($P<0.005$) increase in the amount of Pt/ng exosome (**Figure 3.08**). Considering A2780 cells exhibit a loss of sensitivity to carboplatin upon uptake of CP70-derived exosomes, these data suggest that the uptake of exosomes from more platinum-resistant cells could be enhancing the ability of the recipient cell to export Pt. However, only A2780 cells treated with CP70-derived exosomes exhibited this sort of phenomenon, therefore we investigated additional exosome-mediated mechanisms, which could result in changes in platinum-sensitivity profiles.

Exosomes derived from platinum-resistant cells trigger EMT in platinum-sensitive A2780 cells.

It is now widely accepted that EOC cells utilize EMT as a mechanism of escape from the deleterious effects of platinum-based chemotherapy (Latifi, Abubaker et al. 2011; Kurokawa, Ise et al. 2013; Marchini, Fruscio et al. 2013). In addition, exosomes have been shown to carry cargo which triggers EMT-like changes in breast, colorectal, and urinary cancers (Galindo-Hernandez, Serna-Marquez et al. 2014; Franzen, Blackwell et al. 2015; Philip, Heiler et al. 2015). We therefore chose to investigate the effects of exogenous exosome exposure on changes in EMT characteristics. A2780 cells were treated with exosomes as above, and evaluated for induction of EMT, including mRNA and miRNA expression and morphological changes. A2780 cells exposed to platinum-

resistant exosomes for 24 hours demonstrated a 2-5-fold down-regulation in the expression of epithelial markers *dystroglycan* and *E-cadherin* and a 2-fold decrease in *EpCAM* as compared to control (**Figure 3.09**). We also observed significant ($P < 0.05$) increases in the mesenchymal markers *occludin*, *paladan*, and *twist*, as well as a 70-fold increase in *vimentin* in cells treated with C200-derived exosomes (**Figure 3.09**). In addition, we observed A2780 cells treated with exosomes from platinum-resistant cells displayed a 2-5-fold decrease in *KLF4* (which has been shown to regulate EMT in multiple types of cancers) (Cui, Shi et al. 2013; Tiwari, Meyer-Schaller et al. 2013; Chen, Wang et al. 2014; Li, Wang et al. 2014) as compared to control (**Figure 3.09**).

MicroRNA21 (miR-21) has also been shown to regulate EMT in cancers, such as breast, lung, and clear cell renal (Luo, Ji et al. 2014; De Mattos-Arruda, Bottai et al. 2015; Cao, Liu et al. 2016). We found that CP70, C30, and OVCAR10 cells have 18-, 5-, and 6-fold increases in miR-21 as compared to A2780 cell lines, respectively (**Figure 3.10**). Importantly, A2780 cells treated with CP70- and OVCAR10-derived exosomes have nearly a 4-fold increase in miR-21 as compared to cells treated with PBS or autologous-derived exosomes (**Figure 3.10**). Furthermore, there were no significant changes in primary miR-21 (pri-miR-21) levels (**Figure 3.10**), suggesting elevated miR-21 levels are induced following uptake of exogenous exosomes.

We further observed A2780 cells displayed an increase in spindle-like mesenchymal morphology only when exposed to C30- and C200-derived exosomes, and to a lesser extent, CP70-derived exosomes (**Figure 3.11**). Taken together these data demonstrate

the transfer of a resistant phenotype may be driven, in part, through transfer or upregulation of factors, which contribute to EMT.

Alterations in TGF- β /SMAD signaling effect platinum sensitivity through activation of EMT.

To identify genomic alterations potentially associated with platinum-sensitivity in EOC, DNA sequencing was performed using a panel of cancer associated genes (TruSeq[®] Amplicon Cancer Panel; Illumina) in a series of EOC cell lines, including our experimental lines mentioned above (**Table 3.02**). We observed several mutations specific to platinum-resistant cells including: VEGF receptor 2 (*KDR*) G1348, which was present in OVCAR10, C30), and CP70 cells, Epidermal Growth Factor Receptor (EGFR) C797S and KRAS V125L and D132E which were observed in OVCAR10 cells (**Table 3.02**). Most interestingly, and the basis of much of this thesis, we identified previously unreported somatic mutations in the MH2 domain of *SMAD4*, but only in the three highly resistant cell lines, CP70, C30, and OVCAR10 (**Table 3.02**). *SMAD4* is a key component of the Transforming Growth Factor- β /SMAD signaling pathway and is a known mediator of EMT responses (**Figure 3.12**) (Valcourt, Kowanetz et al. 2005). Interestingly the receptor complex is taken in to the cell via endocytosis and many components of TGF- β /SMAD signaling can be found within and on exosomes (**Figure 3.13**). All three of our most platinum-resistant cell lines contained a common *SMAD4*^{S344I} mutation (OVCAR10 was homozygous for the S344I mutation), while C30 and CP70 each harbor an additional acquired mutation (*SMAD*^{S411C} and *SMAD*^{G508A}, respectively). Because these mutations were only identified in highly resistant cell lines

with prior exposure to cisplatin these initial findings suggest that a loss of function in SMAD4 may contribute or correspond with a loss of platinum sensitivity.

We next used *in silico* analysis to determine the potential functional impact of these mutations (Reva, Antipin et al. 2011). *SMAD4* Q388R which is a missense change discovered in the platinum-sensitive cell line OVCAR4 received a score of 0.54 and, therefore, was predicted to have a neutral (no) effect on protein function. The *SMAD4*^{S344I} mutation, which is common to all 3 platinum resistant cell lines, was assigned a value of 2.765 and predicted to have a considerable effect on function. The second acquired mutations identified only in CP70 (G508A) and C30 (S411C) were predicted to have a high impact on SMAD4 function (ranked at 3.365 and 3.395, respectively) (**Figure 3.14**).

Mutant SMAD4 cell lines and exosomes elicit a resistant phenotype in recipient cells.

To provide direct evidence that mutations in *SMAD4* contribute towards a loss of platinum-sensitivity, we generated SMAD4 mutation specific plasmids using the Quick Change II Site-Directed Mutagenesis Kit (Agilent) (**Figure 3.14**). We exogenously overexpressed these plasmids *SMAD4*^{S344I}, *SMAD4*^{S411C}, or *SMAD4*^{WT} in A2780 cell to generate A2780^{S344I}, A2780^{S411C}, and A2780^{WT} cell lines, respectively (**Figure 3.14-3.15**). Of these cell lines A2780^{S344I} demonstrated a significant increase (P<0.05) in viability at 20 and 40 μM concentrations of carboplatin as compared to A2780^{WT} (**Figure 3.16**). A2780^{S344I} cells also exhibited an increase in mesenchymal markers N-Cadherin and ZEB1 as well as a decrease in the mediator of apoptosis, Programmer of Cell

Death 4 (PDCD4) (**Figure 3.16**), which is known to regulate EMT (Wang, Zhu et al. 2013). We also observed a more mesenchymal phenotype upon exposure of A2780^{S344I} cells to 20 and 40 μ M carboplatin as compared to A2780^{WT} (**Figure 3.17**).

To determine if SMAD4^{mut} cell lines produce exosomes capable of transferring a platinum-resistant phenotype, we isolated exosomes from A2780^{WT} and A2780^{S344I} cells as described above. Parental A2780 cells were then exposed to A2780^{S344I}-derived exosomes as well as A2780^{WT}-derived exosomes, or PBS. Cells exposed to A2780^{S344I}-derived exosomes exhibited a 3-fold increase in viability over both controls after exposure to 10 μ M carboplatin for 72 hours (**Figure 3.18**). In addition, A2780^{S344I}-exosome exposed cells exhibited a nearly 7-fold increase in their IC₅₀ values as compared to cells treated with A2780^{WT}-exosomes (8.2 μ M vs. 55.5 μ M, respectively) ($p=0.0212$) (**Figure 3.18**).

Taken together, these data provide the first evidence that mutations, specifically within *SMAD4*, enhance the native chemo-resistance profile of EOC. Specifically, cells harboring these mutations have survival advantages by way of increased EMT in response to carboplatin. In addition, these cells generate and secrete exosomes capable of modifying the platinum-sensitivity profile of surrounding neoplastic cells, which enhances the development of platinum-resistant disease.

DISCUSSION

Because the ovarian tumor mass is composed of a heterogeneous population of cells with a high degree of individual morphologies and genomic instability the development of platinum-based chemotherapy resistance is multifactorial and frequently due to a variety of changes in specific proteins, genes, or in gene regulation that are advantageous to chemotherapy resistance (Stewart 2007; Eckstein, Servan et al. 2009; Eitan, Kushnir et al. 2009; Cohen, Bruchim et al. 2012; Guddati 2012; Mir, Tortosa et al. 2012; Shang, Lin et al. 2012; Barr, Gray et al. 2013; Diaz-Padilla 2013). It is commonly believed that chemotherapeutic treatment for cancer causes or selects for intrinsically resistant populations of cells, which possess one or more of these advantages. For example, Stronach and colleagues have shown that in response to cisplatin, DNA-dependent protein kinase (DNA-PK) selectively phosphorylates AKT-S473 in the nucleus of platinum-resistant, but not sensitive cells, leading to the clinical development of platinum-resistant disease (Stronach, Chen et al. 2011). Whether by selection of resistant clones or infliction of secondary aberrations, some tumor cells survive primary rounds of therapy, and eventually give rise to new cells, which form more therapy resistant tumors. Here, we have investigated how drug resistant populations of cells perpetuate the development of platinum-resistant disease in ovarian cancer via exosomal mediated cell-cell communication. It is well understood that intracellular transport of genetic material including miRNAs and mRNAs as well as biologically active proteins from one cell to another occurs via microvesicles (Boelens, Wu et al. 2014; Chen, Cai et al. 2014; Chen, Liu et al. 2014; Federici, Petrucci et al. 2014; Lv, Zhu et al.

2014; Takahashi, Yan et al. 2014). We and others have shown that extracellular vesicles may be involved in transformation of surrounding cells via transport of miRNAs and oncogenic proteins (Roberson, Atay et al. 2010; Atay, Banskota et al. 2014; Rodriguez, Silva et al. 2015; Zhang, Zhang et al. 2015; Maida, Takakura et al. 2016). Our current findings suggest that, as an ovarian tumor develops and evolves both genetically and epigenetically, it acquires properties that allow it to escape therapeutic attack. We proposed that exosomes may be a previously unappreciated factor, which contribute to the advancement of drug resistant disease and thus disease progression.

We present evidence demonstrating that exosomes derived from platinum-resistant EOC cells can transfer a portion of their chemo-resistant phenotype to platinum-sensitive cells and this increase in resistance corresponds with EMT and mutations in *SMAD4*. *SMAD4* has been shown to be necessary for the transcription of EMT related genes through *SMAD4/SMAD3* signaling (Do, Kubba et al. 2008) and EMT has been well established as a mediator of drug resistance in ovarian cancer (Helleman, Smid et al. 2010; Latifi, Abubaker et al. 2011; Marchini, Fruscio et al. 2013; Smolle, Taucher et al. 2014). Here we provide the first evidence that mutations within the *SMAD4* gene directly affect the platinum-sensitivity of EOC cells. In addition, we are the first to report that cells harboring these mutations produce exosomes capable of transferring this resistant profile.

TGF- β /SMAD signaling components are found within and on exosomes including SMAD2, SMAD3, SMAD4, TGF β r1 and TGF β r2 (**Figure 3.01 & 3.13**);

however, a momentary transfer of protein alone does not provide sufficient evidence to explain a persistent phenotypic switch. Evidence suggests that the transfer of exosomal mRNAs between cells may lead to more prolonged phenotypic changes (Deregibus, Cantaluppi et al. 2007; Valadi, Ekstrom et al. 2007). We have identified very low levels of the *SMAD4* mRNA within a portion of exosomal samples; however, we were unable to sequence this transcript, suggesting that the whole *SMAD4* mRNA transcript may not be present. Given this information, and the fact that SMAD4 is a transcription factor, we speculate that the exosomal contents responsible for the observed morphological changes may be products of aberrant TGF- β /SMAD signaling.

Current evidence suggests that miR-21 may both be a product of and mediator of TGF- β signaling in many pathological conditions (Garcia, Nistal et al. 2015; Lai, Luo et al. 2015; Han, Wang et al. 2016). We identified a 3- to 4-fold increase in miR-21 expression in platinum-sensitive cells treated with platinum-resistant exosomes and in the parental platinum-resistant cells. Up-regulation of miR-21 in multiple types of cancers, including ovarian, positively correlates with increased EMT (Wang, Gao et al. 2014; Zhang, Pan et al. 2014; Zhao, Tang et al. 2014). Therefore, a complementary mechanism to alter platinum sensitivity is exosome-induced up-regulation of primary miR-21 (pri-miR-21) in recipient cells. Of relevance, recent studies by Davis and colleagues demonstrate that SMAD proteins can bind to the Drosha complex, resulting in the increase processing of pri-miR-21 to pre-miR-21 (Davis, Hilyard et al. 2008). Indeed, we observed increased miR-21 in A2780 cells treated with platinum-resistant exosomes but no significant changes in pri-miR21 suggesting increased miRNA

processing may be an important factor in EOC. Although exciting, further research is warranted to further define the exosomal cargo that is directly responsible for influencing platinum sensitivity.

In summary, we propose a new mechanism by which tumor cell-cell cross talk may actively enhance chemotherapy resistance throughout treatment. It is important to note that the experiments conducted here are limited to a single exosome treatment, and do not mimic the potentially constant exposure to exosomes which likely occurs *in vivo*. This is especially relevant in EOC, where neoplastic cells have enhanced exosomal output as compared to normal epithelial cells (Gercel-Taylor, Atay et al. 2012). Taken together, this work underscores the importance of cell-cell communication in the advancement of platinum resistant disease and provides novel insight as to how exosomal transfer further enhances EMT in response to frontline chemotherapy.

Table 3.01 EOC Cell Line Carboplatin IC₅₀ Values

Cell Line	IC₅₀ (μM)	Fold Change (Over A2780)
A2780	11	1
OVCAR5	44	4
A1847	67	6
CP70	120	11
OVCAR10	200	18
C30	325	30
C200	>500	>50

Table 3.02 Mutations Identified With the TruSeq Amplicon Cancer Panel

	OVCAR 10	AZ780	CP70	C30	A1847	PEO1	PEO4	OVCAR 3	OVCAR 4	OVCAR 5	OVCAR 8	SKOV3
<i>PIK3- CA</i>				Missense _K711R	Missense _K711R					Missense _N444K NC Missense _K711R		Missense _H1047R
<i>KDR</i>	Missense_ G1348E		Missense _G1348E	Missense _G1348E		NC Missense_ Q472H	NC Missense _Q472H		Nonsense _R275X Missense_ L242P		NC Missense _Q472H	NC Missense _Q472H
<i>EGFR</i>	Missense_ C797S											
<i>KRAS</i>	Missense_ V125L Missense_ D132E				Missense _P121H					Missense _G12V	Missense _G12V	
<i>TP53</i>	FS Missense_ V40F		Missense _V40F	Missense _V40F	Missense _P72R	Missense_ G112D	Missense _G112D	Missense _P72R Missense _R116Q	Missense_ L130V Missense_ P72R	Missense _P72R	Missense _P72R	FS
<i>SMAD4</i>	Missense_ S344I		Missense _S344I Missense _G508A	Missense _S344I Missense _S411C				NC	Missense_ Q388R Unknown_ 319			

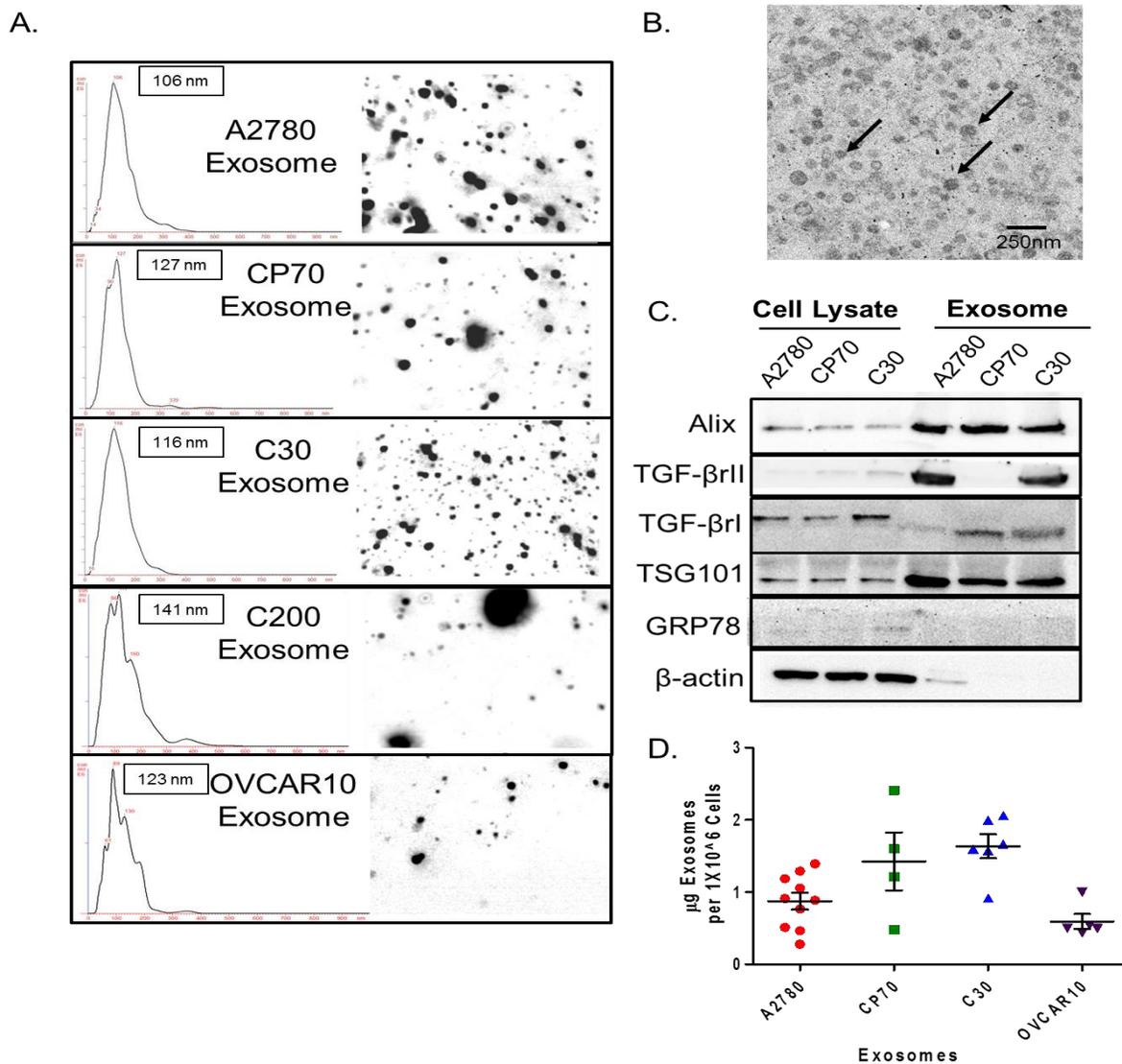


Figure 3.01 Characterization of EOC Derived Exosomes. **A.** Nanoparticle analysis of exosomes derived from EOC cell lines A2780, CP70, C30, C200, and OVCAR10 shows particle size distribution. **B.** TEM of representative exosome sample show exosomes of 80-100 nm (bottom and top arrow respectively) in size. **C.** Western blot analysis of exosome isolates as compared with cellular lysates. β -actin is used as a loading control for cell lysates. **D.** Average concentrations of exosomes in μ g isolated from $\sim 1 \times 10^6$ cells.

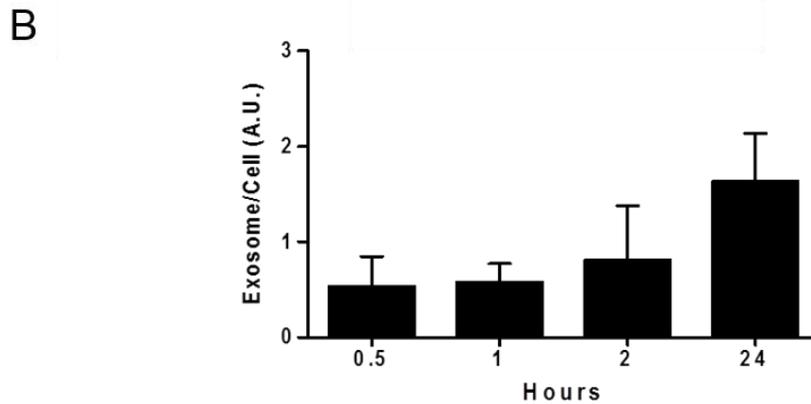
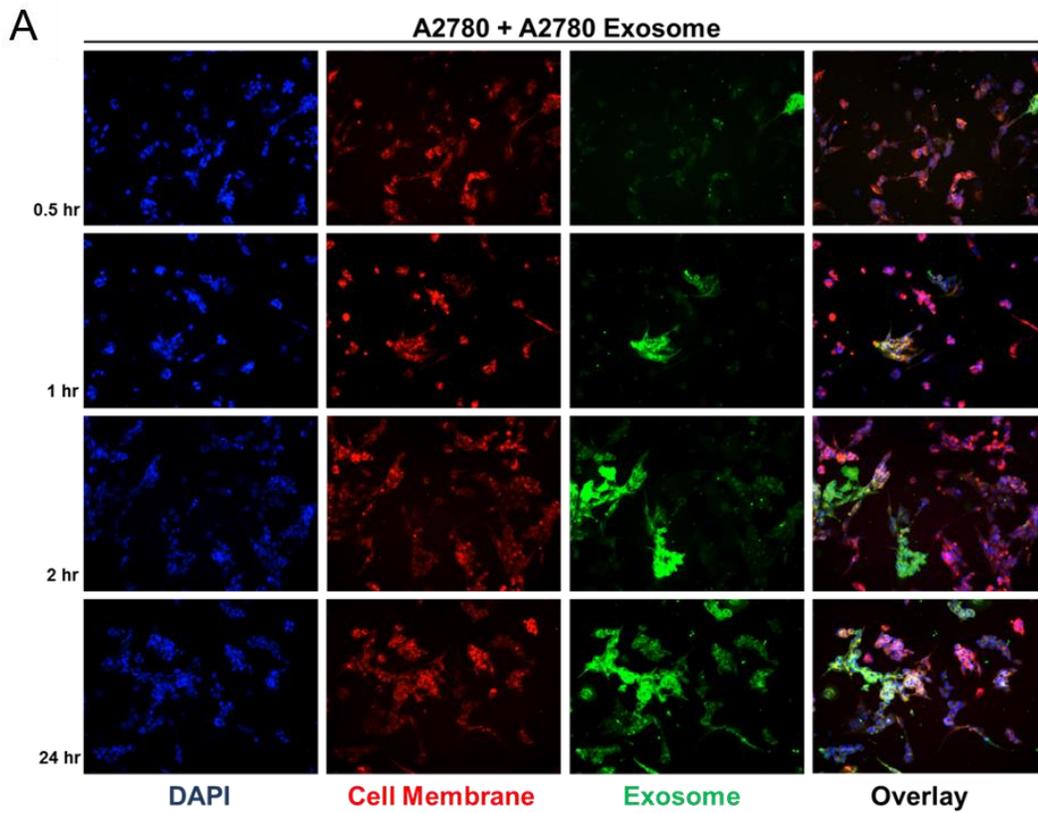


Figure 3.02 A2780 Cell Uptake of A2780-Derived Exosomes. **A.** Representative fluorescent microscopy images of dapi (blue), cell membrane (red), exogenous exosomes (green), after exosome addition at 0.5, 1, 2, and 24 hours. **B.** Average of a minimum of 3 images describing density of exosome/density of cell at each time point. Error bars represent standard error of the mean.

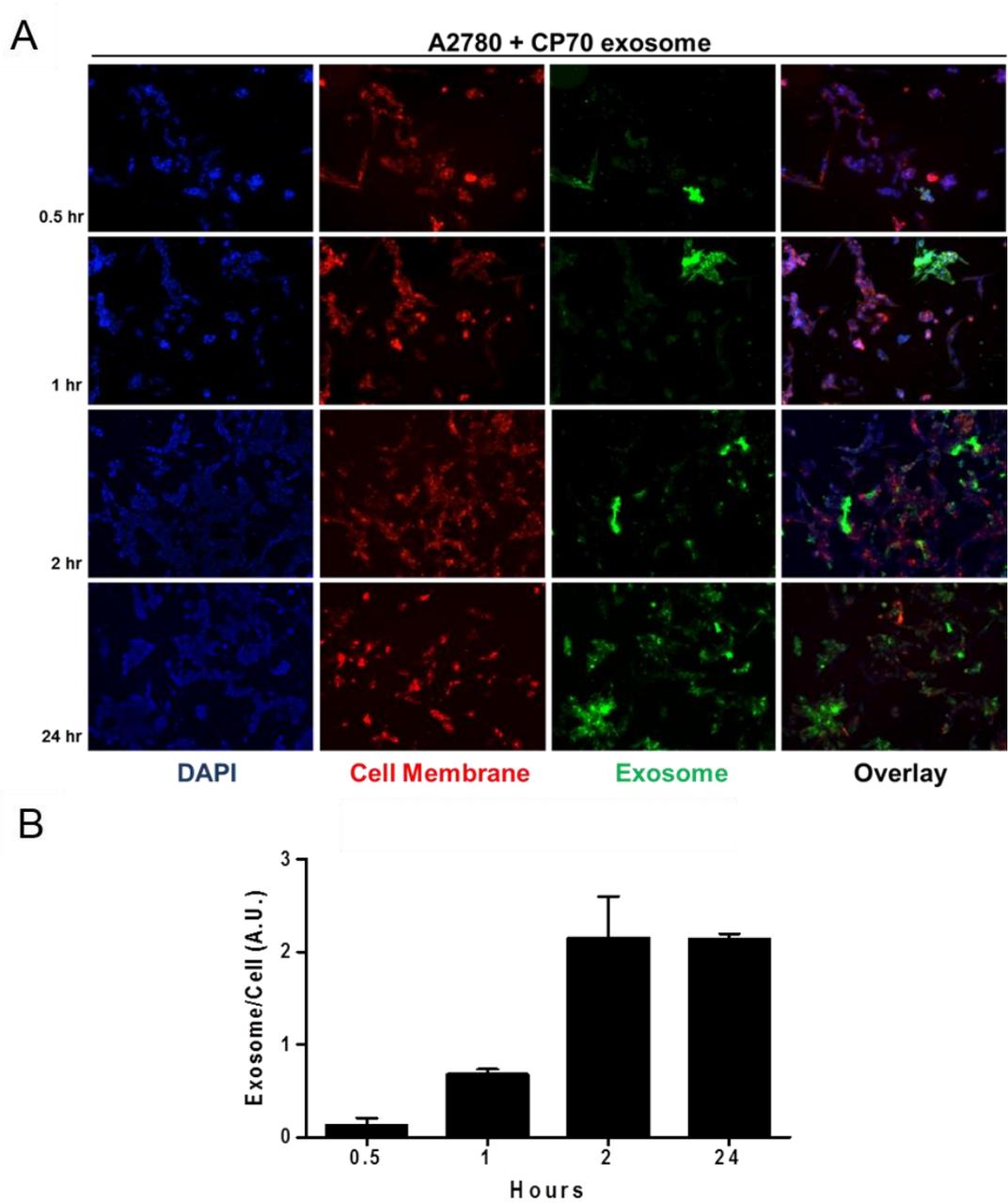


Figure 3.03 A2780 Cell Uptake of CP70-Derived Exosomes. **A.** Representative fluorescent microscopy images of dapi (blue), cell membrane (red), exogenous exosomes (green), after exosome addition at 0.5, 1, 2, and 24 hours. **B.** Average of a minimum of 3 images describing density of exosome/ density of cell at each time point. Error bars represent standard error of the mean.

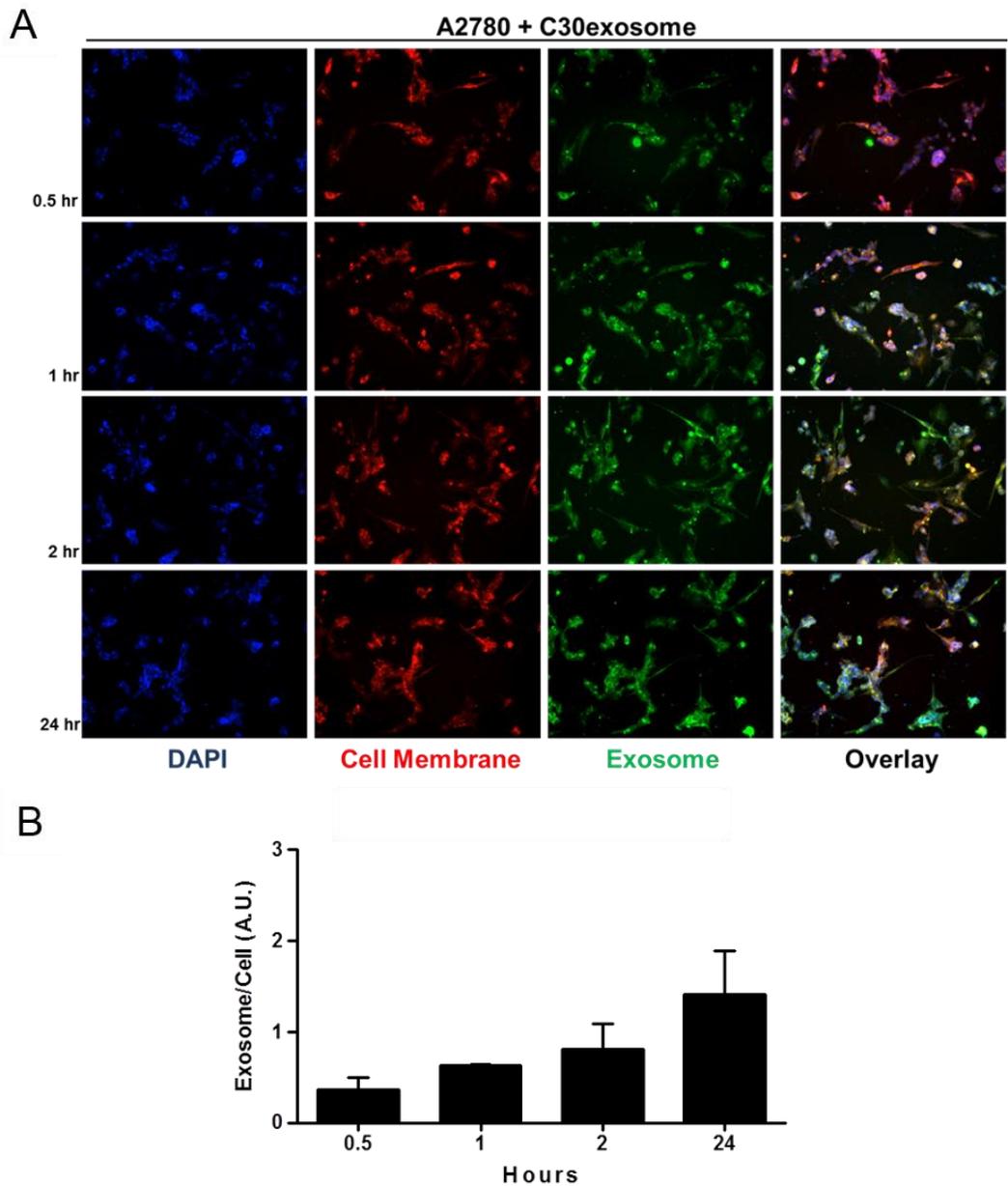


Figure 3.04 A2780 Cell Uptake of C30-Derived Exosomes. **A.** Representative fluorescent microscopy images of dapi (blue), cell membrane (red), exogenous exosomes (green), after exosome addition at 0.5, 1, 2, and 24 hours. **B.** Average of a minimum of 3 images describing density of exosome/ density of cell at each time point. Error bars represent standard error of the mean.

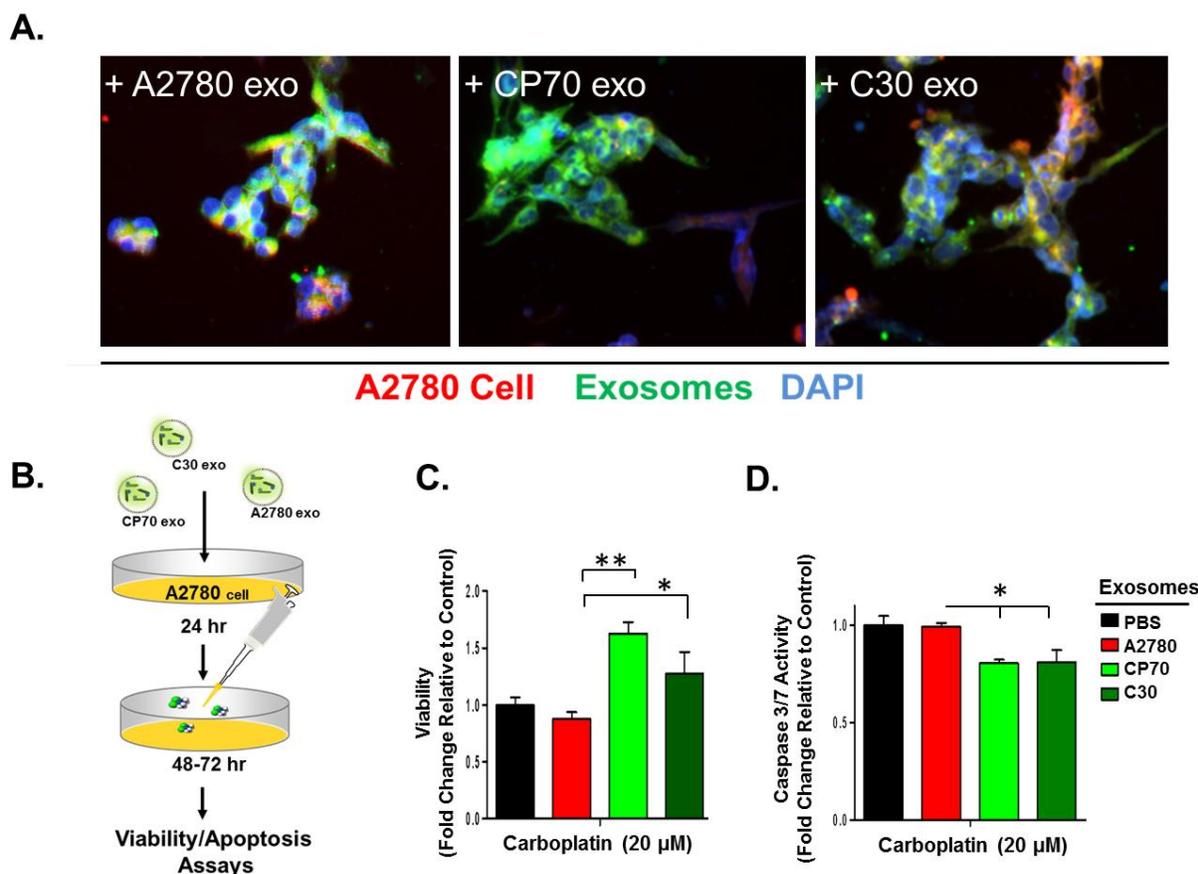


Figure 3.05 Exosome Transfer Corresponds with a Loss of Sensitivity to Platinum. A. Overlay of A2780 cells (Red) exposed to exosomes (Green) derived from A2780, CP70, or C30 cell lines for 24 hours. DAPI (Blue) is included. **B.** Illustration of experimental protocol. **C.** Viability of A2780 cells pre-treated with PBS (Black), or exosomes derived from A2780 (Red), CP70 (Green), or C30 (Dark Green) cell lines followed by exposure to carboplatin. **D.** Caspase 3/7 activity as measured using Caspase Glo[®] luminescent reporter system in A2780 cells exposed to the same conditions as **C.** All values are normalized to vehicle. Fold changes are relative to control (PBS – no exosome group) *P<0.05, **P<0.01

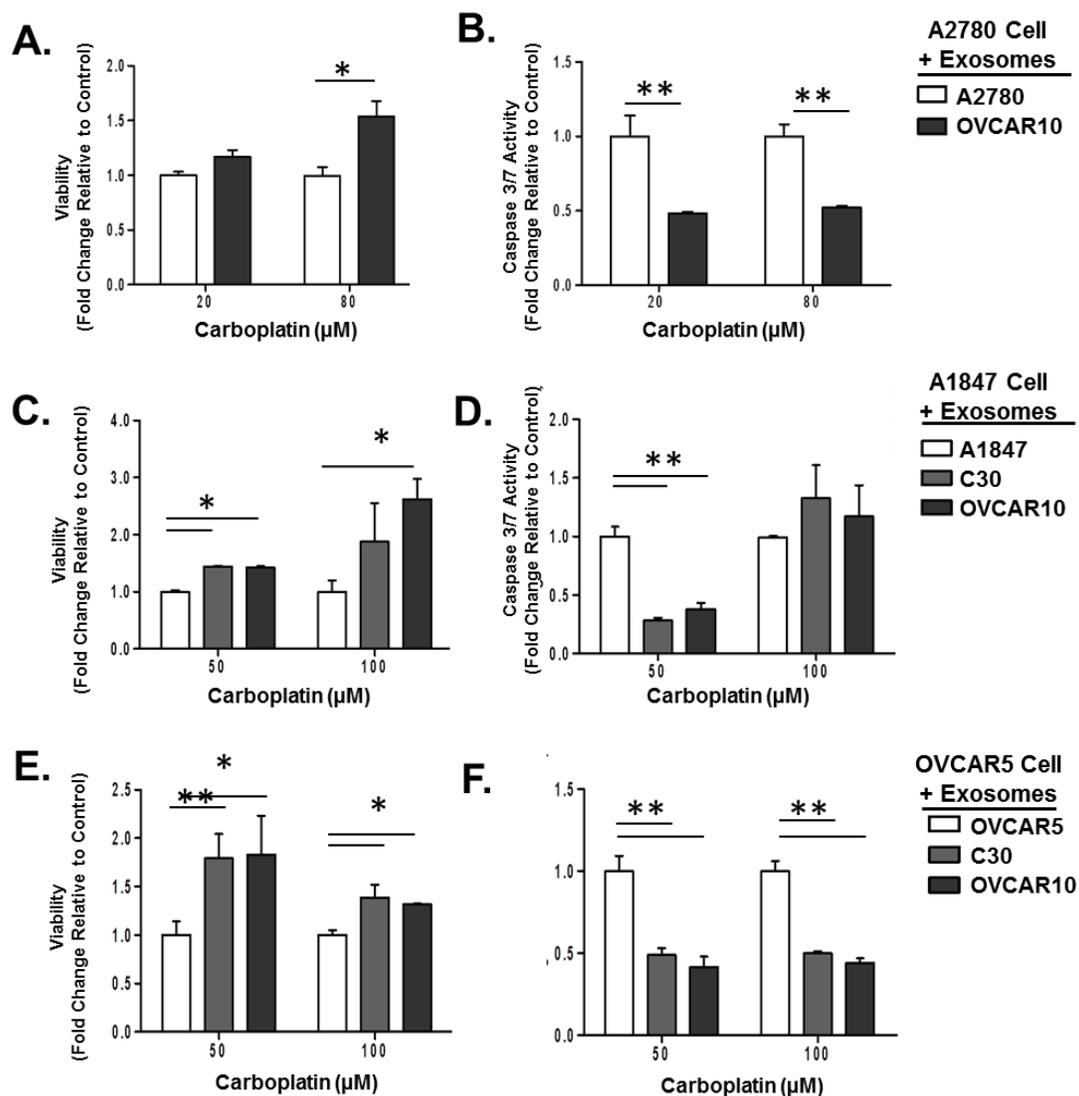


Figure 3.06 Transfer of a Platinum-Resistant Phenotype Extends to Other EOC Cells.

A,C,E. Viability of platinum-sensitive cell lines A2780, A1847 and OVCAR5 with or without exogenous exosomes derived from either self/autologous (A2780, A1847, or OVCAR5) or C30 or OVCAR10 cell lines and treated with carboplatin for 48 hours. All values are normalized to vehicle. Fold-change is relative to control (autologous treated exo group) **B,D,F.** Caspase 3 and 7 activity as measured by Caspase-Glo luminescent assay in the same conditions as above. All values are normalized to viability. Fold-change is calculated relative to control. *P<0.05, **P<0.005

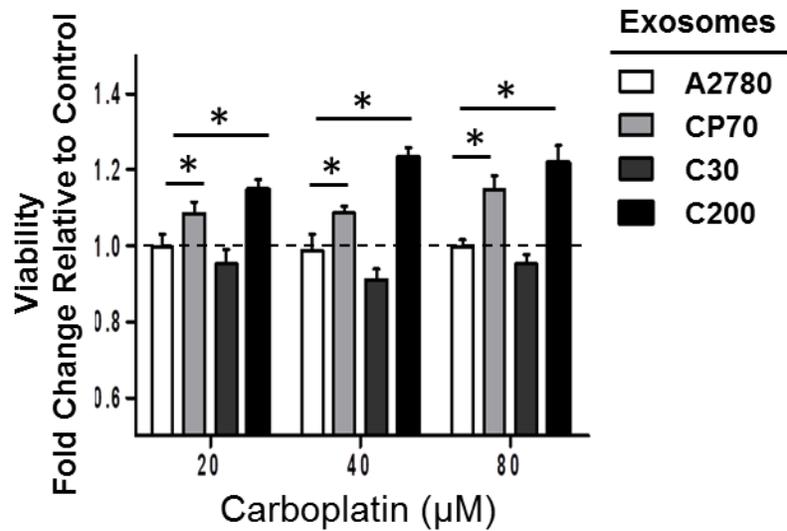


Figure 3.07 Extended Effects of Exosome Uptake. Viability after 72 hours of carboplatin treatment of A2780 cells following a 24 hour exposure to exosomes derived from A2780 (autologous), CP70, C30, or C200 cell lines and normal culture conditions for two weeks. All values are normalized to vehicle. Fold-change is relative to control (A2780-exo group) *P<0.05

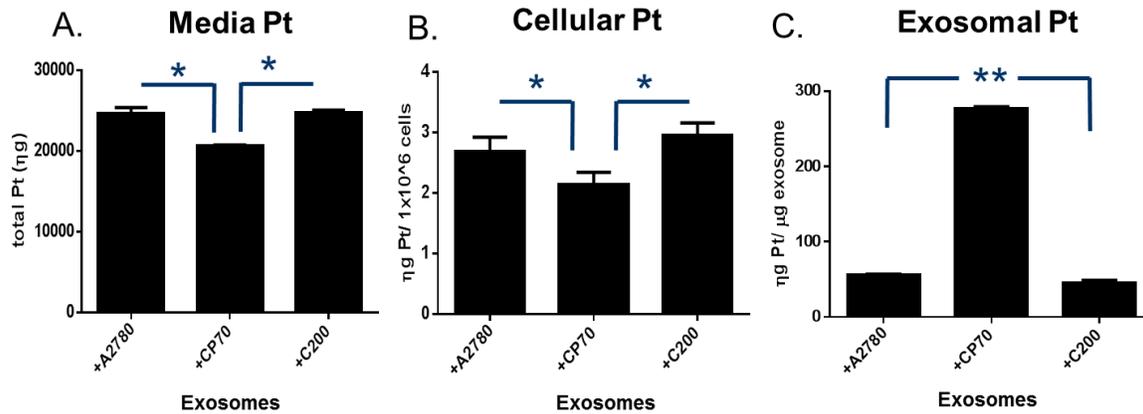


Figure 3.08 A2780 Cells Have Enhanced Exosome-Mediated Pt Export with Prior Exposure to CP70-Exosomes. A2780 cells were treated with either self, CP70, or C200 derived exosomes for 24 hours and then exposed to 100μM Carboplatin for 24 hours. A. ICP-MS analysis of the platinum content in the samples revealed significantly less platinum in the media of A2780+CP70exo than other experimental groups. B. A2780+CP70 exo also had less platinum content per 1×10^6 cells as compared to cells treated with self or C200 derived exosomes. C. Exosomes isolated from the media of A2780+CP70exo group contained approximately 4-fold more platinum per μg exosome than exosomes derived from A2780+self (autologous) or A2780+C200. (* = $P < 0.05$, ** = $P < 0.005$)

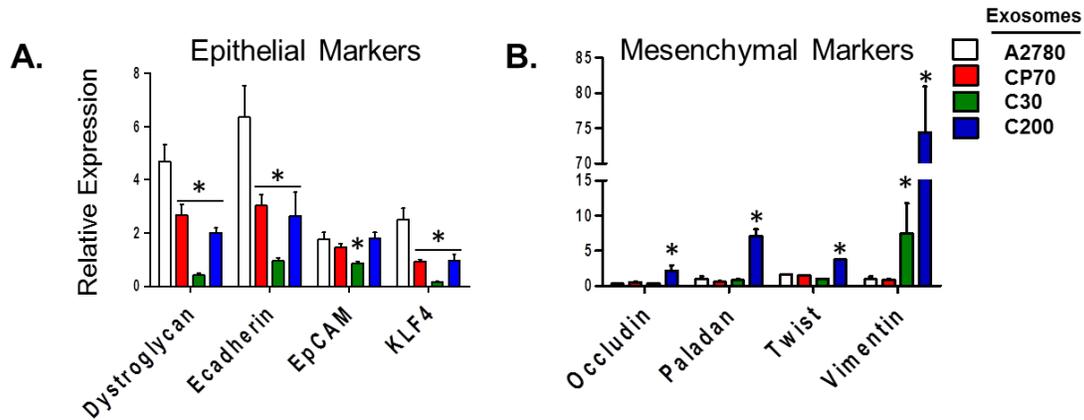


Figure 3.09 A2780 Cells Undergo EMT When Treated with Platinum-Resistant Exosomes. **A,B.** Real-time PCR of epithelial (**A**) and Mesenchymal (**B**) mRNA markers cDNA from A2780 cells treated with either; A2780- (autologous), CP70-, C30-, or C200-derived exosomes for 24 hours. All values are given as cDNA levels (*Dystroglycan*, *ECadherin*, *EpCAM*, *KLF4*, *Occludin*, *Paladan*, *twist*, and *Viamentin*) relative to two housekeeping genes (*GAPDH* and *GUSB*).

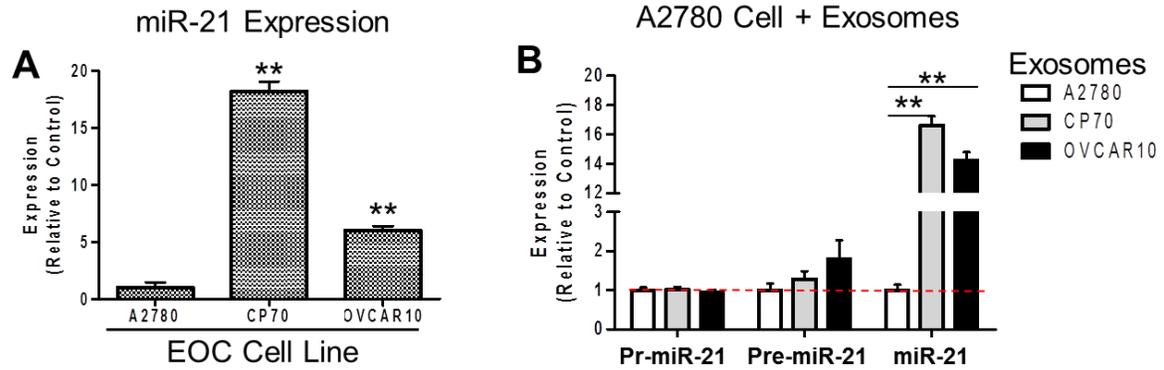


Figure 3.10 Platinum-Resistant EOC Cells Have Altered miRNA21 Expression. **A.** Real-time PCR of mature miR-21 level in A2780, CP70, and OVCAR10 cell lines. **B.** Real-time PCR of primary miR-21, Pre-miR-21, and mature miR-21 levels in A2780 cells treated with exosomes from A2780 (autologous), CP70, or OVCAR10. Primary miR-21 and Pre-miR-21 expression is relative to two housekeeping genes (*GAPDH* and *GUSB*). Mature miR-21 expression is relative to U6. Error bars represent standard error of the mean. *P<0.05, **P<0.01

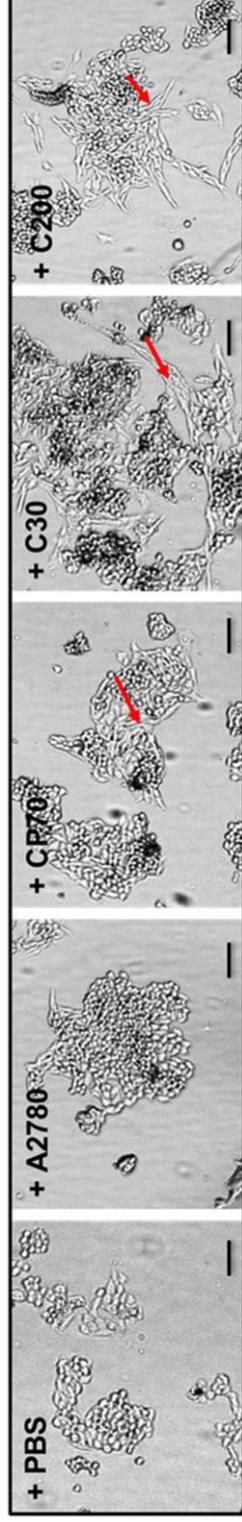


Figure 3.11 A2780 Cells Exhibit a Mesenchymal Phenotype after Exposure to Platinum-Resistant Exosomes. Bright field images of A2780 cells treated with PBS (vehicle) or A2780- (autologous), CP70-, C30-, or C200-derived exosomes detailing enhanced spindle-like and mesenchymal morphology in cells treated with platinum-resistant exosomes as compared to control (arrows). Scale bar = 100 μm .

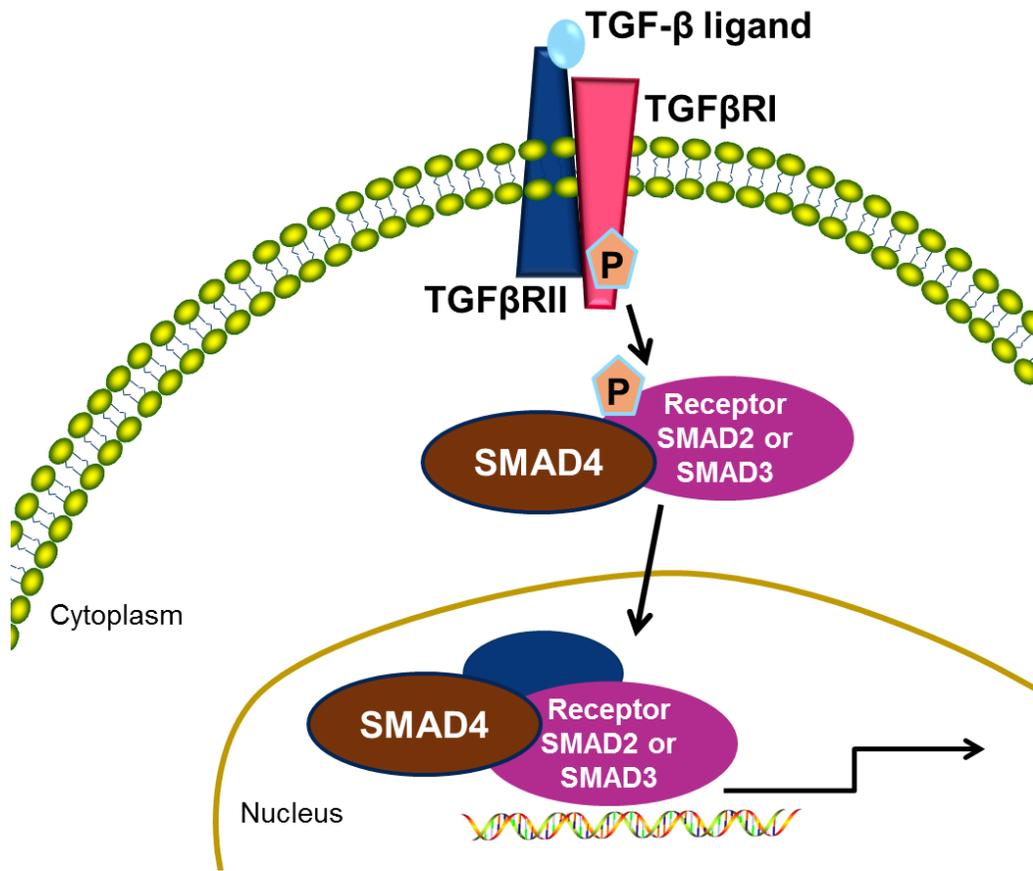


Figure 3.12 The TGF-β/SMAD Signaling Pathway. Schematic representation of TGF-β/SMAD Signaling.

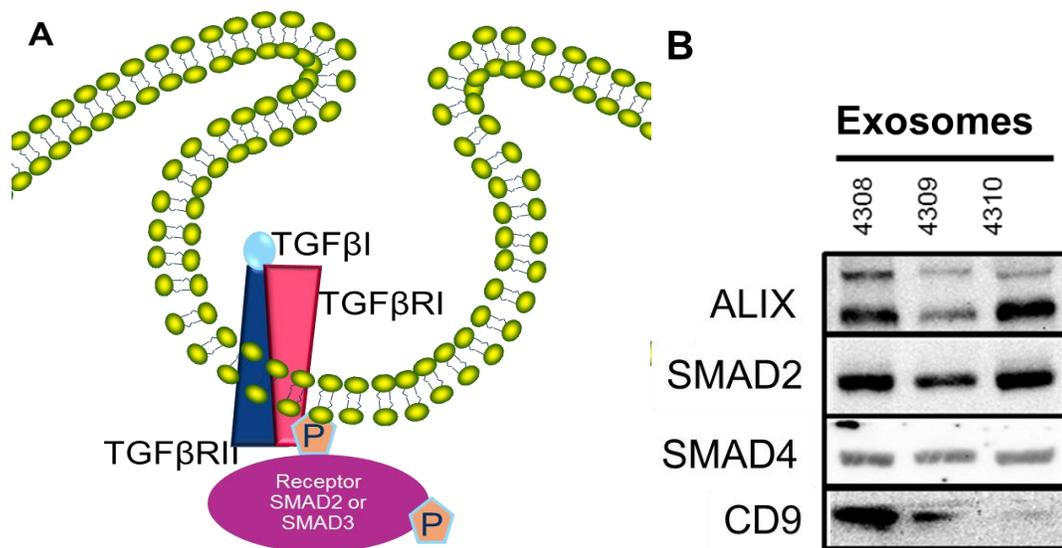


Figure 3.13 SMAD4 Components Within Exosomal Samples. **A.** The TGF- β /SMAD signaling complex is taken in by endocytosis (Anders, Arline et al. 1997). **B.** Western blot analysis of SMAD4 and SMAD2 on exosomes isolated from healthy human serum, n=3. ALIX and CD9 are exosomal markers.

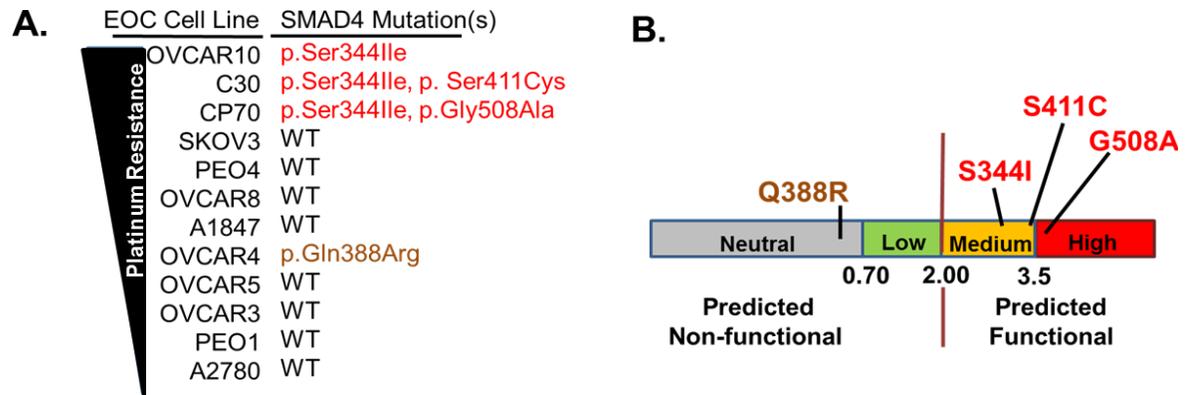


Figure 3.14 Mutations in SMAD4 are Associated with Platinum Resistance. **A.** Illustration of SMAD4 mutations identified in EOC cell lines ranked according to platinum-resistance (Top – most resistant, Bottom – least resistant). **B.** Functional impact scores as predicted using Mutation Assessor, which mathematically assesses functional impact based upon an algorithm combining the overall conservation of the gene with the specificity of the mutation. Values <0.70 are considered neutral, Values between 0.71 and 2.0 are considered of low and values between 2.1 and 3.5 and >3.6 are considered to have medium and high impact, respectively.

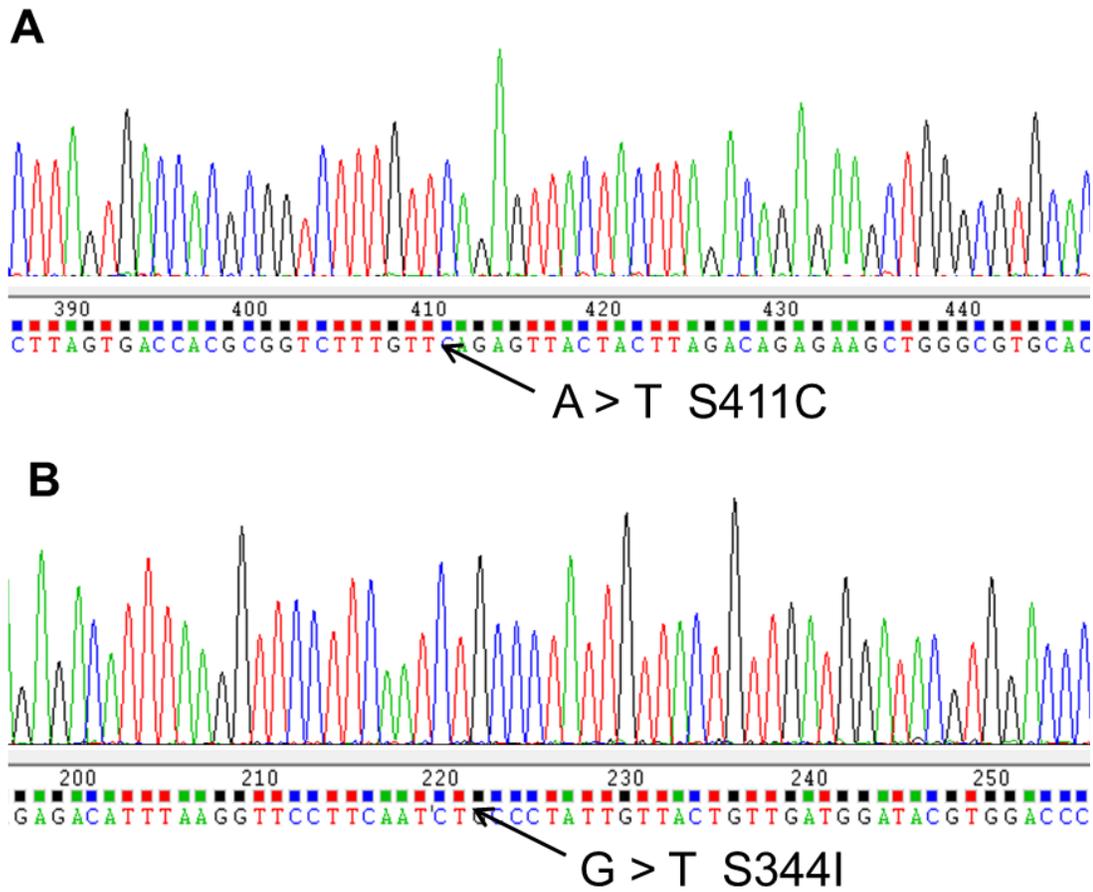


Figure 3.15 Validation of Mutant Plasmids. Specific point mutations were introduced into a SMAD4 plasmids using the Quick Change II sight directed mutagenesis technology (Agilent) to replicate the mutations found in the platinum-resistant cell lines. Sanger sequencing results demonstrate successful mutagenesis replicating S411C (**A**) and S344I (**B**) specific point mutations.

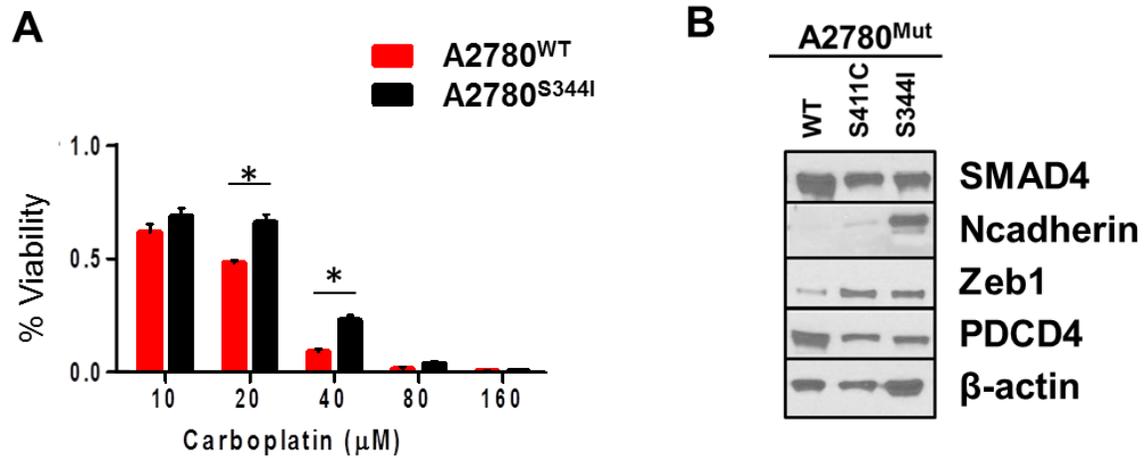


Figure 3.16 Mutations in SMAD4 Contribute to Platinum Resistance and EMT. **A.** Viability of A2780^{WT} and A2780^{S344I} after exposure to 20, 40, 80, 160, and 320 µM carboplatin for 72 hours. Values are normalized to vehicle (PBS). **B.** Western blot analysis of EMT markers in A2780^{WT}, A2780^{S411C}, and A2780^{S344I} cell lysates. **c.** Bright field images of A2780^{WT} and A2780^{S344I} after exposure to 0, 20, and 40 µM carboplatin for 24 hours. Arrows indicate changes in morphology. *P<0.05

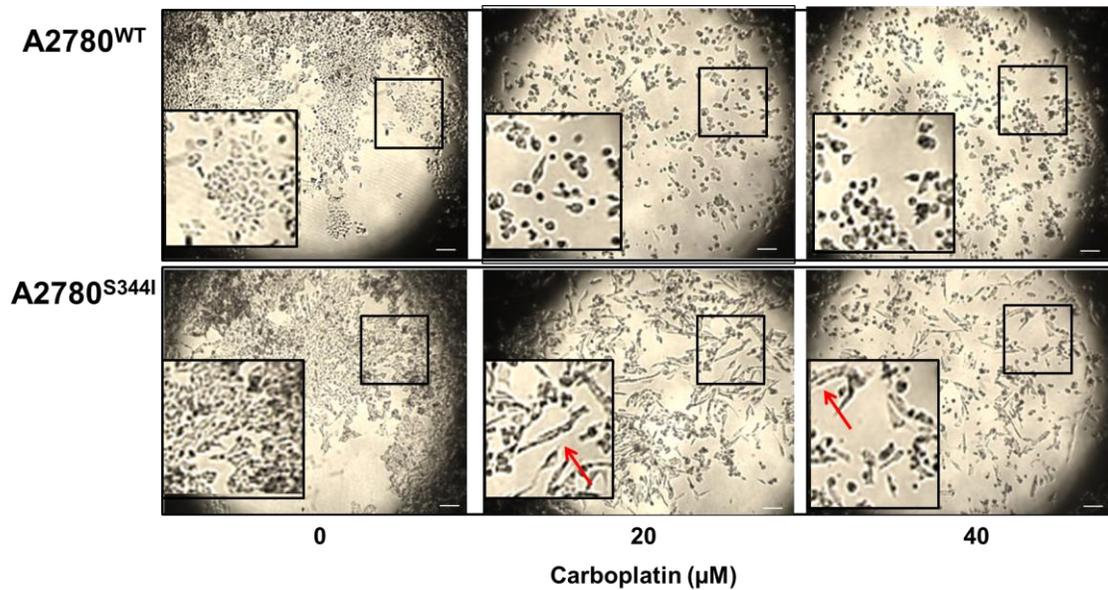


Figure 3.17 A2780^{MUT} Cells Undergo EMT in Response to Carboplatin. Bright field images of A2780^{WT} (top) and A2780^{S344I} (bottom,) after exposure to 0, 20, and 40 μM carboplatin for 24 hours. Arrows indicate changes in morphology.

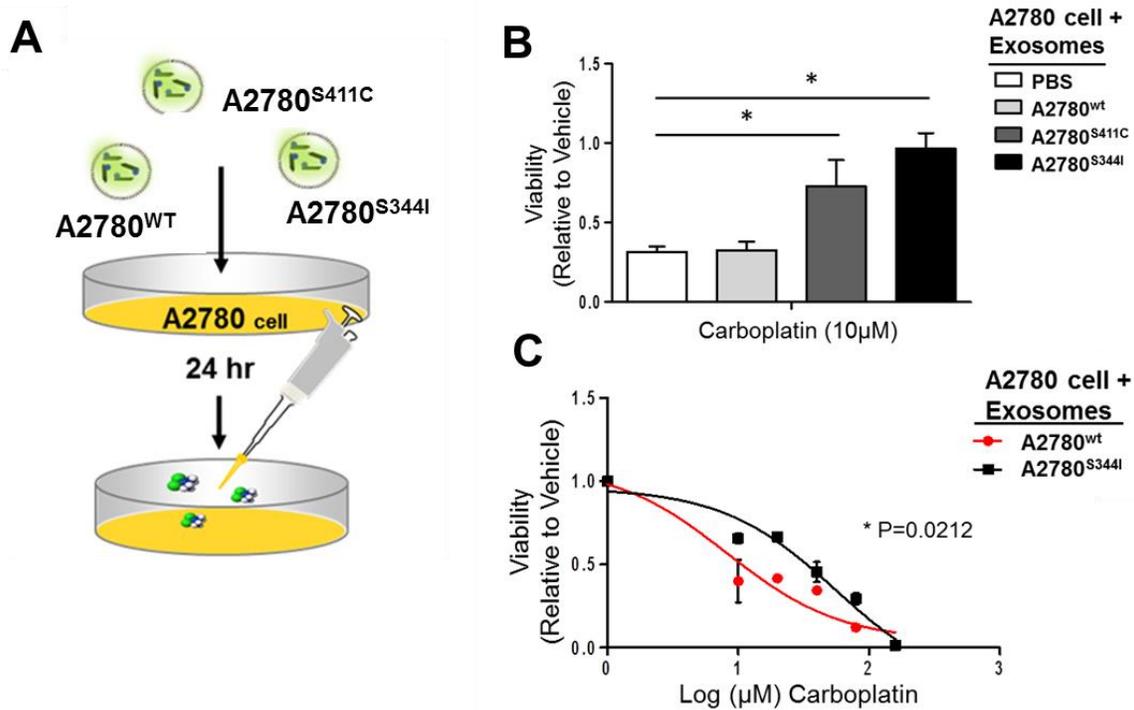


Figure 3.18 Exosomes from Mutant SMAD4 Cell Lines Transfer Platinum Resistance. **A.** Schematic of the experimental procedure. **B.** Viability of A2780 cells exposed to PBS or exosomes derived from A2780^{WT}, A2780^{S411C}, and A2780^{S344I} cell lines for 24 hours prior to carboplatin treatment (10 μM for 72 hours). Viability is normalized to vehicle within each exosome group. **C.** Dose-response curves of A2780 cells pre-exposed to exosomes derived from A2780^{WT} or A2780^{S344I} cell lines for 24 hours followed by carboplatin treatment (0, 10, 20, 40, 80, and 160 μM) for 72 hours. *P<0.05

CHAPTER 4 - TARGETING TGF- β /SMAD SIGNALING TO OVERCOME PLATINUM-RESISTANCE IN OVARIAN CANCER

INTRODUCTION

The TGF- β /SMAD signaling pathway is composed of the serine/threonine protein kinase TGF- β receptors I and II (TGF β rI/II) which are activated when TGF- β ligands (TGF- β I, II, or III) bind TGF β rII which in turn binds and phosphorylates TGF β rI. This activation triggers recruitment and subsequent phosphorylation and activation of receptor SMADs (SMAD2 or SMAD3). Activated receptor SMADs form a heterodimer with SMAD4 and translocate to the nucleus where they bind additional cofactors as well as the SMAD Binding Element (SBE) and initiate transcription of target genes (Derynck, Gelbart et al. 1996; Lagna, Hata et al. 1996; Zhang, Feng et al. 1996). The TGF- β /SMAD signaling pathway is important in the regulation of several physiological processes including cellular proliferation, differentiation, and wound repair (Massague 2012). This wide variety of functions is largely due to the extensive network of cofactors capable of binding receptor SMADs within the nucleus. In addition, SMAD proteins have been implicated in functions outside of gene regulation including processing of miRNA, which was discussed in Chapter 3. Given the fact that this pathway is a key regulator of processes such as apoptosis and EMT, it is of no surprise that it is frequently dysregulated in cancer (Siegel and Massague 2003; Massague 2008).

TGF- β /SMAD signaling has been considered a 'double edged sword' in tumorigenesis (Raiborg, Rusten et al. 2003). Early in tumor development, products of the TGF- β /SMAD signaling pathway are considered to be largely anti-tumorigenic in nature, however, as cancer progresses the pathway is hijacked to be tumor-supportive (de

Caestecker, Piek et al. 2000). Through decades of research has focused on this pathway, current ideas have evolved which view this switch as a dynamic event, involving specific regulation of key TGF- β /SMAD pathway components, such as TGF β RI and TGF β RII (Meulmeester and Ten Dijke 2011; Yu, Bardia et al. 2013). Additionally, while it has historically been thought that SMAD2/SMAD4 and SMAD3/SMAD4 complexes had redundant roles in gene regulation, the most recent work highlights very different roles of these complexes in cellular processes such as apoptosis (SMAD2) (Yang, Wahdan-Alaswad et al. 2009) and EMT (SMAD3) (Valcourt, Kowanetz et al. 2005; Wang, Gao et al. 2014). Disruption of the balance between SMAD2/SMAD4 and SMAD3/SMAD4 signaling has been implicated as a driver of invasion, metastasis, and poor clinical outcome in multiple types of cancer including gastric, breast, skin, and prostate (Yang, Wahdan-Alaswad et al. 2009; Petersen, Pardali et al. 2010; Wu, Li et al. 2012). In Chapter 2, I discussed how EOC cell lines harboring mutations in *SMAD4* exhibit a loss of sensitivity to carboplatin and this, at least in part, is mediated through an increase in EMT. This being said, we hypothesize that cells harboring loss of function mutations in *SMAD4* may have dysregulation of the balance between SMAD2 and SMAD3 signaling. In addition, given the fact that there is a crippling lack of treatment options for women with recurrent or resistant epithelial ovarian cancer, we provide a novel therapeutic strategy involving combination treatment with carboplatin and inhibition of the SMAD4/SMAD3 signaling arm of the TGF- β /SMAD pathway to re-sensitize cells to platinum.

MATERIALS AND METHODS

Cells and Culture Conditions. In these studies we utilized human ovarian cancer cells A2780, CP70, C30, OVCAR4, and OVCAR10 (Hamilton, Young et al. 1984; Behrens, Hamilton et al. 1987). All cell lines were cultured in RPMI-1640 (Gibco, Thermo Fisher) media supplemented with 10% (v/v) exosome-depleted FBS, 2 mM L-glutamine, 0.2 units/mL human insulin, and 100 units/mL penicillin-streptomycin at 37°C with 5% CO₂.

Therapeutic Agents. Cells were treated with carboplatin (SelleckChem, Houston, TX), Specific Inhibitor of SMAD3 (SIS3; SelleckChem), and Halaven® (eribulin mesylate; Eisai, Inc). Carboplatin was diluted from a 20 mM stock in PBS. SIS3 was diluted from a 10 mM stock in DMSO. Eribulin was diluted in PBS to a stock concentration of 10 µM.

Cell Viability Assays. EOC cells were plated at 5,000 cells/well in black walled 96 well plates. Cell viability was assessed using Cell Titer Blue (ThermoFisher), as previously published (Sethi, Pathak et al. 2012). Fluorescence signals were detected using the Tecan Plate reader by 560/590 excitation/emission spectra. Viability was determined by normalizing each treatment group to vehicle control. Statistical significance between experimental and control groups was determined using Student's T-test. Values of < 0.05 were considered significant.

Combination Index and Drug Synergy. CalcuSyn (Biosoft) software was used to determine combination index values. The software performs multiple drug dose-effect

calculations using the Median Effect methods as described by T-C Chou and P. Talalay (Chou and Talalay 1984). 5000 cells/well were plated in an 8x8 matrix and increasing doses of carboplatin (0-100 μ M or 0-640 μ M, vertical axis) + SIS3 (0-10 μ M, horizontal axis) or carboplatin (0-100 μ M or 0-640 μ M, vertical axis) + Eribulin (0-10 nM horizontal axis) were administered. After 72 hours viability was determined using Cell Titer[®] Blue and normalized to vehicle controls. All assays were run in duplicate. Values < 0.75 were considered to be synergistic.

SDS Page and Western Blot Analysis. Exosome samples and cell lysates (prepared in RIPA buffer) were separated by adding 40 μ g protein on 10% Mini-PROTEAN[®] TGX[™] Precast Gels, (BioRad, Hercules, CA) and transferred to a supported nitrocellulose membrane (BioRad). The membranes were blocked with 5% non-fat milk for one hour. Membranes were incubated with primary antibodies overnight and washed thrice for 10 minutes before addition of HRP conjugated anti-rabbit or anti-mouse secondary antibody (BioRad) for 1 hour. Membranes were then washed and treated with ECL Western Blotting Substrate (Fisher Scientific) according to manufacturer's instructions.

Real time PCR. Cells were harvested at 80% confluency and RNA was extracted using Trizol and Direct-sol[™] RNA mini-prep system (Zymo Research, Irvine CA). Reverse Transcriptase was performed using the GoScript[™] Reverse Transcriptase System (Promega, Madison, WI) according to manufacturer's instructions. Real time PCR was conducted using SoSoGreen real time PCR Master Mix (BioRad). Primers for EMT-related genes were taken from Yew *et al* (Yew, Crow et al. 2013).

Statistical Analysis. Statistical analysis was performed using the two-tailed Student's t-test on both excel and Graph Pad Prism Programs and one-way ANOVA analysis on Graph Pad Prism.

RESULTS

Dysregulation of TGF- β /SMAD signaling is found in clinical EOC samples.

Mutations leading to a loss of function in SMAD4 are well documented in cancers such as colorectal and pancreatic (**Figure 4.01**). The TCGA reports (4%) of EOC cases possess genetic abnormalities (mutations, deletions, and amplifications) in *SMAD4*, and, when mRNA was evaluated, the percentage of cases with significant alterations in *SMAD4* expression increased to 20% (**Figure 4.02**). When key components of the TGF- β /SMAD signaling pathway (*SMAD4*, *SMAD3*, *SMAD2*, *TGF β 1*, *TGF β 2*, *TGF β 3*, *TGF β RI*, *TGF β RII*, *SMAD7*, *TGF β 1*, *TGF β 2*, and *TGF β 3*) were evaluated together, over 63% (197 of 316) of EOC samples contained alterations in one or more of these genes (**Figure 4.02**) (Cerami, Gao et al. 2012; Gao, Aksoy et al. 2013). In addition, enrichment analysis of samples with miRNA sequencing data and loss of *SMAD4* (via gene deletion of mRNA down-regulation) (n=61) revealed subsets of miRNAs, which are differentially expressed (**Figure 4.03**). Importantly, miR-21, which was previously found to be upregulated in platinum-resistant cell lines and is a well-documented product of SMAD activity (Davis, Hilyard et al. 2008; Garcia, Nistal et al. 2015), was significantly (p=0.00417) overexpressed in cases with a loss of *SMAD4* expression (**Figure 4.03**). This data is interesting due to the fact that miR-21 is a known regulator of EMT, and has been reported to be upregulated in many types of cancer (Han, Xu et al. 2015; Qu, Lin et al. 2016; Wu, Tao et al. 2016) and it is suggested that miRNA-21 expression could correlate with platinum-resistance in ovarian cancer (Echevarria-Vargas, Valiyeva et al. 2014). We continued by investigating correlations between *SMAD2*, *SMAD3*, and

SMAD4 expression and overall survival. Out of 316 cases with complete data, no significant change in OS was observed in cases harboring *SMAD4* (n=61) or *SMAD2* (n=81) down-regulation or deletions; however, patients exhibiting a loss of *SMAD3* (n=16) expression exhibited a significant increase in OS (~21.5 months, p=0.0386) further demonstrating the complexity and importance of TGF- β /SMAD signaling, specifically via SMAD3 and SMAD4, in EOC progression (**Figure 4.04**). Lastly, in Chapter 3, we reported novel *SMAD4* missense mutations in our most platinum-resistant cell lines (CP70, C30, and OVCAR10). To expand this analysis, we conducted mRNAseq on A2780, CP70, C30, OVCAR4, and OVCAR10 cell lines. In analyzing the data, we uncovered additional missense mutations in key TGF- β /SMAD signaling components, specific to cells with a loss of platinum-sensitivity. C30, CP70, and OVCAR10 each harbored a shared mutation in *TGF β 1* (R321H) (**Table 1.01**). Additionally, C30 had a unique mutation in *SMAD2* (G423R) (**Table 1.01**). Importantly, we did not observe additional mutations in platinum-sensitive cell lines, A2780 or OVCAR4. Taken together these data further supports the hypothesis that dysregulation in TGF- β /SMAD signaling is correlated with the advent of platinum-resistant disease.

Platinum-Resistant Cell lines have preferential activation of SMAD3. In ovarian cancer, SMAD4 acts in conjunction with receptor SMAD3 to regulate transcription of EMT target genes (Do, Kubba et al. 2008). In Chapter 3 we discussed the role of EMT in platinum-resistance. To gain a deeper understanding of how the TGF- β /SMAD pathway is altered in EOC cells harboring mutations in *SMAD4* we first investigated the presence of *SMAD2*, *SMAD3*, and *SMAD4* at the mRNA and protein levels (**Figure**

4.05). *SMAD4* was present in both the transcript and the protein level in all EOC cells, regardless of *SMAD* mutational status (**Figure 4.05**). We discovered; however, that although *SMAD2* and *SMAD3* were present at the mRNA and protein levels in all cells, (**Figures 4.05, 4.06**) cells lines with mutant *SMAD4* exhibited a near complete loss of receptor activated *SMAD2*, but not *SMAD3*. This was not observed in cell lines WT for *SMAD4* which contained receptor activated forms of both *SMAD2* and *SMAD3* (**Figure 4.06**). These data suggest that a loss of *SMAD2/SMAD4* signaling and maintenance of *SMAD3/SMAD4* signaling, may be important in a cell's response to platinum. Further investigation of the TCGA supported this concept, in that our analysis of platinum-resistant vs. platinum-sensitive EOC samples revealed that over 25% of EOC cases had down-regulation of *SMAD2* at the mRNA level regardless of platinum status (**Figure 4.02**). Interestingly, the platinum-resistant subset (n=197) exhibited an increased loss of *SMAD2* expression over the platinum-sensitive (n=90) subset (32% vs. 24% respectively) (**Figure 4.07**). Importantly, *SMAD3* alterations were minimal (10% total genetic dysregulation in both platinum-sensitive and platinum-resistant subsets). However, there was a noticeable inverse shift between the subpopulations, with the platinum-resistant subset having increased up-regulation of mRNA over the platinum-sensitive set, and vice versa for down-regulation of the gene (**Figure 4.07**). Taken together these data suggest that a loss in *SMAD2* signaling may lead to over-activation of the *SMAD3* arm of the TGF- β /*SMAD* signaling pathway which, subsequently drives upregulation of survival pathways, including EMT, and ultimately a loss in platinum-sensitivity (**Figure 4.08**).

Combination of SMAD4 inhibition with carboplatin increases cell death in platinum-resistant EOC Cell lines. Our group previously conducted an RNA interference lethality screen of the human druggable genome to identify molecular vulnerabilities in epithelial ovarian cancer (Sethi, Pathak et al. 2012). In analyzing data from that published study Sethi *et al.*, observed no effect on tumor viability when blocking the TGF- β /SMAD pathway with siRNA against *SMAD2*, *SMAD3*, or *SMAD4* alone (**Figure 4.09**). Given that SMAD4/SMAD3 signaling seems to be important for resistance to platinum we questioned whether inhibition of SMAD4 would impact viability upon treatment with carboplatin. To test this hypothesis we transiently silenced SMAD4 using siRNA for 48 hours and then treated with either 50 μ M or 200 μ M carboplatin (depending on the sensitivity levels of the cell lines) for 72 hours. We observed that cells with decreased levels of SMAD4 had significantly ($P < 0.05$) lower viability as compared to control (scrambled) siRNA and this effect appears to be more pronounced in platinum-resistant cell lines (**Figure 4.09**).

Combination of SMAD3 inhibition with carboplatin increases EOC cell death. We next questioned the effects of SMAD3 inhibition in combination with carboplatin on cell viability. We utilized the Specific Inhibitor of SMAD3 (SIS3), which selectively inhibits receptor phosphorylation of SMAD3 and SMAD3/SMAD4 oligomerization (Jinnin, Ihn et al. 2006). SIS3 selectively inhibits TGF- β and activin signaling by suppressing SMAD3 phosphorylation without affecting the MAPK/p38, ERK, or PI3-kinase signaling pathways (Jinnin, Ihn et al. 2006). A2780 cells were treated with 3 μ M SIS3 for 0, 1, 2, 6, 12, and 24 hours. A loss of pSMAD3^{Ser423/425} was observed by 6 hours (**Figure 4.10**).

Additionally, inhibition of SMAD3 corresponded with changes in EMT related genes. After 12 hours exposure to 3 μ M SIS3, we observed a 2-fold increase in the epithelial marker *EpCAM* and 3-fold and 2-fold down-regulation of mesenchymal markers *N-cadherin* and *vimentin*, respectively. To address whether this inhibition would be able to sensitize cells to platinum we evaluated cell viability following a combination of SIS3 with carboplatin. A2780, CP70, and C30 cells were plated in an 8x8 matrix and treated with 0-10 μ M SIS3 and 0-100 μ M (A2780) or 0-430 μ M (CP70 and C30) carboplatin. Cells were treated with increasing doses of SIS3, up to 10 μ M, 3 hours prior to carboplatin (0-100 μ M or 0-640 μ M depending on cell line). We observed synergistic effects, which contributed to increases in cell death over the effect of single drugs alone. Interestingly, this effect was observed in (A2780) as well as platinum-resistant SMAD4 mutant cell lines (CP70 and C30) cell lines (**Figure 4.11**). It is important to recall that both mutant and WT SMAD4 cell lines exhibited activated SMAD3/SMAD4 signaling, therefore the synergistic effects suggest that SMAD4/SMAD3 may be important in the cell's response to platinum-based chemotherapy and that this treatment strategy may be applicable in both a platinum-sensitive and platinum-resistant setting.

Combination of eribulin mesylate and carboplatin acts synergistically to enhance cell death. Given the fact that SIS3 is not approved for patients we next sought to find a more clinically relevant drug capable of inhibiting the activity of SMAD3 and thus, evaluated eribulin mesylate (Halaven®, Eisai, Inc.). Eribulin mesylate is a synthetic analogue of halichondrin B, a product isolated the Japanese sea sponge *Halichondria okadai* (McBride and Butler 2012). Eribulin differs from other anti-microtubule agents in

that it can bind to the microtubule cap and inhibit tubulin polymerization, leading to microtubule arrest (McBride and Butler 2012). Eribulin has been FDA approved for use in patients with metastatic breast cancer and liposarcoma (a specific type of soft tissue sarcoma) (Newman 2007; Vahdat, Pruitt et al. 2009; Twelves, Cortes et al. 2010; Mok, Geater et al. 2014; 2016). Eribulin has been shown to prevent EMT by preventing receptor SMAD phosphorylation and subsequent activation with a seemingly stronger effect on SMAD3 than SMAD2 (Yoshida, Ozawa et al. 2014). Maximum tolerated doses of eribulin mesylate in combination with carboplatin have been established for patients with non-small cell lung cancer (ClinicalTrials.gov Identifier: NCT00268905); however, clinical trials for eribulin in ovarian cancer have been limited to use as a single agent. In a phase II trial, eribulin alone achieved partial response in 5.5% of women with platinum-resistant, recurrent ovarian cancer and in 19% of women with platinum-sensitive disease. The median progression-free survival was 1.8 months in the platinum-resistant group and 4.1 months in the platinum-sensitive group (Hensley, Kravetz et al. 2012). Given our preliminary data supporting synergetic effects between SMAD3 inhibition and carboplatin, we explored the ability of eribulin to act in synergy with carboplatin. To examine this hypothesis we plated OVCAR4 and OVCAR10 cells in an 8x8 matrix and exposed them to 0-64 nM eribulin for 3 hours followed by 0-100 μ M (OVCAR4) and 0-640 μ M (OVCAR10) carboplatin. Excitingly, we observed significant synergy and enhanced cell death in cells with combination treatment over single agent alone (**Figure 4.12**) suggesting that this treatment strategy may be an effective option for women with platinum-resistant disease.

DISCUSSION

Increased exposure to platinum contributes to the development of platinum-resistant disease and the current body of work suggests that the advent of resistant disease can be held off by increasing the platinum-free interval (PFI), either by extending the drug-free time between platinum dosing or by alternating with non-platinum chemotherapy (*i.e.*, pegylated liposomal doxorubicin) (Gordon, Fleagle et al. 2001; Fung-Kee-Fung, Oliver et al. 2007; Naumann and Coleman 2011). These observations suggest that EOC is a dynamic disease and platinum resistance is not necessarily a stable phenomenon but may be inducible and perhaps reversible (Kuczynski, Sargent et al. 2013). We hypothesize that EMT is responsible, in part, for the development of platinum resistance; however, how the transition to a more mesenchymal phenotype contributes to protection from chemotherapy is still unknown. The challenge, therefore, lies in identifying a druggable mediator of EMT across a large subset of EOC tumors. In platinum-exposed and platinum-resistant EOC cell lines we have identified, for the first time, a dysregulation of SMAD2/SMAD3 signaling which favors SMAD3 over SMAD2. This is seen both in down-regulation of *SMAD2* mRNA as well as a loss of receptor-activated SMAD. Importantly, SMAD3 is not affected. In addition, survival data from the TCGA reports that patients with down-regulation of *SMAD3* mRNA have improved OS although the n value for this study was low it further supports the theory that SMAD4/SMAD3 signaling is important to the development of platinum resistant disease. There currently are several TGF- β inhibitors including antibodies, antisense oligonucleotides, and receptor kinase inhibitors which have been introduced into clinical

trials for the treatment of a variety of malignancies (Buijs, Stayrook et al. 2012). A sampling of the most recent include trabedersen (Isarna Therapeutics), a phosphorothioate antisense oligodeoxynucleotide specific for TGF- β II (Schlingensiepen, Schlingensiepen et al. 2006; Schlingensiepen, Fischer-Blass et al. 2008), galunisertib (Eli Lilly), a TGF β r1 inhibitor (Herbertz, Sawyer et al. 2015; Rodon, Carducci et al. 2015), and PF-03446962 (Pfizer), a monoclonal antibody against TGF β rI (Necchi, Giannatempo et al. 2014). Interestingly, there are no current studies focused on inhibiting TGF- β /SMAD signaling in combination with carboplatin or cisplatin for treatment of platinum-resistant ovarian cancer. We believe, given the above preliminary data that this novel strategy may advance our ability to treat platinum-resistant disease and/or prolong disease-free survival.

Table 4.01 Additional Mutations in Platinum-Resistant EOC Cell Lines

Gene	Mutation	Observation	
		<i>Cell Line</i>	<i>Percentage</i>
TGFβ1	Chr:101908829 CGT>CAT [Arg>His] AA321	C30	31%
		CP70	56%
		OVCAR10	49%
SMAD2	Chr18:4537172 TCC>TCT [Gly>Arg] AA423	C30	46%

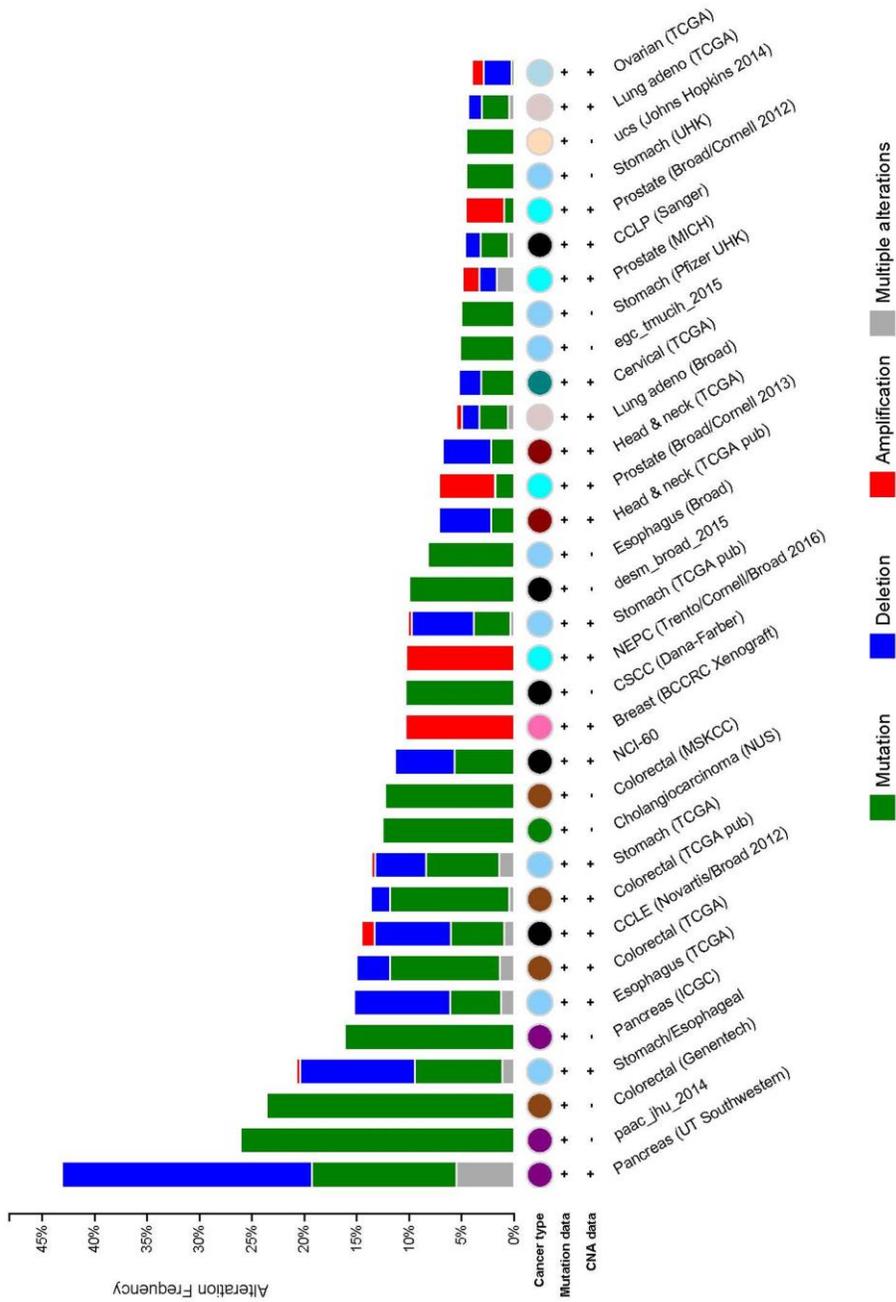


Figure 4.01 SMAD4 Alterations Across All TCGA Cancers. TCGA data representing the frequency of SMAD4 mutations across all cancers in the TCGA. Data presented is cancers with >4% alterations in SMAD4. Ovarian cancer is on the far right and is altered in 4% of the cases, primarily represented by deletions of the gene.

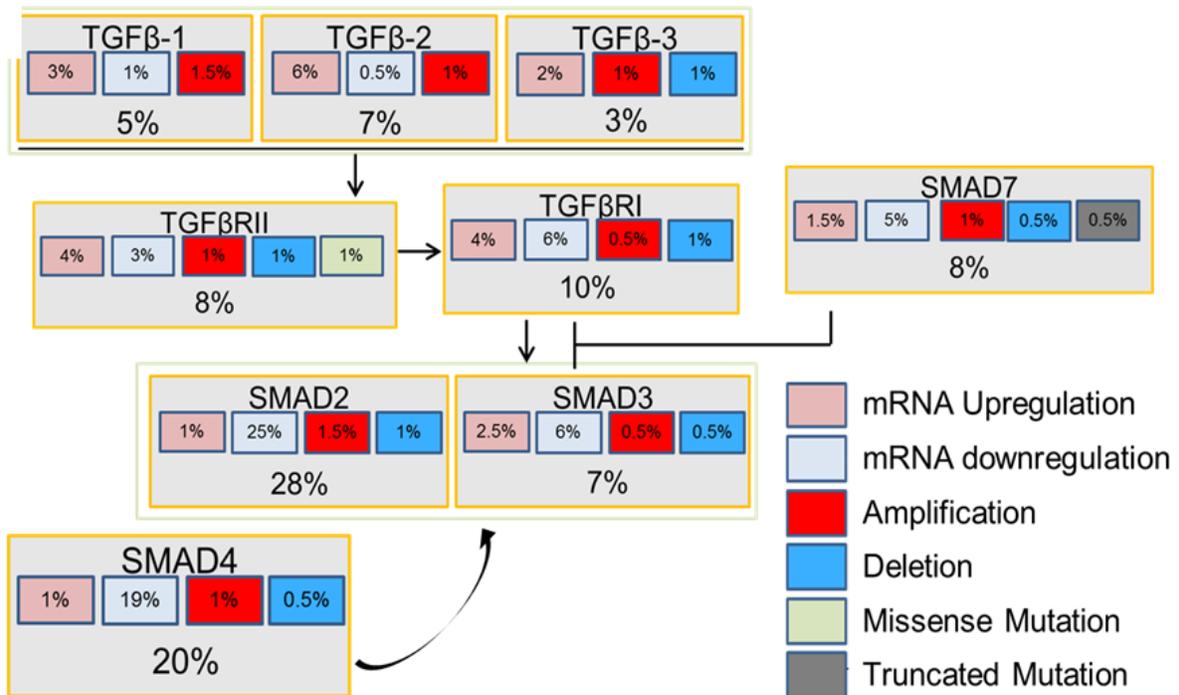


Figure 4.02 Dysregulation of TGF-β/SMAD Signaling Components in TCGA Samples. Data from the TCGA reveals mRNA upregulation (pink), mRNA downregulation (light blue), amplification (red), deletion (blue), missense mutations (green), and truncated mutations (gray) in components of the TGF-β/SMAD signaling pathway in 316 EOC samples with complete data (mRNA, CAN, and sequencing).

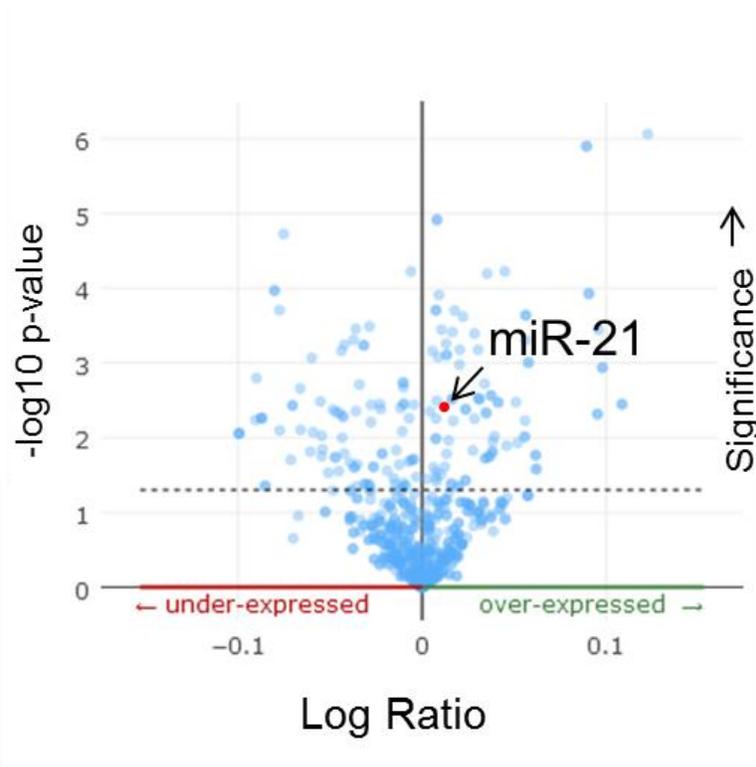


Figure 4.03 Dysregulation of miR-21 in TCGA Samples. Volcano plot of TCGA EOC samples with a loss of expression in *SMAD4* by either mRNA down-regulation or gene deletion (n=61) demonstrating that the miR-21 levels (red dot) are significantly ($p=0.00417$) enhanced in this population of samples.

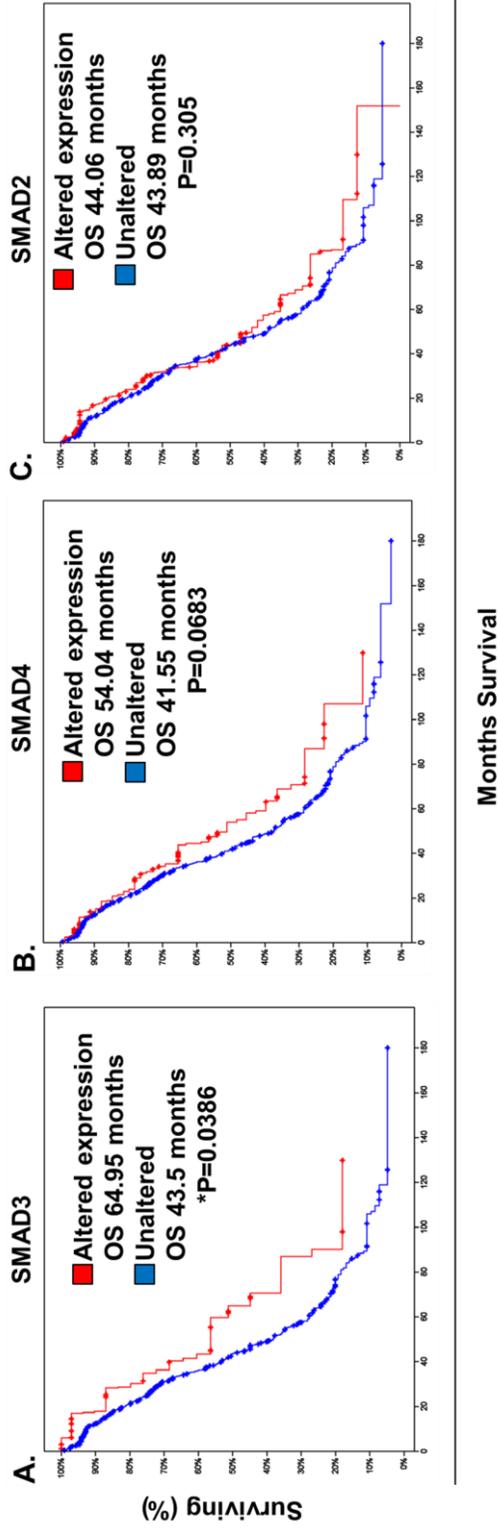


Figure 4.04 Dysregulation of SMAD3 impacts Overall Survival. OS plotted as Kaplan-Meier curves that compare patients with down-regulation or deletion of SMAD3 n= 16(A), SMAD4 n=61 (B), or SMAD2 n=81 (C), as compared to unaltered cases (total cases = 316) with complete data. Patients with a loss of SMAD3 expression have a significant increase (64 months vs 43 months) P=0.0386 in OS and patients with a loss of wild-type SMAD4 expression have an increased OS of ~12.5 months. P values are determined by log-rank test. P values < 0.05 are considered significant.

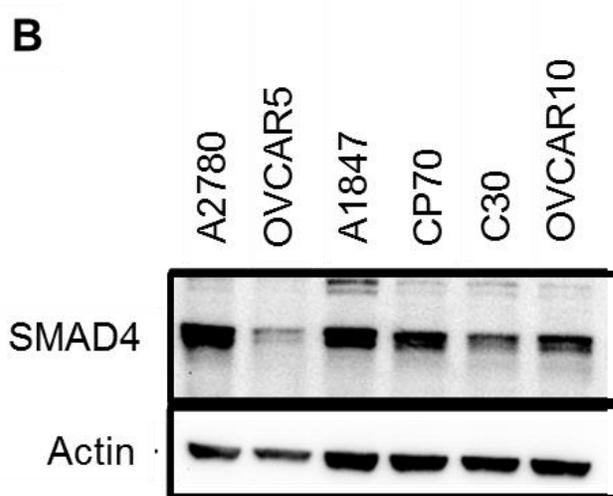
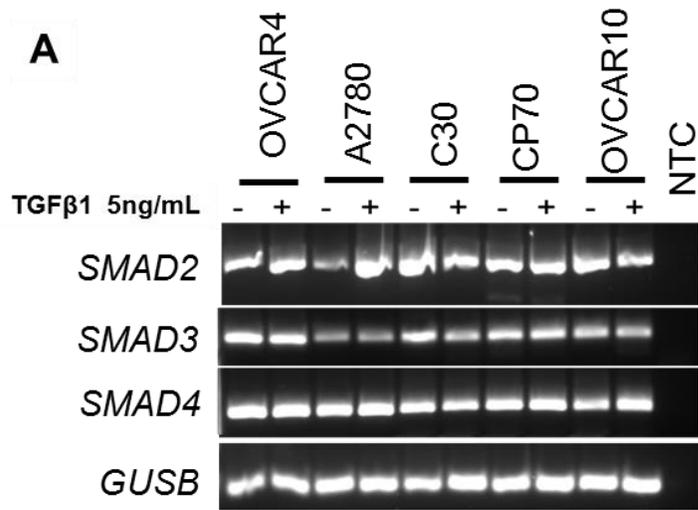


Figure 4.05 SMAD4 is Present in EOC Cell Lines. A. Gel electrophoresis of amplicon generated with target gene-specific primers (SMAD4, SMAD3, and SMAD2) demonstrates the presence of transcript mRNA in EOC cell lines. B. Western Blot analysis of SMAD4 shows presence of the protein in EOC cell lines.

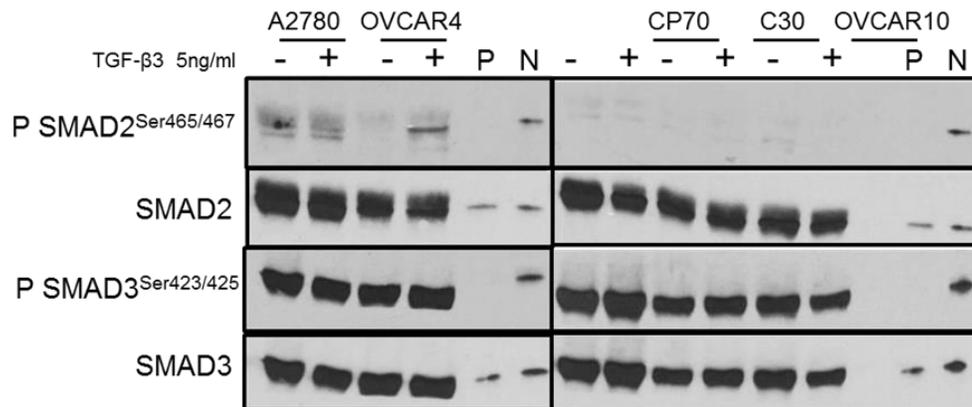


Figure 4.06 Loss of SMAD2 Activation Corresponds with a Loss of Sensitivity to Platinum. Western blot analysis of EOC cells platinum-sensitive (A2780 and OVCAR4) and platinum-resistant (CP70, C30, and OVCAR10) both with and without TGFβ stimulation.

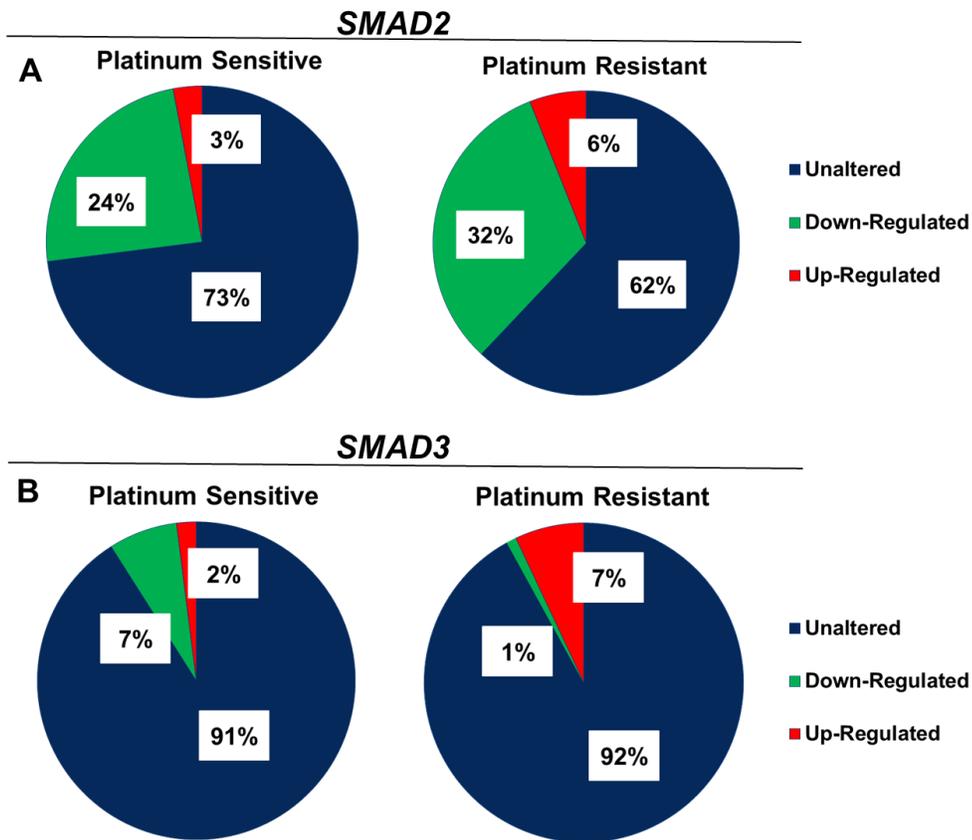


Figure 4.07 Loss of SMAD2 Expression Corresponds with a Platinum-Resistant Phenotype. **A)** TCGA data analyzed to show variations in regulation of *SMAD2* mRNA between platinum-resistant (n=197) and platinum-sensitive (n=90) patient tumor samples. **B)** TCGA data analyzed to show variations in regulation of *SMAD3* mRNA between platinum-resistant (n=197) and platinum-sensitive (n=90) patient tumor samples.

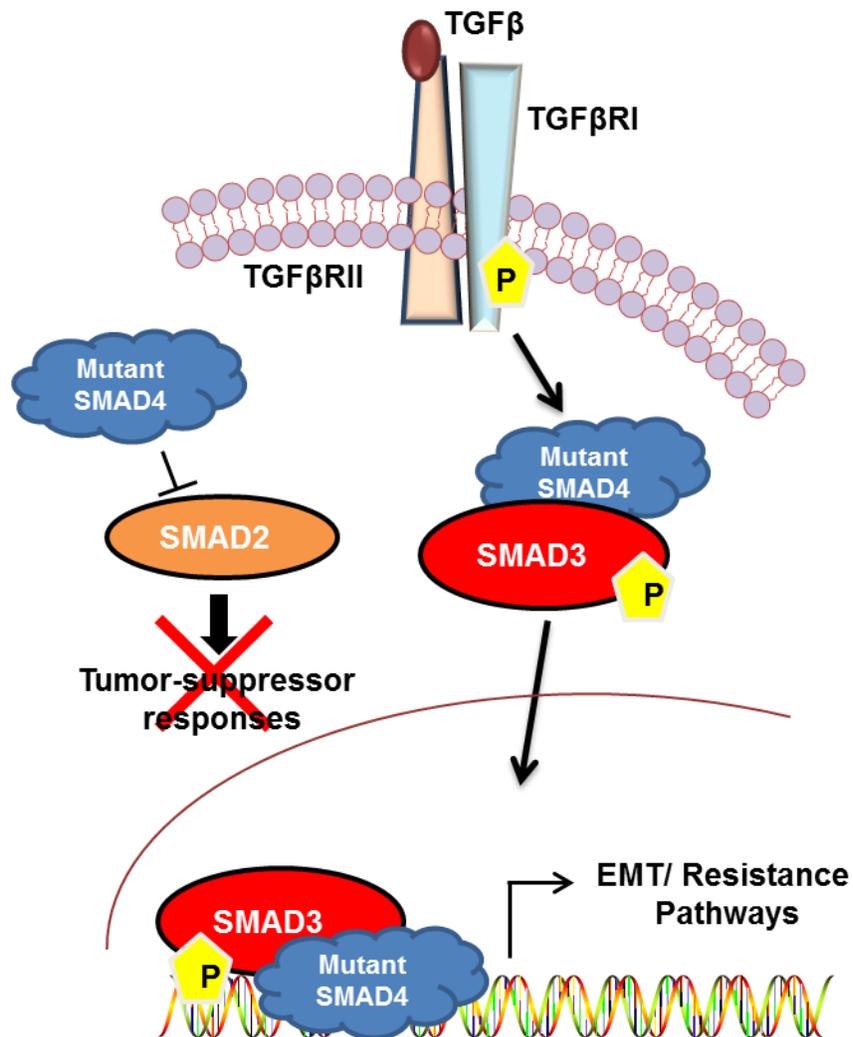


Figure 4.08 Working Model. Loss of SMAD2 leads to over-activation of SMAD3-mediated signaling, which in turn, leads to increases in EMT related gene expression and a loss of platinum-sensitivity.

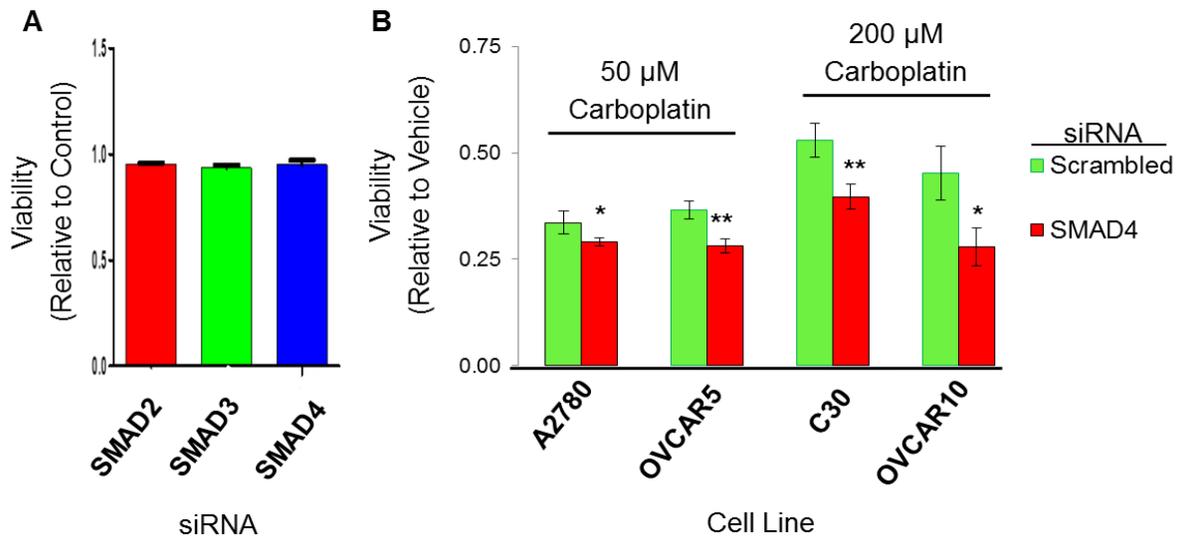


Figure 4.09..Combination of SMAD4 inhibition with Carboplatin Enhances Cell Death. A. Viability relative to control siRNA after A1847 cell treatment with siRNA against SMAD2, SMAD3, and SMAD4 (Sethi and Godwin, 2012). B. Viability relative to vehicle of A2780, OVCAR5, C30, and OVCAR10 cells after treatment with control or SMAD4 siRNA and carboplatin. (OVCAR5 & A2780 50 μM, C30 & OVCAR10 200 μM). Viability is determined by Cell Titer Blue® (Promega) in each assay. **P<0.01 *P<0.05.

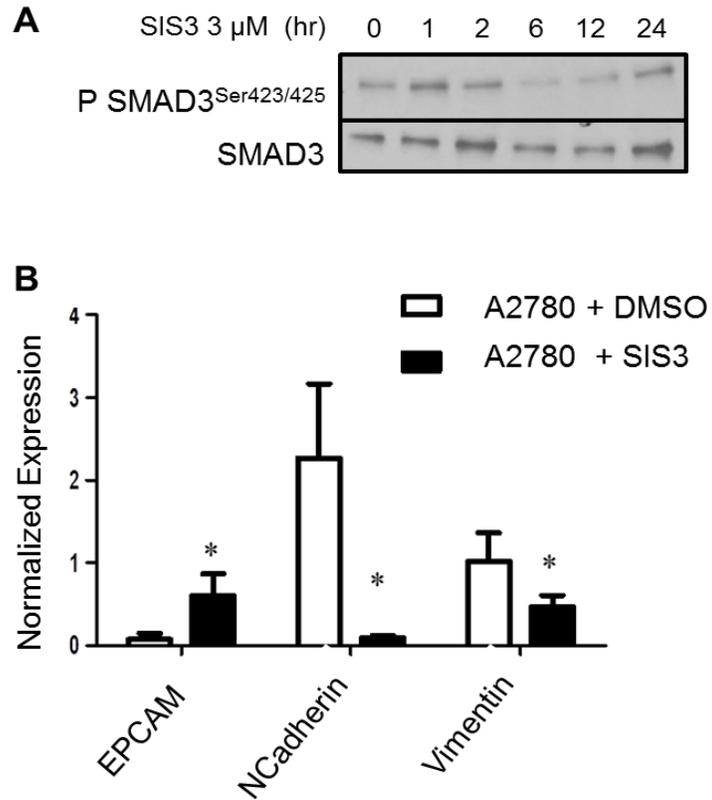


Figure 4.10 SIS3 Inhibits Phosphorylation of SMAD3 and Triggers EMT. A. Western blot analysis of A2780 cells after 3 μ M exposure to SIS3 shows a loss of phosphorylated SMAD3 by 6 hr. B. A2780 cells relative mRNA levels after 12 hr exposure to SIS3. RNA levels are normalized to both GAPDH and GUSB. *=P<0.05.

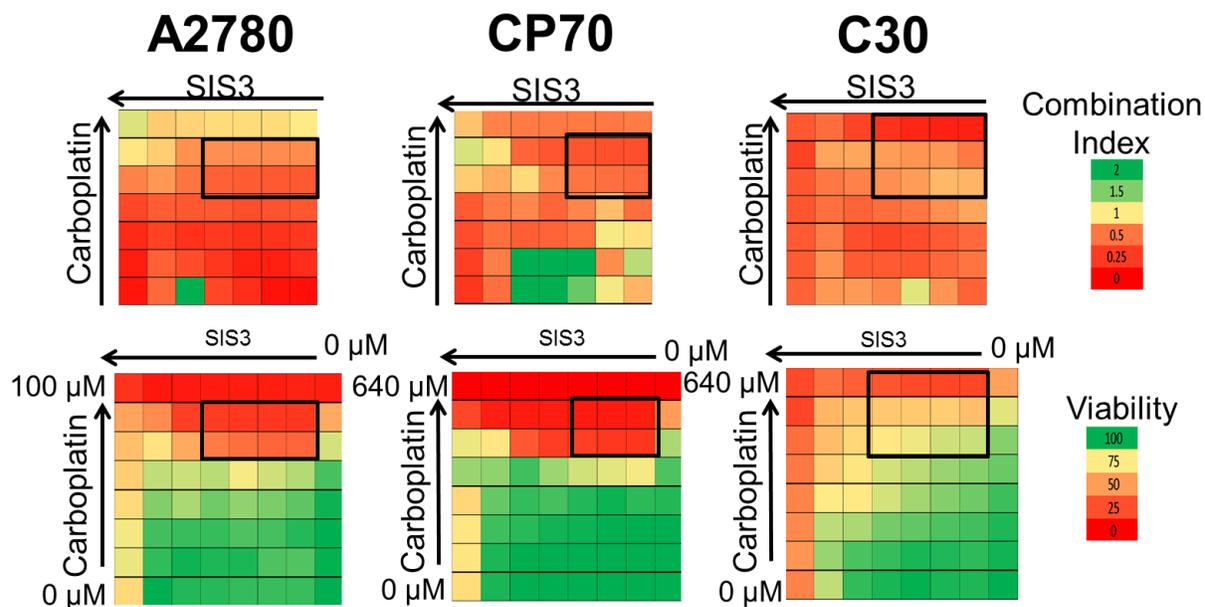


Figure 4.11 Synergistic Effects of SIS3 and Carboplatin. Viability (bottom) and synergy (top) 8x8 drug matrix of response to combinations of SIS3 and carboplatin. Combination index scores as determined using CalcuSyn (BioSoft®) of <1 indicate synergistic effects (orange – red). Viability is normalized to untreated control and scored as 100% (green) to 0% (red) indicating the amount of cell death as determined by Cell Titer Blue®. Boxes indicate corresponding values within each cell line. Each matrix is representative of duplicate experiments.

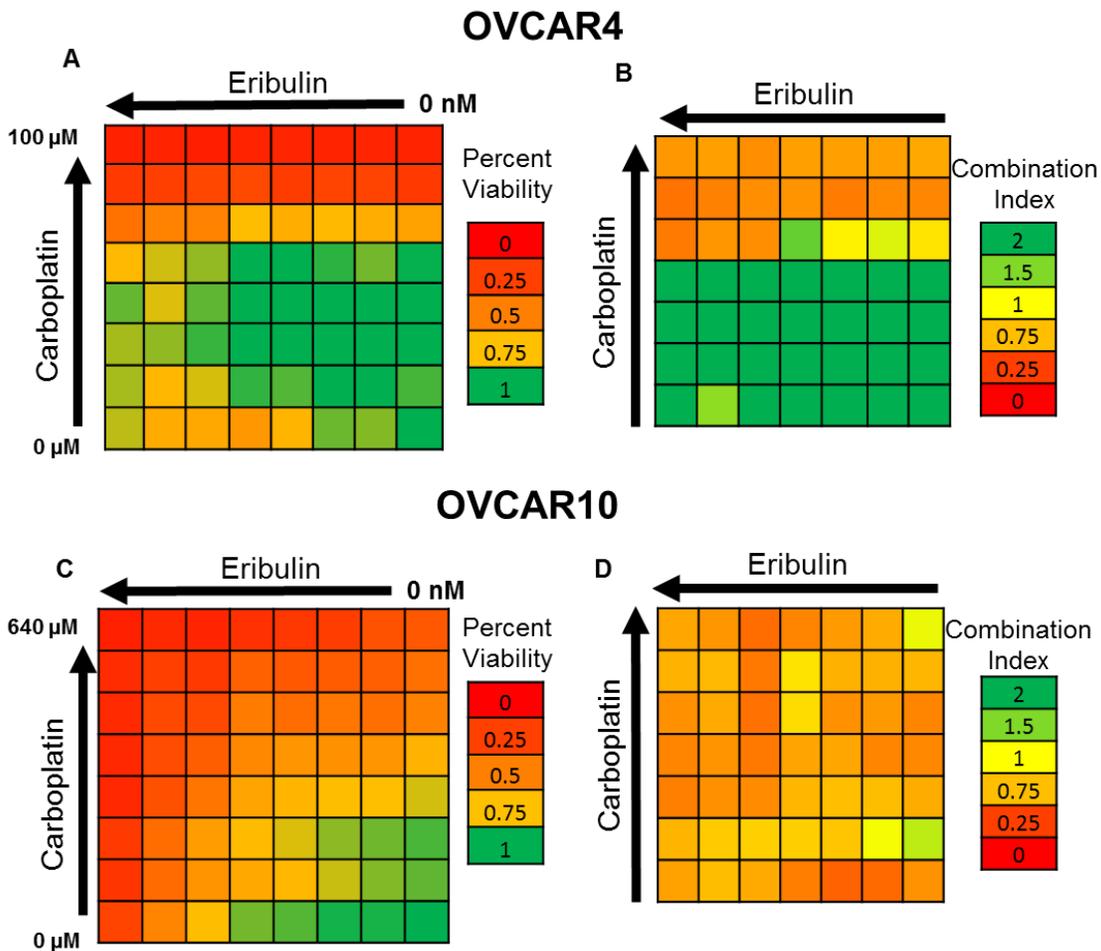


Figure 4.12 Combination Therapy of Eribulin and Carboplatin is Synergistic. **A & C**) 8x8 drug matrix of viability response to combinations of eribulin and carboplatin in OVCAR4 (**A**) and OVCAR10 (**C**) EOC cell lines. Viability is normalized to untreated control and scored as 100% (green) to 0% (red) indicating the amount of cell death as determined by Cell Titer Blue®. **B & D**) Corresponding combination index scores as determined using CalcuSyn (BioSoft®) in OVCAR4 (**B**) and OVCAR10 (**D**). CI values <1 indicate synergistic effects (orange – red). Boxes indicate corresponding values within each cell line. Each matrix is representative of duplicate experiments.

CHAPTER 5 - CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

We are sitting at a new frontier of cancer research. The rapid advancement of inexpensive, specific, and sensitive genetic sequencing coupled with novel screening techniques to identify candidate drugs has made the concept of 'personalized medicine' a reality. The addition of exosomes to this matrix elevates the utility of this approach to previously unthought of levels. Our ability to catch a glimpse inside the innerworking of cancer cells, in real-time, and with minimally invasive procedures means that clinicians now have the possibility to interact more completely with the disease they are treating. In some ways it becomes a sort of conversation. The clinician treats the tumor with a specific type of drug, the tumor then responds. The clinician interprets the message sent via exosomes and responds by altering the therapy. I feel, interrogation of this sophisticated exchange is one of the primary goals of personalized medicine and our increasing understanding of extracellular vesicles, such as exosomes will assist us in realizing this ideal.

The use of exosomes as predictive biomarkers is currently being investigated for a number of malignancies including breast, ovarian, and prostate cancers (Kharaziha, Chioureas et al. 2015; Lowry, Gallagher et al. 2015). Researchers are attracted to these 'mini-me' versions of cells (this reference is credited to my mentor, Dr. Godwin) due to their unique makeup and relative abundance in biological fluids (up to 50-fold more) in disease states as compared to that of healthy controls (Gercel-Taylor, Atay et al. 2012; He, Crow et al. 2014). However, our understanding of exosomes is still

rudimentary at best and the molecular decoding of these nanovesicles remains very challenging due to their unique properties. For example, exosomes differ according to their cell of origin and their properties are highly dynamic, depending on cellular states, environmental factors or activating stimulus (Ge, Tan et al. 2012; Ohno, Ishikawa et al. 2013; Phuyal, Skotland et al. 2015; Jelonek, Widlak et al. 2016). Also, recent studies have revealed enormously diverse proteins (~42,000) and nucleic acids (>7,540 RNAs) in exosomes (Keerthikumar, Chisanga et al. 2016) especially those found in human blood (Zarovni, Corrado et al. 2015; Freedman, Gerstein et al. 2016; Willms, Johansson et al. 2016).

A third confounding factor is the emerging disagreement on how to best effectively characterize and 'prove' that isolated exosomal populations are truly pure. While exosomes have a unique endosomal biogenesis pathway (as discussed in Chapter 2), and, are believed to have a type of organized shuttling of content, their size distribution overlaps with that of other secreted vesicles such as microvesicles and apoptotic bodies (Raposo and Stoorvogel 2013). Therefore, isolation solely based upon size (or ability to pellet at 100,000xg as compared to 10,000xg) is potentially a flawed technique, albeit it currently the most accepted technique in the field of microvesicles. Several bodies of thought suggest that the characteristic lipid dense membrane of these vesicles allow them to be further purified after initial isolation by centrifugation on a sucrose gradient, and this addition to centrifugation provides the most 'pure' and consistent exosomal preparation as reviewed by Abramowicz *et al.* (Abramowicz, Widlak et al. 2016). The trouble then lies in how to best apply this type of technique within the clinical setting

where the sample volume is often small, turnaround time for lab results is of the essence, and ease of use is mandatory.

I have previously published data demonstrating the isolation of exosomal populations via a microfluidic platform (as discussed in Chapter 2) (He, Crow et al. 2014). While a population of reviewers would suggest (as rightfully so) that the vesicles isolated from this and other microfluidic devices include vesicles not of endosomal origin it must be noted that common exosomal markers (such as CD63 and CD9) are found on a variety of EVs and both types of vesicles have been considered for biomarker development (Ohno, Ishikawa et al. 2013; Raposo and Stoorvogel 2013). I suggest that the larger aim of one's research be taken into account when considering whether to use exosomes or total EVs. While additional purification may be relevant, and necessary in exosome-specific characterization investigations, a larger body of EV-based work focuses on how these vesicles contribute to disease and can be utilized to detect and combat maladies such as cancer. That being said I pose a question, "If the overall goal of the research is to isolate vesicles secreted from a specific cell type for the detection and monitoring of disease, does it really matter if the preparation contains vesicles of mixed origin?" These sorts of questions are becoming increasingly more common as we expand our understanding of the biology of secreted vesicles and should always be taken into consideration when reviewing or preparing for future studies.

FUTURE DIRECTIONS

The utility of EV removal in combating platinum-resistant EOC. In understanding the importance of the impact exosomes and other EVs have in the development of cancer progression, removal of these vesicles is an attractive therapeutic option. As previously discussed in Chapter 2, devices such as Aethlon ADAPT™ (trade name Hemopurifier®) may be of use in combating metastatic disease (Marleau, Chen et al. 2012). This device utilizes a plasmapheresis cartridge, which allows cells to pass through, but captures vesicles < 200 nm with a custom designed affinity matrix. It received IDE approval from the FDA to start clinical trials and the first patient completed the Hemopurifier Clinical study for the removal of Hepatitis C virus in February of 2015 (Marleau, Chen et al. 2012). I would suggest that this could be taken a step further and added to the primary standard backbone therapeutic regimen for women diagnosed with ovarian cancer. As discussed in Chapter 1, successful cytoreductive surgery has a tremendous impact on OS in EOC. As stated, this involves removal of as much of the visible tumor as possible (at least all tumor > 1cm) (Peng, Yan et al. 2011). Tumor-derived exosomes have also been implicated in the establishment of new tumor niches and in neoplastic transformation (as discussed in Chapter 2). In fact, exosomes derived from the ascites fluid of EOC patients were shown to inhibit the cytotoxic effects of peripheral blood mononuclear cells, thus contributing to a less tumor suppressive environment (Peng, Yan et al. 2011). Given that exosomes and microvesicles are nano-sized versions of the parental cell, and can function in lieu of their cell of origin, I hypothesize that the removal of these circulating vesicles from the blood of women

undergoing primary therapy could further limit the potential of tumor reoccurrence. While the Athelon has stated to have interest in working towards the use of the Hemopurifier[®] for the treatment of cancer, there are no clinical trials or pre-clinical data to date. In addition, the use of exosomes for the treatment of cancer in clinical trials is limited to delivery of therapeutics or in immune system 'priming' (Clinical Trials.gov). One hurdle to address, which has been discussed in Chapter 3, is how, exactly to target and capture all ovarian cancer-associated exosomes and EVs.

Exosomal use as biomarkers in EOC. Recent advancements in the molecular annotation of tumors have elevated the utility of personalized medicine in the clinical setting. In addition, the development of databases such as The Cancer Genome Atlas Network, led by the National Institutes of Health, have streamlined the process of identifying novel therapeutic and prognostic targets thus shortening the duration between transitioning of therapeutic strategies from bench to bedside. As was previously discussed, there is a dire lack of biomarkers for the early detection and monitoring of disease states in EOC and work from our lab and others suggests that isolation and characterization of EOC-specific EVs may prove clinically beneficial in both the identification of disease at earlier stages and/or in determining how the tumor responsiveness to a specific therapy (He, Crow et al. 2014). *Currently there are no comprehensive data sets analyzing the evolution of exosomal cargo throughout the development of chemotherapy resistance in EOC* (Safaei, Larson et al. 2005; Yin, Yan et al. 2012; Pink, Samuel et al. 2015). A tremendous hurdle in ovarian cancer research is a lack of tissue samples beyond primary cytoreductive surgery. This is due to the fact

that most women will not undergo successive rounds of surgery upon relapse. The ability to isolate tumor-derived exosomes from plasma and ascites therefore is exciting in that it provides researchers with more direct view of molecular changes, which occur with disease progression. In addition, because exosomes can be isolated from patient samples the results can be more directly translated into the clinical setting. Perhaps the most promising notion for ovarian cancer patients, as discussed at the beginning of this chapter, is that by evaluating changes in tumor-derived exosomal content, such as proteins and miRNAs, throughout a the course of the disease, we may be able predict if and when specific molecularly-targeted therapies would be advantageous. Given that components of the TGF- β /SMAD signaling pathway are found directly within and on exosomes this work may prove useful as a foundation for research focused on applying exosomal biomarkers as a mechanism for predicting therapeutic response to platinum-based anti-neoplastic agents. In fact, recent work by Scjanik and colleagues has reported that exosomes derived from the plasma of patients with ovarian cancer had elevated levels of TGF- β 1, which distinguished them from the exosomes derived from patients with benign tumors and normal controls (Szajnik, Derbis et al. 2013). While the “n” value of this study was relatively small, the data supports the multifaceted role of TGF- β signaling in ovarian cancer and the importance of further examination of this pathway for biomarker applications.

Targeting TGF- β in Cancer

The concept of targeting the specific molecular abnormalities of malignancies has proven to have significant impacts on progression free survival and overall survival in cancers such melanoma and lung adenocarcinoma. For example, the discovery of *BRAF V600E* mutation, which is present in approximately 50% of melanoma cases led to a significant increase in overall survival by using targeted therapies (Mora, Alvarez-Cubela et al. 2016). Additionally the use of tyrosine kinase inhibitors such as erlotinib, gefitinib, and afatinib have resulted in increased overall survival by as much as 20 months in patients with NSCLC harboring mutations in EGFR (Mora, Alvarez-Cubela et al. 2016). While EOC cannot be defined by specific molecular drivers, which can cover the majority of patients, this work has identified genetic vulnerabilities in TGF- β /SMAD signaling which have the potential, when targeted in the clinical setting, to potentially increase both PFS and OS. While TGF- β specific targeting agents are currently being used or have been examined for a wide variety of cancers, these agents act to suppress the pathway as a whole. In this work we present a novel concept of targeting just a specific arm (SMAD3/SMAD4) of the TGF- β /SMAD signaling pathway. Given that this pathway has roles in suppressing and promoting tumorigenesis, we believe that a more select inhibition of the SMAD3-EMT tumor promoting arm could prove more therapeutically beneficial in the clinical setting. While SIS3 is an ideal candidate for this novel strategy due to its specific targeting of SMAD3, a more clinically relevant alternative is much needed. Even though not specific for TGF- β /SMAD signaling, we propose the use of eribulin mesylate (HALAVEN[®]; Eisai Inc.) as a FDA approved option.

In addition, this work provides a solid foundation to justify a larger pharmaceutical screen to detect an even greater variety of SMAD3 inhibitors.

Overall impact of this research on ovarian cancer. There are numerous reasons that EOC remains such a difficult disease to treat. I have discussed the late stage of diagnosis, general heterogeneity, and an incomplete knowledge of how cells lose sensitivity to platinum-based chemotherapy. From detection of disease, preventing the advent of chemotherapy-resistance, and to re-sensitizing cells to chemotherapy this body of work addresses a full circle of the challenges facing clinicians, basic scientists, and, most importantly the patients. We eagerly await the next decade as we advance the translation of exosome biology further into cancer management strategies and intensify the war on cancer.

This page is intentionally left blank

REFERENCES

- Cancer Facts & Figures 2016. Atlanta: American Cancer Society. A. C. Society.
- (2011). "Integrated genomic analyses of ovarian carcinoma." Nature **474**(7353): 609-615.
- (2014). "Taking a daily aspirin might reduce ovarian cancer risk." Harv Womens Health Watch **21**(9): 8.
- (2016). "FDA Approves Eribulin for Advanced Liposarcoma." Cancer Discov **6**(3): OF1.
- Abd Elmageed, Z. Y., Y. Yang, et al. (2014). "Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes." Stem Cells **32**(4): 983-997.
- Abramowicz, A., P. Widlak, et al. (2016). "Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation." Mol Biosyst **12**(5): 1407-1419.
- Abusamra, A. J., Z. Zhong, et al. (2005). "Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis." Blood Cells Mol Dis **35**(2): 169-173.
- ACS, T. A. C. S. (2016). "What are the risk factors for ovarian cancer." from <http://www.cancer.org/cancer/ovariancancer>
- Al-Nedawi, K., B. Meehan, et al. (2008). "Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells." Nat Cell Biol **10**(5): 619-624.
- Alberts, D. S., S. Green, et al. (1992). "Improved therapeutic index of carboplatin plus cyclophosphamide versus cisplatin plus cyclophosphamide: final report by the Southwest Oncology Group of a phase III randomized trial in stages III and IV ovarian cancer." J Clin Oncol **10**(5): 706-717.
- Alegre, E., L. Zubiri, et al. (2016). "Circulating melanoma exosomes as diagnostic and prognosis biomarkers." Clin Chim Acta **454**: 28-32.
- Anders, R. A., S. L. Arline, et al. (1997). "Distinct endocytic responses of heteromeric and homomeric transforming growth factor beta receptors." Mol Biol Cell **8**(11): 2133-2143.
- Andre Mdo, R., A. Pedro, et al. (2016). "Cancer Exosomes as Mediators of Drug Resistance." Methods Mol Biol **1395**: 229-239.

- Anglesio, M. S., M. S. Carey, et al. (2011). "Clear cell carcinoma of the ovary: a report from the first Ovarian Clear Cell Symposium, June 24th, 2010." Gynecol Oncol **121**(2): 407-415.
- ASCO, t. A. S. o. C. O. (2015). "Ovarian Cancer: Risk Factors and Prevention." from <http://www.cancer.net/cancer-types/ovarian-cancer>.
- Atay, S., S. Banskota, et al. (2014). "Oncogenic KIT-containing exosomes increase gastrointestinal stromal tumor cell invasion." Proc Natl Acad Sci U S A **111**(2): 711-716.
- Atay, S. and A. K. Godwin (2014). "Tumor-derived exosomes: A message delivery system for tumor progression." Commun Integr Biol **7**(1): e28231.
- Audeh, M. W., J. Carmichael, et al. (2010). "Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial." Lancet **376**(9737): 245-251.
- Baandrup, L. (2015). "Drugs with potential chemopreventive properties in relation to epithelial ovarian cancer--a nationwide case-control study." Dan Med J **62**(7).
- Babst, M., D. J. Katzmann, et al. (2002). "Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting." Dev Cell **3**(2): 271-282.
- Babst, M., D. J. Katzmann, et al. (2002). "Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body." Dev Cell **3**(2): 283-289.
- Baietti, M. F., Z. Zhang, et al. (2012). "Syndecan-syntenin-ALIX regulates the biogenesis of exosomes." Nat Cell Biol.
- Baran, J., M. Baj-Krzyworzeka, et al. (2010). "Circulating tumour-derived microvesicles in plasma of gastric cancer patients." Cancer Immunol Immunother **59**(6): 841-850.
- Barr, M. P., S. G. Gray, et al. (2013). "Generation and characterisation of cisplatin-resistant non-small cell lung cancer cell lines displaying a stem-like signature." PLoS One **8**(1): e54193.
- Battke, C., R. Ruiss, et al. (2011). "Tumour exosomes inhibit binding of tumour-reactive antibodies to tumour cells and reduce ADCC." Cancer Immunol Immunother **60**(5): 639-648.

- Baynes, R. D., Y. J. Shih, et al. (1991). "Characterization of transferrin receptor released by K562 erythroleukemia cells." Proc Soc Exp Biol Med **197**(4): 416-423.
- Behrens, B. C., T. C. Hamilton, et al. (1987). "Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues." Cancer Res **47**(2): 414-418.
- Berrondo, C., J. Flax, et al. (2016). "Expression of the Long Non-Coding RNA HOTAIR Correlates with Disease Progression in Bladder Cancer and Is Contained in Bladder Cancer Patient Urinary Exosomes." PLoS One **11**(1): e0147236.
- Berruti, A., M. Tampellini, et al. (1994). "Prognostic value in predicting overall survival of two mucinous markers: CA 15-3 and CA 125 in breast cancer patients at first relapse of disease." Eur J Cancer **30A**(14): 2082-2084.
- Bilodeau, P. S., S. C. Winistorfer, et al. (2003). "Vps27-Hse1 and ESCRT-I complexes cooperate to increase efficiency of sorting ubiquitinated proteins at the endosome." J Cell Biol **163**(2): 237-243.
- Bischof, P., L. Tseng, et al. (1986). "Cancer antigen 125 is produced by human endometrial stromal cells." Hum Reprod **1**(7): 423-426.
- Bobrie, A., S. Krumeich, et al. (2012). "Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression." Cancer Res.
- Boelens, M. C., T. J. Wu, et al. (2014). "Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways." Cell **159**(3): 499-513.
- Bristow, R. E., R. S. Tomacruz, et al. (2002). "Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis." J Clin Oncol **20**(5): 1248-1259.
- Brose, M. S., T. R. Rebbeck, et al. (2002). "Cancer risk estimates for BRCA1 mutation carriers identified in a risk evaluation program." J Natl Cancer Inst **94**(18): 1365-1372.
- Buijs, J. T., K. R. Stayrook, et al. (2012). "The role of TGF-beta in bone metastasis: novel therapeutic perspectives." Bonekey Rep **1**: 96.
- Cao, J., J. Liu, et al. (2016). "MicroRNA-21 stimulates epithelial-to-mesenchymal transition and tumorigenesis in clear cell renal cells." Mol Med Rep **13**(1): 75-82.
- Carbotti, G., A. M. Orengo, et al. (2013). "Activated leukocyte cell adhesion molecule soluble form: a potential biomarker of epithelial ovarian cancer is increased in type II tumors." Int J Cancer **132**(11): 2597-2605.

- Casey, G., S. Plummer, et al. (1993). "Functional evidence for a breast cancer growth suppressor gene on chromosome 17." Hum Mol Genet **2**(11): 1921-1927.
- Cerami, E., J. Gao, et al. (2012). "The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data." Cancer Discov **2**(5): 401-404.
- Challagundla, K. B., P. M. Wise, et al. (2015). "Exosome-mediated transfer of microRNAs within the tumor microenvironment and neuroblastoma resistance to chemotherapy." J Natl Cancer Inst **107**(7).
- Chen, W. X., Y. Q. Cai, et al. (2014). "Exosomes from docetaxel-resistant breast cancer cells alter chemosensitivity by delivering microRNAs." Tumour Biol **35**(10): 9649-9659.
- Chen, W. X., X. M. Liu, et al. (2014). "Exosomes from Drug-Resistant Breast Cancer Cells Transmit Chemoresistance by a Horizontal Transfer of MicroRNAs." PLoS One **9**(4): e95240.
- Chen, Z., Y. Wang, et al. (2014). "Doxycycline inducible Kruppel-like factor 4 lentiviral vector mediates mesenchymal to epithelial transition in ovarian cancer cells." PLoS One **9**(8): e105331.
- Cho, J. A., H. Park, et al. (2011). "Exosomes from ovarian cancer cells induce adipose tissue-derived mesenchymal stem cells to acquire the physical and functional characteristics of tumor-supporting myofibroblasts." Gynecol Oncol **123**(2): 379-386.
- Chou, T. C. and P. Talalay (1984). "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors." Adv Enzyme Regul **22**: 27-55.
- Clayton, A., J. P. Mitchell, et al. (2007). "Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2." Cancer Res **67**(15): 7458-7466.
- Clayton, A. and Z. Tabi (2005). "Exosomes and the MICA-NKG2D system in cancer." Blood Cells Mol Dis **34**(3): 206-213.
- Coffman, L. G., Y. J. Choi, et al. (2016). "Human carcinoma-associated mesenchymal stem cells promote ovarian cancer chemotherapy resistance via a BMP4/HH signaling loop." Oncotarget.
- Cohen, S., I. Bruchim, et al. (2012). "Platinum-resistance in ovarian cancer cells is mediated by IL-6 secretion via the increased expression of its target cIAP-2." J Mol Med (Berl).

- Coleman, R. L., B. J. Monk, et al. (2013). "Latest research and treatment of advanced-stage epithelial ovarian cancer." Nat Rev Clin Oncol **10**(4): 211-224.
- Cooper, J. M., P. B. Wiklander, et al. (2014). "Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice." Mov Disord **29**(12): 1476-1485.
- Corcoran, C., S. Rani, et al. (2012). "Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes." PLoS One **7**(12): e50999.
- Cordani, M., R. Pacchiana, et al. (2016). "Mutant p53 proteins alter cancer cell secretome and tumour microenvironment: involvement in cancer invasion and metastasis." Cancer Lett.
- Crijnen, T. E., M. L. Janssen-Heijnen, et al. (2005). "Survival of patients with ovarian cancer due to a mismatch repair defect." Fam Cancer **4**(4): 301-305.
- CSSOCR, C. o. t. S. o. T. S. i. O. C. R., Ed. (2016). Ovarian Cancer: Evolving Paradigms in Research and Care.
- Cui, J., M. Shi, et al. (2013). "Regulation of EMT by KLF4 in gastrointestinal cancer." Curr Cancer Drug Targets **13**(9): 986-995.
- Davis, B. N., A. C. Hilyard, et al. (2008). "SMAD proteins control DROSHA-mediated microRNA maturation." Nature **454**(7200): 56-61.
- de Caestecker, M. P., E. Piek, et al. (2000). "Role of transforming growth factor-beta signaling in cancer." J Natl Cancer Inst **92**(17): 1388-1402.
- de la Fuente, A., L. Alonso-Alconada, et al. (2015). "M-Trap: Exosome-Based Capture of Tumor Cells as a New Technology in Peritoneal Metastasis." J Natl Cancer Inst **107**(9).
- De Mattos-Arruda, L., G. Bottai, et al. (2015). "MicroRNA-21 links epithelial-to-mesenchymal transition and inflammatory signals to confer resistance to neoadjuvant trastuzumab and chemotherapy in HER2-positive breast cancer patients." Oncotarget **6**(35): 37269-37280.
- Demory Beckler, M., J. N. Higginbotham, et al. (2013). "Proteomic analysis of exosomes from mutant KRAS colon cancer cells identifies intercellular transfer of mutant KRAS." Mol Cell Proteomics **12**(2): 343-355.

- Deng, Z., Z. Cheng, et al. (2012). "Tumor cell cross talk with tumor-associated leukocytes leads to induction of tumor exosomal fibronectin and promotes tumor progression." Am J Pathol **180**(1): 390-398.
- Deregibus, M. C., V. Cantaluppi, et al. (2007). "Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA." Blood **110**(7): 2440-2448.
- Derynck, R., W. M. Gelbart, et al. (1996). "Nomenclature: vertebrate mediators of TGFbeta family signals." Cell **87**(2): 173.
- Diaz-Padilla, G. T. a. I. (2013). *Molecular Mechanisms of Platinum Resistance in Ovarian Cancer, Ovarian Cancer- A Clinical and Translational Update*. D. I. Diaz-Padilla, ISBN.
- Dijkgraaf, E. M., M. Heusinkveld, et al. (2013). "Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment." Cancer Res **73**(8): 2480-2492.
- Do, T. V., L. A. Kubba, et al. (2008). "Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition." Mol Cancer Res **6**(5): 695-705.
- Domchek, S. M., T. M. Friebel, et al. (2010). "Association of risk-reducing surgery in BRCA1 or BRCA2 mutation carriers with cancer risk and mortality." JAMA **304**(9): 967-975.
- Drew, Y., E. A. Mulligan, et al. (2011). "Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2." J Natl Cancer Inst **103**(4): 334-346.
- du Bois, A., A. Reuss, et al. (2009). "Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO)." Cancer **115**(6): 1234-1244.
- Eagle, K. and J. A. Ledermann (1997). "Tumor Markers in Ovarian Malignancies." Oncologist **2**(5): 324-329.
- Echevarria-Vargas, I. M., F. Valiyeva, et al. (2014). "Upregulation of miR-21 in cisplatin resistant ovarian cancer via JNK-1/c-Jun pathway." PLoS One **9**(5): e97094.

- Eckstein, N., K. Servan, et al. (2009). "Hyperactivation of the insulin-like growth factor receptor I signaling pathway is an essential event for cisplatin resistance of ovarian cancer cells." Cancer Res **69**(7): 2996-3003.
- Eichelser, C., I. Stuckrath, et al. (2014). "Increased serum levels of circulating exosomal microRNA-373 in receptor-negative breast cancer patients." Oncotarget **5**(20): 9650-9663.
- Eitan, R., M. Kushnir, et al. (2009). "Tumor microRNA expression patterns associated with resistance to platinum based chemotherapy and survival in ovarian cancer patients." Gynecol Oncol **114**(2): 253-259.
- Eva, A., K. C. Robbins, et al. (1982). "Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells." Nature **295**(5845): 116-119.
- Evans, B. D., K. S. Raju, et al. (1983). "Phase II study of JM8, a new platinum analog, in advanced ovarian carcinoma." Cancer Treat Rep **67**(11): 997-1000.
- Fabbri, M., A. Paone, et al. (2012). "MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response." Proc Natl Acad Sci U S A **109**(31): E2110-2116.
- Fathalla, M. F. (1971). "Incessant ovulation--a factor in ovarian neoplasia?" Lancet **2**(7716): 163.
- Federici, C., F. Petrucci, et al. (2014). "Exosome release and low pH belong to a framework of resistance of human melanoma cells to cisplatin." PLoS One **9**(2): e88193.
- Feng, Z. (2010). "p53 regulation of the IGF-1/AKT/mTOR pathways and the endosomal compartment." Cold Spring Harb Perspect Biol **2**(2): a001057.
- Ferrone, C. R., D. A. Levine, et al. (2009). "BRCA germline mutations in Jewish patients with pancreatic adenocarcinoma." J Clin Oncol **27**(3): 433-438.
- Ferry, K. V., T. C. Hamilton, et al. (2000). "Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: role of ERCC1-XPF." Biochem Pharmacol **60**(9): 1305-1313.
- Fink, D., S. Nebel, et al. (1996). "The role of DNA mismatch repair in platinum drug resistance." Cancer Res **56**(21): 4881-4886.
- Fischer, K. R., A. Durrans, et al. (2015). "Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance." Nature **527**(7579): 472-476.

- Fong, M. Y., W. Zhou, et al. (2015). "Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis." Nat Cell Biol **17**(2): 183-194.
- Fong, P. C., T. A. Yap, et al. (2010). "Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval." J Clin Oncol **28**(15): 2512-2519.
- Franzen, C. A., R. H. Blackwell, et al. (2015). "Urothelial cells undergo epithelial-to-mesenchymal transition after exposure to muscle invasive bladder cancer exosomes." Oncogenesis **4**: e163.
- Freedman, J. E., M. Gerstein, et al. (2016). "Diverse human extracellular RNAs are widely detected in human plasma." Nat Commun **7**: 11106.
- Fung-Kee-Fung, M., T. Oliver, et al. (2007). "Optimal chemotherapy treatment for women with recurrent ovarian cancer." Curr Oncol **14**(5): 195-208.
- Galindo-Hernandez, O., N. Serna-Marquez, et al. (2014). "Extracellular vesicles from MDA-MB-231 breast cancer cells stimulated with linoleic acid promote an EMT-like process in MCF10A cells." Prostaglandins Leukot Essent Fatty Acids **91**(6): 299-310.
- Galluzzi, L., L. Senovilla, et al. (2012). "Molecular mechanisms of cisplatin resistance." Oncogene **31**(15): 1869-1883.
- Gao, J., B. A. Aksoy, et al. (2013). "Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal." Sci Signal **6**(269): p11.
- Garcia, R., J. F. Nistal, et al. (2015). "p-SMAD2/3 and DICER promote pre-miR-21 processing during pressure overload-associated myocardial remodeling." Biochim Biophys Acta **1852**(7): 1520-1530.
- Ge, R., E. Tan, et al. (2012). "Exosomes in Cancer Microenvironment and Beyond: have we Overlooked these Extracellular Messengers?" Cancer Microenviron **5**(3): 323-332.
- Gercel-Taylor, C., S. Atay, et al. (2012). "Nanoparticle analysis of circulating cell-derived vesicles in ovarian cancer patients." Anal Biochem **428**(1): 44-53.
- Gercel-Taylor, C., S. Atay, et al. (2012). "Nanoparticle analysis of circulating cell-derived vesicles in ovarian cancer patients." Anal Biochem.
- Gernapudi, R., Y. Yao, et al. (2015). "Targeting exosomes from preadipocytes inhibits preadipocyte to cancer stem cell signaling in early-stage breast cancer." Breast Cancer Res Treat **150**(3): 685-695.

- Godwin, A. K., A. Meister, et al. (1992). "High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis." Proc Natl Acad Sci U S A **89**(7): 3070-3074.
- Godwin, A. K., J. R. Testa, et al. (1992). "Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer." J Natl Cancer Inst **84**(8): 592-601.
- Gorczynski, R. M., N. Erin, et al. (2016). "Serum-derived exosomes from mice with highly metastatic breast cancer transfer increased metastatic capacity to a poorly metastatic tumor." Cancer Med **5**(2): 325-336.
- Gordon, A. N., J. T. Fleagle, et al. (2001). "Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin versus topotecan." J Clin Oncol **19**(14): 3312-3322.
- Gourley, C., J. Farley, et al. (2014). "Gynecologic Cancer InterGroup (GCIg) consensus review for ovarian and primary peritoneal low-grade serous carcinomas." Int J Gynecol Cancer **24**(9 Suppl 3): S9-13.
- Guddati, A. K. (2012). "Ovarian cancer stem cells: elusive targets for chemotherapy." Med Oncol.
- Gunderson, C. C. and K. N. Moore (2015). "BRACAnalysis CDx as a companion diagnostic tool for Lynparza." Expert Rev Mol Diagn **15**(9): 1111-1116.
- Hamilton, T. C., G. M. Lai, et al. (1989). "Mechanisms of resistance to cisplatin and alkylating agents." Cancer Treat Res **48**: 151-169.
- Hamilton, T. C., R. C. Young, et al. (1984). "Experimental model systems of ovarian cancer: applications to the design and evaluation of new treatment approaches." Semin Oncol **11**(3): 285-298.
- Han, M., F. Wang, et al. (2016). "MicroRNA-21 induces breast cancer cell invasion and migration by suppressing smad7 via EGF and TGF-beta pathways." Oncol Rep **35**(1): 73-80.
- Han, Y., G. X. Xu, et al. (2015). "Dysregulation of miRNA-21 and their potential as biomarkers for the diagnosis of cervical cancer." Int J Clin Exp Pathol **8**(6): 7131-7139.
- Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

- Harding, C., J. Heuser, et al. (1983). "Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes." J Cell Biol **97**(2): 329-339.
- Haug, B. H., O. H. Hald, et al. (2015). "Exosome-like Extracellular Vesicles from MYCN-amplified Neuroblastoma Cells Contain Oncogenic miRNAs." Anticancer Res **35**(5): 2521-2530.
- He, M., J. Crow, et al. (2014). "Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology." Lab Chip **14**(19): 3773-3780.
- Hector, S., W. Bolanowska-Higdon, et al. (2001). "In vitro studies on the mechanisms of oxaliplatin resistance." Cancer Chemother Pharmacol **48**(5): 398-406.
- Helleday, T., E. Petermann, et al. (2008). "DNA repair pathways as targets for cancer therapy." Nat Rev Cancer **8**(3): 193-204.
- Helleman, J., M. Smid, et al. (2010). "Pathway analysis of gene lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture." Gynecol Oncol **117**(2): 170-176.
- Hendriks, R. J., S. Dijkstra, et al. (2016). "Comparative analysis of prostate cancer specific biomarkers PCA3 and ERG in whole urine, urinary sediments and exosomes." Clin Chem Lab Med **54**(3): 483-492.
- Hendrix, A., G. Braems, et al. (2010). "The secretory small GTPase Rab27B as a marker for breast cancer progression." Oncotarget **1**(4): 304-308.
- Hendrix, A., D. Maynard, et al. (2010). "Effect of the secretory small GTPase Rab27B on breast cancer growth, invasion, and metastasis." J Natl Cancer Inst **102**(12): 866-880.
- Hensley, M. L., S. Kravetz, et al. (2012). "Eribulin mesylate (halichondrin B analog E7389) in platinum-resistant and platinum-sensitive ovarian cancer: a 2-cohort, phase 2 study." Cancer **118**(9): 2403-2410.
- Herbertz, S., J. S. Sawyer, et al. (2015). "Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway." Drug Des Devel Ther **9**: 4479-4499.
- Herreros-Villanueva, M. and L. Bujanda (2016). "Glypican-1 in exosomes as biomarker for early detection of pancreatic cancer." Ann Transl Med **4**(4): 64.
- Higginbotham, J. N., M. Demory Beckler, et al. (2011). "Amphiregulin exosomes increase cancer cell invasion." Curr Biol **21**(9): 779-786.

- Hong, B. S., J. H. Cho, et al. (2009). "Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells." BMC Genomics **10**: 556.
- Hood, J. L., R. S. San, et al. (2011). "Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis." Cancer Res **71**(11): 3792-3801.
- Howlander N, N., AM, Krapcho M, Garshell J, Miller D, Alterkruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich A, Mariotto A, Lewis DR, dhen HS, Feuer EJ, Cronin KA (2015). SEER cancer statistics review, 1975-2011, National Cancer Institute.
- Hsu, C., Y. Morohashi, et al. (2010). "Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C." J Cell Biol **189**(2): 223-232.
- Huang, X., T. Yuan, et al. (2015). "Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer." Eur Urol **67**(1): 33-41.
- Husain, A., Y. Wang, et al. (2016). "Independent Radiologic Review of AURELIA, A Phase 3 Trial of Bevacizumab Plus Chemotherapy for Platinum-Resistant Recurrent Ovarian Cancer." Gynecol Oncol.
- Ishida, S., J. Lee, et al. (2002). "Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals." Proc Natl Acad Sci U S A **99**(22): 14298-14302.
- Ishikawa, T. (1992). "The ATP-dependent glutathione S-conjugate export pump." Trends Biochem Sci **17**(11): 463-468.
- Jacobs, I. J., U. Menon, et al. (2016). "Ovarian cancer screening and mortality in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial." Lancet **387**(10022): 945-956.
- Janowska-Wieczorek, A., M. Wysoczynski, et al. (2005). "Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer." Int J Cancer **113**(5): 752-760.
- Jelonek, K., P. Widlak, et al. (2016). "The Influence of Ionizing Radiation on Exosome Composition, Secretion and Intercellular Communication." Protein Pept Lett.
- Jinnin, M., H. Ihn, et al. (2006). "Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression." Mol Pharmacol **69**(2): 597-607.
- Johnstone, R. M. (2006). "Exosomes biological significance: A concise review." Blood Cells Mol Dis **36**(2): 315-321.

- Johnstone, R. M., M. Adam, et al. (1987). "Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)." J Biol Chem **262**(19): 9412-9420.
- Johnstone, R. M., A. Bianchini, et al. (1989). "Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions." Blood **74**(5): 1844-1851.
- Joss, R. A., S. Kaplan, et al. (1984). "A phase I trial of cis-diammine-1,1-cyclobutane dicarboxylate platinum II (Carboplatin, CBDCA, JM-8) with a single dose every five week-schedule." Invest New Drugs **2**(3): 297-304.
- Kahlert, C., S. A. Melo, et al. (2014). "Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer." J Biol Chem **289**(7): 3869-3875.
- Katano, K., A. Kondo, et al. (2002). "Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper." Cancer Res **62**(22): 6559-6565.
- Katzmann, D. J., M. Babst, et al. (2001). "Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I." Cell **106**(2): 145-155.
- Katzmann, D. J., C. J. Stefan, et al. (2003). "Vps27 recruits ESCRT machinery to endosomes during MVB sorting." J Cell Biol **162**(3): 413-423.
- Keerthikumar, S., D. Chisanga, et al. (2016). "ExoCarta: A Web-Based Compendium of Exosomal Cargo." J Mol Biol **428**(4): 688-692.
- Kelland, L. (2007). "The resurgence of platinum-based cancer chemotherapy." Nat Rev Cancer **7**(8): 573-584.
- Keller, S., A. K. Konig, et al. (2009). "Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes." Cancer Lett **278**(1): 73-81.
- Keller, S., M. P. Sanderson, et al. (2006). "Exosomes: from biogenesis and secretion to biological function." Immunol Lett **107**(2): 102-108.
- Khan, S., J. M. Jutzy, et al. (2011). "Survivin is released from cancer cells via exosomes." Apoptosis **16**(1): 1-12.
- Kharaziha, P., D. Chioureas, et al. (2015). "Molecular profiling of prostate cancer derived exosomes may reveal a predictive signature for response to docetaxel." Oncotarget **6**(25): 21740-21754.

- Kim, G., G. Ison, et al. (2015). "FDA Approval Summary: Olaparib Monotherapy in Patients with Deleterious Germline BRCA-Mutated Advanced Ovarian Cancer Treated with Three or More Lines of Chemotherapy." Clin Cancer Res **21**(19): 4257-4261.
- King, H. W., M. Z. Michael, et al. (2012). "Hypoxic enhancement of exosome release by breast cancer cells." BMC Cancer **12**: 421.
- Knox, R. J., F. Friedlos, et al. (1986). "Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA." Cancer Res **46**(4 Pt 2): 1972-1979.
- Kogure, T., W. L. Lin, et al. (2011). "Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth." Hepatology **54**(4): 1237-1248.
- Koppers-Lalic, D., M. M. Hogenboom, et al. (2013). "Virus-modified exosomes for targeted RNA delivery; a new approach in nanomedicine." Adv Drug Deliv Rev **65**(3): 348-356.
- Kucharzewska, P., H. C. Christianson, et al. (2013). "Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development." Proc Natl Acad Sci U S A **110**(18): 7312-7317.
- Kuczynski, E. A., D. J. Sargent, et al. (2013). "Drug rechallenge and treatment beyond progression--implications for drug resistance." Nat Rev Clin Oncol **10**(10): 571-587.
- Kurman, R. J. and M. Shih le (2010). "The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory." Am J Surg Pathol **34**(3): 433-443.
- Kurokawa, M., N. Ise, et al. (2013). "Cisplatin influences acquisition of resistance to molecular-targeted agents through epithelial-mesenchymal transition-like changes." Cancer Sci **104**(7): 904-911.
- Kwon, Y., B. D. Smith, et al. (2015). "Effective inhibition of c-MET-mediated signaling, growth and migration of ovarian cancer cells is influenced by the ovarian tissue microenvironment." Oncogene **34**(2): 144-153.
- Kyrgiou, M., G. Salanti, et al. (2006). "Survival benefits with diverse chemotherapy regimens for ovarian cancer: meta-analysis of multiple treatments." J Natl Cancer Inst **98**(22): 1655-1663.
- Lagna, G., A. Hata, et al. (1996). "Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways." Nature **383**(6603): 832-836.

- Lai, J. Y., J. Luo, et al. (2015). "MicroRNA-21 in glomerular injury." J Am Soc Nephrol **26**(4): 805-816.
- Latifi, A., K. Abubaker, et al. (2011). "Cisplatin treatment of primary and metastatic epithelial ovarian carcinomas generates residual cells with mesenchymal stem cell-like profile." J Cell Biochem **112**(10): 2850-2864.
- Lau, C., Y. Kim, et al. (2013). "Role of pancreatic cancer-derived exosomes in salivary biomarker development." J Biol Chem **288**(37): 26888-26897.
- Laulagnier, K., D. Grand, et al. (2004). "PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes." FEBS Lett **572**(1-3): 11-14.
- Ledermann, J. A. and R. S. Kristeleit (2010). "Optimal treatment for relapsing ovarian cancer." Ann Oncol **21 Suppl 7**: vii218-222.
- Lee, K. R. and R. H. Young (2003). "The distinction between primary and metastatic mucinous carcinomas of the ovary: gross and histologic findings in 50 cases." Am J Surg Pathol **27**(3): 281-292.
- Lee, Y. S., S. H. Kim, et al. (2011). "Introduction of the CIITA gene into tumor cells produces exosomes with enhanced anti-tumor effects." Exp Mol Med **43**(5): 281-290.
- Levine, A. J., N. Reich, et al. (1983). "The regulation of a cellular protein, p53, in normal and transformed cells." Prog Clin Biol Res **119**: 159-169.
- Levy-Lahad, E. and E. Friedman (2007). "Cancer risks among BRCA1 and BRCA2 mutation carriers." Br J Cancer **96**(1): 11-15.
- Li, H., J. Wang, et al. (2014). "Epigenetic inactivation of KLF4 is associated with urothelial cancer progression and early recurrence." J Urol **191**(2): 493-501.
- Li, W., Y. Hu, et al. (2014). "Rab27A regulates exosome secretion from lung adenocarcinoma cells A549: involvement of EPI64." APMIS **122**(11): 1080-1087.
- Lim, P. K., S. A. Bliss, et al. (2011). "Gap junction-mediated import of microRNA from bone marrow stromal cells can elicit cell cycle quiescence in breast cancer cells." Cancer Res **71**(5): 1550-1560.
- Liu, J., H. Sun, et al. (2014). "Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer." Int J Mol Sci **15**(1): 758-773.

- Louie, K. G., B. C. Behrens, et al. (1985). "Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine." Cancer Res **45**(5): 2110-2115.
- Lowry, M. C., W. M. Gallagher, et al. (2015). "The Role of Exosomes in Breast Cancer." Clin Chem **61**(12): 1457-1465.
- Luga, V. and J. L. Wrana (2013). "Tumor-stroma interaction: Revealing fibroblast-secreted exosomes as potent regulators of Wnt-planar cell polarity signaling in cancer metastasis." Cancer Res **73**(23): 6843-6847.
- Luo, F., J. Ji, et al. (2014). "MicroRNA-21, up-regulated by arsenite, directs the epithelial-mesenchymal transition and enhances the invasive potential of transformed human bronchial epithelial cells by targeting PDCD4." Toxicol Lett **232**(1): 301-309.
- Luvero, D., A. Milani, et al. (2014). "Treatment options in recurrent ovarian cancer: latest evidence and clinical potential." Ther Adv Med Oncol **6**(5): 229-239.
- Lv, M. M., X. Y. Zhu, et al. (2014). "Exosomes mediate drug resistance transfer in MCF-7 breast cancer cells and a probable mechanism is delivery of P-glycoprotein." Tumour Biol **35**(11): 10773-10779.
- Lynch, H. T., M. J. Casey, et al. (2009). "Hereditary ovarian carcinoma: heterogeneity, molecular genetics, pathology, and management." Mol Oncol **3**(2): 97-137.
- Lynch, H. T. and P. M. Lynch (1979). "Tumor variation in the cancer family syndrome: ovarian cancer." Am J Surg **138**(3): 439-442.
- Madhavan, B., S. Yue, et al. (2015). "Combined evaluation of a panel of protein and miRNA serum-exosome biomarkers for pancreatic cancer diagnosis increases sensitivity and specificity." Int J Cancer **136**(11): 2616-2627.
- Maida, Y., M. Takakura, et al. (2016). "Exosomal transfer of functional small RNAs mediates cancer-stroma communication in human endometrium." Cancer Med **5**(2): 304-314.
- Marchini, S., R. Fruscio, et al. (2013). "Resistance to platinum-based chemotherapy is associated with epithelial to mesenchymal transition in epithelial ovarian cancer." Eur J Cancer **49**(2): 520-530.
- Marchini, S., R. Fruscio, et al. (2013). "Resistance to platinum-based chemotherapy is associated with epithelial to mesenchymal transition in epithelial ovarian cancer." Eur J Cancer **49**(2): 520-530.

- Markman, M., J. Blessing, et al. (2006). "Phase II trial of weekly paclitaxel (80 mg/m²) in platinum and paclitaxel-resistant ovarian and primary peritoneal cancers: a Gynecologic Oncology Group study." Gynecol Oncol **101**(3): 436-440.
- Marleau, A. M., C. S. Chen, et al. (2012). "Exosome removal as a therapeutic adjuvant in cancer." J Transl Med **10**: 134.
- Marquez, R. T., K. A. Baggerly, et al. (2005). "Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon." Clin Cancer Res **11**(17): 6116-6126.
- Massague, J. (2008). "TGFbeta in Cancer." Cell **134**(2): 215-230.
- Massague, J. (2012). "TGFbeta signalling in context." Nat Rev Mol Cell Biol **13**(10): 616-630.
- Matsumura, T., K. Sugimachi, et al. (2015). "Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer." Br J Cancer **113**(2): 275-281.
- Mavaddat, N., D. Barrowdale, et al. (2012). "Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA)." Cancer Epidemiol Biomarkers Prev **21**(1): 134-147.
- Mavaddat, N., S. Peock, et al. (2013). "Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE." J Natl Cancer Inst **105**(11): 812-822.
- McBride, A. and S. K. Butler (2012). "Eribulin mesylate: a novel halichondrin B analogue for the treatment of metastatic breast cancer." Am J Health Syst Pharm **69**(9): 745-755.
- McCluggage, W. G. (2011). "Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis." Pathology **43**(5): 420-432.
- McGuire, W. P., W. J. Hoskins, et al. (1996). "Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer." N Engl J Med **334**(1): 1-6.
- McLemore, M. R., C. Miaskowski, et al. (2009). "Epidemiological and genetic factors associated with ovarian cancer." Cancer Nurs **32**(4): 281-288; quiz 289-290.
- Melo, S. A., L. B. Luecke, et al. (2015). "Glypican-1 identifies cancer exosomes and detects early pancreatic cancer." Nature **523**(7559): 177-182.

- Melo, S. A., H. Sugimoto, et al. (2014). "Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis." Cancer Cell **26**(5): 707-721.
- Merendino, A. M., F. Bucchieri, et al. (2010). "Hsp60 is actively secreted by human tumor cells." PLoS One **5**(2): e9247.
- Meulmeester, E. and P. Ten Dijke (2011). "The dynamic roles of TGF-beta in cancer." J Pathol **223**(2): 205-218.
- Mir, R., A. Tortosa, et al. (2012). "Mdm2 antagonists induce apoptosis and synergize with cisplatin overcoming chemoresistance in TP53 wild type ovarian cancer cells." Int J Cancer.
- Mistry, P., L. R. Kelland, et al. (1991). "The relationships between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines." Br J Cancer **64**(2): 215-220.
- Mitchell, P. J., J. Welton, et al. (2009). "Can urinary exosomes act as treatment response markers in prostate cancer?" J Transl Med **7**: 4.
- Mok, T. S., S. L. Geater, et al. (2014). "Randomized phase II study of two intercalated combinations of eribulin mesylate and erlotinib in patients with previously treated advanced non-small-cell lung cancer." Ann Oncol **25**(8): 1578-1584.
- Mora, E. M., S. Alvarez-Cubela, et al. (2016). "Biobanking of Exosomes in the Era of Precision Medicine: Are We There Yet?" Int J Mol Sci **17**(1).
- Morse, M. A., J. Garst, et al. (2005). "A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer." J Transl Med **3**(1): 9.
- Muggia, F. M., A. Bonetti, et al. (2015). "Platinum Antitumor Complexes: 50 Years Since Barnett Rosenberg's Discovery." J Clin Oncol **33**(35): 4219-4226.
- Munagala, R., F. Aqil, et al. (2016). "Bovine milk-derived exosomes for drug delivery." Cancer Lett **371**(1): 48-61.
- Naumann, R. W. and R. L. Coleman (2011). "Management strategies for recurrent platinum-resistant ovarian cancer." Drugs **71**(11): 1397-1412.
- Nazarenko, I., S. Rana, et al. (2010). "Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation." Cancer Res **70**(4): 1668-1678.
- Necchi, A., P. Giannatempo, et al. (2014). "PF-03446962, a fully-human monoclonal antibody against transforming growth-factor beta (TGFbeta) receptor ALK1, in pre-treated patients with urothelial cancer: an open label, single-group, phase 2 trial." Invest New Drugs **32**(3): 555-560.

- Newman, S. (2007). "Eribulin, a simplified ketone analog of the tubulin inhibitor halichondrin B, for the potential treatment of cancer." Curr Opin Investig Drugs **8**(12): 1057-1066.
- Nicolson, G. L. (2015). "Cell membrane fluid-mosaic structure and cancer metastasis." Cancer Res **75**(7): 1169-1176.
- Ogata-Kawata, H., M. Izumiya, et al. (2014). "Circulating exosomal microRNAs as biomarkers of colon cancer." PLoS One **9**(4): e92921.
- Ogorevc, E., V. Kralj-Iglic, et al. (2013). "The role of extracellular vesicles in phenotypic cancer transformation." Radiol Oncol **47**(3): 197-205.
- Ohishi, Y., Y. Oda, et al. (2002). "ATP-binding cassette superfamily transporter gene expression in human primary ovarian carcinoma." Clin Cancer Res **8**(12): 3767-3775.
- Ohno, S., A. Ishikawa, et al. (2013). "Roles of exosomes and microvesicles in disease pathogenesis." Adv Drug Deliv Rev **65**(3): 398-401.
- Ohno, S., M. Takanashi, et al. (2013). "Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells." Mol Ther **21**(1): 185-191.
- Ostenfeld, M. S., D. K. Jeppesen, et al. (2014). "Cellular disposal of miR23b by RAB27-dependent exosome release is linked to acquisition of metastatic properties." Cancer Res **74**(20): 5758-5771.
- Ostrowski, M., N. B. Carmo, et al. (2010). "Rab27a and Rab27b control different steps of the exosome secretion pathway." Nat Cell Biol **12**(1): 19-30; sup pp 11-13.
- Paduch, R. (2016). "The role of lymphangiogenesis and angiogenesis in tumor metastasis." Cell Oncol (Dordr).
- Pan, B. T., K. Teng, et al. (1985). "Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes." J Cell Biol **101**(3): 942-948.
- Park, J. E., H. S. Tan, et al. (2010). "Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes." Mol Cell Proteomics **9**(6): 1085-1099.
- Peinado, H., M. Aleckovic, et al. (2012). "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET." Nat Med **18**(6): 883-891.

- Peinado, H., M. Aleckovic, et al. (2012). "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET." Nat Med.
- Peng, P., Y. Yan, et al. (2011). "Exosomes in the ascites of ovarian cancer patients: origin and effects on anti-tumor immunity." Oncol Rep **25**(3): 749-762.
- Perez-Torres, M., B. L. Valle, et al. (2008). "Shedding of epidermal growth factor receptor is a regulated process that occurs with overexpression in malignant cells." Exp Cell Res **314**(16): 2907-2918.
- Perez, R. P., T. C. Hamilton, et al. (1990). "Resistance to alkylating agents and cisplatin: insights from ovarian carcinoma model systems." Pharmacol Ther **48**(1): 19-27.
- Perez, R. P., K. M. Perez, et al. (1992). "In vitro interactions between platinum analogues in human ovarian-carcinoma cell lines." Cancer Chemother Pharmacol **29**(6): 430-434.
- Perren, T. J., A. M. Swart, et al. (2011). "A phase 3 trial of bevacizumab in ovarian cancer." N Engl J Med **365**(26): 2484-2496.
- Petersen, M., E. Pardali, et al. (2010). "Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis." Oncogene **29**(9): 1351-1361.
- Philip, R., S. Heiler, et al. (2015). "Claudin-7 promotes the epithelial-mesenchymal transition in human colorectal cancer." Oncotarget **6**(4): 2046-2063.
- Phuyal, S., T. Skotland, et al. (2015). "The ether lipid precursor hexadecylglycerol stimulates the release and changes the composition of exosomes derived from PC-3 cells." J Biol Chem **290**(7): 4225-4237.
- Piccart, M. J., K. Bertelsen, et al. (2000). "Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results." J Natl Cancer Inst **92**(9): 699-708.
- Pink, R. C., P. Samuel, et al. (2015). "The passenger strand, miR-21-3p, plays a role in mediating cisplatin resistance in ovarian cancer cells." Gynecol Oncol **137**(1): 143-151.
- Pittaway, D. E. and J. A. Fayez (1986). "The use of CA-125 in the diagnosis and management of endometriosis." Fertil Steril **46**(5): 790-795.
- Prat, J. (2015). "FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication." J Gynecol Oncol **26**(2): 87-89.

- Qu, K., T. Lin, et al. (2016). "Extracellular miRNA-21 as a novel biomarker in glioma: Evidence from meta-analysis, clinical validation and experimental investigations." Oncotarget.
- Rabinowits, G., C. Gerdel-Taylor, et al. (2009). "Exosomal microRNA: a diagnostic marker for lung cancer." Clin Lung Cancer **10**(1): 42-46.
- Raiborg, C., T. E. Rusten, et al. (2003). "Protein sorting into multivesicular endosomes." Curr Opin Cell Biol **15**(4): 446-455.
- Ramteke, A., H. Ting, et al. (2015). "Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules." Mol Carcinog **54**(7): 554-565.
- Raposo, G. and W. Stoorvogel (2013). "Extracellular vesicles: exosomes, microvesicles, and friends." J Cell Biol **200**(4): 373-383.
- Raposo, G., D. Tenza, et al. (1997). "Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation." Mol Biol Cell **8**(12): 2631-2645.
- Rebbeck, T. R., N. Mitra, et al. (2015). "Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer." JAMA **313**(13): 1347-1361.
- Reva, B., Y. Antipin, et al. (2011). "Predicting the functional impact of protein mutations: application to cancer genomics." Nucleic Acids Res **39**(17): e118.
- Robbins, P. D. and A. E. Morelli (2014). "Regulation of immune responses by extracellular vesicles." Nat Rev Immunol **14**(3): 195-208.
- Roberson, C. D., S. Atay, et al. (2010). "Tumor-derived exosomes as mediators of disease and potential diagnostic biomarkers." Cancer Biomark **8**(4-5): 281-291.
- Rodon, J., M. Carducci, et al. (2015). "Pharmacokinetic, pharmacodynamic and biomarker evaluation of transforming growth factor-beta receptor I kinase inhibitor, galunisertib, in phase 1 study in patients with advanced cancer." Invest New Drugs **33**(2): 357-370.
- Rodriguez, M., J. Silva, et al. (2015). "Exosomes enriched in stemness/metastatic-related mRNAs promote oncogenic potential in breast cancer." Oncotarget **6**(38): 40575-40587.
- Rooj, A. K., M. Mineo, et al. (2016). "MicroRNA and extracellular vesicles in glioblastoma: small but powerful." Brain Tumor Pathol **33**(2): 77-88.

- Rosenberg, B., L. Vancamp, et al. (1965). "INHIBITION OF CELL DIVISION IN ESCHERICHIA COLI BY ELECTROLYSIS PRODUCTS FROM A PLATINUM ELECTRODE." Nature **205**: 698-699.
- Rosenberg, B., L. VanCamp, et al. (1969). "Platinum compounds: a new class of potent antitumour agents." Nature **222**(5191): 385-386.
- Rossof, A. H., R. W. Talley, et al. (1979). "Phase II evaluation of cis-dichlorodiammineplatinum(II) in advanced malignancies of the genitourinary and gynecologic organs: a Southwest Oncology Group Study." Cancer Treat Rep **63**(9-10): 1557-1564.
- Runz, S., S. Keller, et al. (2007). "Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM." Gynecol Oncol **107**(3): 563-571.
- Safaei, R., B. J. Larson, et al. (2005). "Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells." Mol Cancer Ther **4**(10): 1595-1604.
- Salani, R., R. J. Kurman, et al. (2008). "Assessment of TP53 mutation using purified tissue samples of ovarian serous carcinomas reveals a higher mutation rate than previously reported and does not correlate with drug resistance." Int J Gynecol Cancer **18**(3): 487-491.
- Schlingensiepen, K. H., B. Fischer-Blass, et al. (2008). "Antisense therapeutics for tumor treatment: the TGF-beta2 inhibitor AP 12009 in clinical development against malignant tumors." Recent Results Cancer Res **177**: 137-150.
- Schlingensiepen, K. H., R. Schlingensiepen, et al. (2006). "Targeted tumor therapy with the TGF-beta 2 antisense compound AP 12009." Cytokine Growth Factor Rev **17**(1-2): 129-139.
- Seidman, J. D., I. Horkayne-Szakaly, et al. (2004). "The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin." Int J Gynecol Pathol **23**(1): 41-44.
- Sethi, G., H. B. Pathak, et al. (2012). "An RNA interference lethality screen of the human druggable genome to identify molecular vulnerabilities in epithelial ovarian cancer." PLoS One **7**(10): e47086.
- Shang, X., X. Lin, et al. (2012). "Claudin-3 and Claudin-4 Regulate Sensitivity to Cisplatin by Controlling Expression of the Copper and Cisplatin Influx Ransporter CTR1." Mol Pharmacol.
- Sharma, S., B. M. Gillespie, et al. (2011). "Quantitative nanostructural and single-molecule force spectroscopy biomolecular analysis of human-saliva-derived exosomes." Langmuir **27**(23): 14394-14400.

- Shtam, T. A., R. A. Kovalev, et al. (2013). "Exosomes are natural carriers of exogenous siRNA to human cells in vitro." Cell Commun Signal **11**: 88.
- Shweiki, D., A. Itin, et al. (1992). "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis." Nature **359**(6398): 843-845.
- Siegel, P. M. and J. Massague (2003). "Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer." Nat Rev Cancer **3**(11): 807-821.
- Siegel, R., J. Ma, et al. (2014). "Cancer statistics, 2014." CA Cancer J Clin **64**(1): 9-29.
- Singer, G., R. J. Kurman, et al. (2002). "Diverse tumorigenic pathways in ovarian serous carcinoma." Am J Pathol **160**(4): 1223-1228.
- Singh, R., R. Pochampally, et al. (2014). "Exosome-mediated transfer of miR-10b promotes cell invasion in breast cancer." Mol Cancer **13**: 256.
- Sivadasan, P., M. K. Gupta, et al. (2015). "Human salivary proteome--a resource of potential biomarkers for oral cancer." J Proteomics **127**(Pt A): 89-95.
- Skog, J., T. Wurdinger, et al. (2008). "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers." Nat Cell Biol **10**(12): 1470-1476.
- Smolle, E., V. Taucher, et al. (2014). "Targeted treatment of ovarian cancer--the multiple - kinase - inhibitor sorafenib as a potential option." Anticancer Res **34**(4): 1519-1530.
- Stewart, D. J. (2007). "Mechanisms of resistance to cisplatin and carboplatin." Crit Rev Oncol Hematol **63**(1): 12-31.
- Stoeck, A., S. Keller, et al. (2006). "A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44." Biochem J **393**(Pt 3): 609-618.
- Storey, D. J., R. Rush, et al. (2008). "Endometrioid epithelial ovarian cancer : 20 years of prospectively collected data from a single center." Cancer **112**(10): 2211-2220.
- Strauss, K., C. Goebel, et al. (2010). "Exosome secretion ameliorates lysosomal storage of cholesterol in Niemann-Pick type C disease." J Biol Chem **285**(34): 26279-26288.
- Stronach, E. A., M. Chen, et al. (2011). "DNA-PK mediates AKT activation and apoptosis inhibition in clinically acquired platinum resistance." Neoplasia **13**(11): 1069-1080.

- Sugimachi, K., T. Matsumura, et al. (2015). "Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation." Br J Cancer **112**(3): 532-538.
- Sun, D., X. Zhuang, et al. (2010). "A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes." Mol Ther **18**(9): 1606-1614.
- Sundar, S., R. D. Neal, et al. (2015). "Diagnosis of ovarian cancer." BMJ **351**: h4443.
- Sung, P. L., Y. H. Jan, et al. (2015). "Periostin in tumor microenvironment is associated with poor prognosis and platinum resistance in epithelial ovarian carcinoma." Oncotarget.
- Svensson, K. J., P. Kucharzewska, et al. (2011). "Hypoxia triggers a proangiogenic pathway involving cancer cell microvesicles and PAR-2-mediated heparin-binding EGF signaling in endothelial cells." Proc Natl Acad Sci U S A **108**(32): 13147-13152.
- Swisher, E. M., W. Sakai, et al. (2008). "Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance." Cancer Res **68**(8): 2581-2586.
- Szajnik, M., M. Derbis, et al. (2013). "Exosomes in Plasma of Patients with Ovarian Carcinoma: Potential Biomarkers of Tumor Progression and Response to Therapy." Gynecol Obstet (Sunnyvale) **Suppl 4**: 3.
- Tadokoro, H., T. Umezumi, et al. (2013). "Exosomes derived from hypoxic leukemia cells enhance tube formation in endothelial cells." J Biol Chem **288**(48): 34343-34351.
- Takahashi, K., I. K. Yan, et al. (2014). "Extracellular vesicle-mediated transfer of long non-coding RNA ROR modulates chemosensitivity in human hepatocellular cancer." FEBS Open Bio **4**: 458-467.
- Tan, A., H. De La Pena, et al. (2010). "The application of exosomes as a nanoscale cancer vaccine." Int J Nanomedicine **5**: 889-900.
- Tanaka, Y., H. Kamohara, et al. (2013). "Clinical impact of serum exosomal microRNA-21 as a clinical biomarker in human esophageal squamous cell carcinoma." Cancer **119**(6): 1159-1167.
- Tarn, C. and A. K. Godwin (2006). "The molecular pathogenesis of gastrointestinal stromal tumors." Clin Colorectal Cancer **6 Suppl 1**: S7-17.
- Taverna, S., M. Giallombardo, et al. (2016). "Exosomes isolation and characterization in serum is feasible in non-small cell lung cancer patients: critical analysis of evidence and potential role in clinical practice." Oncotarget.

- Taylor, D. D. and G. J. Doellgast (1979). "Quantitation of peroxidase-antibody binding to membrane fragments using column chromatography." Anal Biochem **98**(1): 53-59.
- Taylor, D. D. and C. Gercel-Taylor (2008). "MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer." Gynecol Oncol **110**(1): 13-21.
- Taylor, D. D., H. D. Homesley, et al. (1980). "Binding of specific peroxidase-labeled antibody to placental-type phosphatase on tumor-derived membrane fragments." Cancer Res **40**(11): 4064-4069.
- Terry, K. L., H. Schock, et al. (2016). "A prospective evaluation of early detection biomarkers for ovarian cancer in the European EPIC cohort." Clin Cancer Res.
- Thery, C., S. Amigorena, et al. (2006). "Isolation and characterization of exosomes from cell culture supernatants and biological fluids." Curr Protoc Cell Biol **Chapter 3**: Unit 3 22.
- Thery, C., M. Ostrowski, et al. (2009). "Membrane vesicles as conveyors of immune responses." Nat Rev Immunol **9**(8): 581-593.
- Thery, C., L. Zitvogel, et al. (2002). "Exosomes: composition, biogenesis and function." Nat Rev Immunol **2**(8): 569-579.
- Thigpen, T., H. Shingleton, et al. (1979). "cis-Dichlorodiammineplatinum(II) in the treatment of gynecologic malignancies: phase II trials by the Gynecologic Oncology Group." Cancer Treat Rep **63**(9-10): 1549-1555.
- Tian, Y., S. Li, et al. (2014). "A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy." Biomaterials **35**(7): 2383-2390.
- Tiwari, N., N. Meyer-Schaller, et al. (2013). "Klf4 is a transcriptional regulator of genes critical for EMT, including Jnk1 (Mapk8)." PLoS One **8**(2): e57329.
- Tokuhisa, M., Y. Ichikawa, et al. (2015). "Exosomal miRNAs from Peritoneum Lavage Fluid as Potential Prognostic Biomarkers of Peritoneal Metastasis in Gastric Cancer." PLoS One **10**(7): e0130472.
- Toss, A., C. Tomasello, et al. (2015). "Hereditary ovarian cancer: not only BRCA 1 and 2 genes." Biomed Res Int **2015**: 341723.
- Trajkovic, K., C. Hsu, et al. (2008). "Ceramide triggers budding of exosome vesicles into multivesicular endosomes." Science **319**(5867): 1244-1247.

- Trams, E. G., C. J. Lauter, et al. (1981). "Exfoliation of membrane ecto-enzymes in the form of micro-vesicles." Biochim Biophys Acta **645**(1): 63-70.
- Turner, N., A. Tutt, et al. (2004). "Hallmarks of 'BRCAness' in sporadic cancers." Nat Rev Cancer **4**(10): 814-819.
- Twelves, C., J. Cortes, et al. (2010). "Phase III trials of eribulin mesylate (E7389) in extensively pretreated patients with locally recurrent or metastatic breast cancer." Clin Breast Cancer **10**(2): 160-163.
- Umezumi, T., K. Ohyashiki, et al. (2013). "Leukemia cell to endothelial cell communication via exosomal miRNAs." Oncogene **32**(22): 2747-2755.
- Umezumi, T., H. Tadokoro, et al. (2014). "Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1." Blood **124**(25): 3748-3757.
- Vahdat, L. T., B. Pruitt, et al. (2009). "Phase II study of eribulin mesylate, a halichondrin B analog, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane." J Clin Oncol **27**(18): 2954-2961.
- Valadi, H., K. Ekstrom, et al. (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nat Cell Biol **9**(6): 654-659.
- Valcourt, U., M. Kowanetz, et al. (2005). "TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition." Mol Biol Cell **16**(4): 1987-2002.
- van der Vos, K. E., E. R. Abels, et al. (2016). "Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain." Neuro Oncol **18**(1): 58-69.
- van Meurs, H. S., P. Tadjik, et al. (2013). "Which patients benefit most from primary surgery or neoadjuvant chemotherapy in stage IIIC or IV ovarian cancer? An exploratory analysis of the European Organisation for Research and Treatment of Cancer 55971 randomised trial." Eur J Cancer **49**(15): 3191-3201.
- Vang, R., M. Shih le, et al. (2009). "Ovarian low-grade and high-grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems." Adv Anat Pathol **16**(5): 267-282.
- Vella, L. J. (2014). "The emerging role of exosomes in epithelial-mesenchymal-transition in cancer." Front Oncol **4**: 361.

- Vinay, D. S., E. P. Ryan, et al. (2015). "Immune evasion in cancer: Mechanistic basis and therapeutic strategies." Semin Cancer Biol **35 Suppl**: S185-198.
- Wahlgren, J., L. K. T. De, et al. (2012). "Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes." Nucleic Acids Res **40**(17): e130.
- Wang, H., L. Hou, et al. (2014). "Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma." Biomed Res Int **2014**: 864894.
- Wang, J. J., Z. Y. Wang, et al. (2015). "Macrophage-secreted Exosomes Delivering miRNA-21 Inhibitor can Regulate BGC-823 Cell Proliferation." Asian Pac J Cancer Prev **16**(10): 4203-4209.
- Wang, J. Y., Y. B. Gao, et al. (2014). "miR-21 overexpression enhances TGF-beta1-induced epithelial-to-mesenchymal transition by target smad7 and aggravates renal damage in diabetic nephropathy." Mol Cell Endocrinol **392**(1-2): 163-172.
- Wang, M., C. Zhao, et al. (2014). "Deregulated microRNAs in gastric cancer tissue-derived mesenchymal stem cells: novel biomarkers and a mechanism for gastric cancer." Br J Cancer **110**(5): 1199-1210.
- Wang, Q., J. Zhu, et al. (2013). "Down-regulation of programmed cell death 4 leads to epithelial to mesenchymal transition and promotes metastasis in mice." Eur J Cancer **49**(7): 1761-1770.
- Wang, T., D. M. Gilkes, et al. (2014). "Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis." Proc Natl Acad Sci U S A **111**(31): E3234-3242.
- Wang, W. and W. D. Figg (2008). "Secondary BRCA1 and BRCA2 alterations and acquired chemoresistance." Cancer Biol Ther **7**(7): 1004-1005.
- Wang, Y., X. L. Niu, et al. (2015). "IL6 induces TAM resistance via kinase-specific phosphorylation of ERalpha in OVCA cells." J Mol Endocrinol **54**(3): 351-361.
- Warnecke-Eberz, U., S. H. Chon, et al. (2015). "Exosomal onco-miRs from serum of patients with adenocarcinoma of the esophagus: comparison of miRNA profiles of exosomes and matching tumor." Tumour Biol **36**(6): 4643-4653.
- Webber, J., R. Steadman, et al. (2010). "Cancer exosomes trigger fibroblast to myfibroblast differentiation." Cancer Res **70**(23): 9621-9630.
- Wei, Y., X. Lai, et al. (2014). "Exosomal miR-221/222 enhances tamoxifen resistance in recipient ER-positive breast cancer cells." Breast Cancer Res Treat **147**(2): 423-431.

- Wheate, N. J., S. Walker, et al. (2010). "The status of platinum anticancer drugs in the clinic and in clinical trials." Dalton Trans **39**(35): 8113-8127.
- Willms, E., H. J. Johansson, et al. (2016). "Cells release subpopulations of exosomes with distinct molecular and biological properties." Sci Rep **6**: 22519.
- Wolfers, J., A. Lozier, et al. (2001). "Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming." Nat Med **7**(3): 297-303.
- Wu, Y., Q. Li, et al. (2012). "Decreased levels of active SMAD2 correlate with poor prognosis in gastric cancer." PLoS One **7**(4): e35684.
- Wu, Z. H., Z. H. Tao, et al. (2016). "MiRNA-21 induces epithelial to mesenchymal transition and gemcitabine resistance via the PTEN/AKT pathway in breast cancer." Tumour Biol **37**(6): 7245-7254.
- Wubbolts, R., R. S. Leckie, et al. (2003). "Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation." J Biol Chem **278**(13): 10963-10972.
- Xiao, X., S. Yu, et al. (2014). "Exosomes: decreased sensitivity of lung cancer A549 cells to cisplatin." PLoS One **9**(2): e89534.
- Yang, J., W. Liu, et al. (2015). "High expression of small GTPase Rab3D promotes cancer progression and metastasis." Oncotarget **6**(13): 11125-11138.
- Yang, J., R. Wahdan-Alaswad, et al. (2009). "Critical role of Smad2 in tumor suppression and transforming growth factor-beta-induced apoptosis of prostate epithelial cells." Cancer Res **69**(6): 2185-2190.
- Yang, J., F. Wei, et al. (2014). "Detection of tumor cell-specific mRNA and protein in exosome-like microvesicles from blood and saliva." PLoS One **9**(11): e110641.
- Yang, L., X. H. Wu, et al. (2013). "Bladder cancer cell-derived exosomes inhibit tumor cell apoptosis and induce cell proliferation in vitro." Mol Med Rep **8**(4): 1272-1278.
- Yang, M., J. Chen, et al. (2011). "Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells." Mol Cancer **10**: 117.
- Yang, M., J. Chen, et al. (2011). "Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells." Mol Cancer **10**: 117.
- Ye, S. B., Z. L. Li, et al. (2014). "Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma." Oncotarget **5**(14): 5439-5452.

- Yew, K. H., J. Crow, et al. (2013). "Epimorphin-induced MET sensitizes ovarian cancer cells to platinum." PLoS One **8**(9): e72637.
- Yin, J., X. Yan, et al. (2012). "Secretion of annexin A3 from ovarian cancer cells and its association with platinum resistance in ovarian cancer patients." J Cell Mol Med **16**(2): 337-348.
- Yoshida, T., Y. Ozawa, et al. (2014). "Eribulin mesilate suppresses experimental metastasis of breast cancer cells by reversing phenotype from epithelial-mesenchymal transition (EMT) to mesenchymal-epithelial transition (MET) states." Br J Cancer **110**(6): 1497-1505.
- Yu, M., A. Bardia, et al. (2013). "Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition." Science **339**(6119): 580-584.
- Yu, X., S. L. Harris, et al. (2006). "The regulation of exosome secretion: a novel function of the p53 protein." Cancer Res **66**(9): 4795-4801.
- Zarovni, N., A. Corrado, et al. (2015). "Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches." Methods **87**: 46-58.
- Zhang, L., S. Zhang, et al. (2015). "Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth." Nature **527**(7576): 100-104.
- Zhang, Y., X. Feng, et al. (1996). "Receptor-associated Mad homologues synergize as effectors of the TGF-beta response." Nature **383**(6596): 168-172.
- Zhang, Y., T. Pan, et al. (2014). "Nicotine upregulates microRNA-21 and promotes TGF-beta-dependent epithelial-mesenchymal transition of esophageal cancer cells." Tumour Biol **35**(7): 7063-7072.
- Zhao, J., N. Tang, et al. (2014). "MiR-21 Simultaneously Regulates ERK1 Signaling in HSC Activation and Hepatocyte EMT in Hepatic Fibrosis." PLoS One **9**(10): e108005.
- Zheng, X., J. L. Carstens, et al. (2015). "Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer." Nature **527**(7579): 525-530.
- Zhou, W., M. Y. Fong, et al. (2014). "Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis." Cancer Cell **25**(4): 501-515.

Zhuang, G., X. Wu, et al. (2012). "Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway." Embo j **31**(17): 3513-3523.