

Identification of licofelone through three-dimensional cell culture drug screening as a repurposed agent able to reverse drug resistant and cancer stem cell-like subpopulations in ovarian cancer

By
Jeffrey J. Hirst

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

Chairperson: Andrew K Godwin, Ph.D.

Fariba Behbod, Pharm.D., Ph.D.

Soumen Paul, Ph.D.

Benyi Li, M.D., Ph.D.

Yuxia Zhang, Ph.D.

Adam Krieg, Ph.D.

Date Defended: 23 February 2017

The dissertation committee for Jeffrey J. Hirst certifies that this is the approved version of the following dissertation:

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Chairperson: Andrew K Godwin, Ph.D.

Date Approved: 14 March 2017

Abstract

Since the introduction of paclitaxel in 1992, the overall survival for women diagnosed with ovarian cancer has remained relatively stagnant. This unsatisfactory outcome is in part attributed to the presence of subpopulations of tumor cells with stem cell-like properties, which are resistance to front-line therapies. Efforts to identify unappreciated drugs to combat these resistant cell populations typically have used traditional two-dimensional (2D) cell culture models which do not always reflect the tumor heterogeneity seen in clinical samples including reduced cell proliferation, hypoxia, and cancer stem-like cells. The lack of congruency between *in vitro* screening models and the patient's outcome has limited the number of successful anti-cancer drugs that demonstrate clinical benefit. In order to identify drugs that are more likely to benefit patients, a drug screening model that better represents clinical disease was required. Towards this goal, I developed and characterized a three-dimensional (3D) cell culture model in which ovarian cancer cells were grown as multicellular tumor spheroids (MCTS) and directly compared drug activity in 2D and 3D models.

Compared to a panel of ovarian cancer cell lines grown in 2D, MCTS formation induced many different phenotypes associated with clinical drug resistance including reduced cell proliferation, cellular hypoxia, and stem-like gene expression and function. Cells grown as MCTS better mimicked the response to chemotherapy seen in the clinic as compared to 2D. Specifically, paclitaxel treatment of MCTS revealed significant increases in expression of stem cell-related genes, *e.g.*, *CD133*, *CD44*, *OCT4*, *SOX2*, and *NANOG*. Correspondingly, significant reduction in cell proliferation with accompanying resistance to paclitaxel was observed in MCTS compared to 2D cultures, providing an *in vitro* model that reflects certain aspects of clinical progression and drug resistance. Interestingly, the drug resistance and stem-like properties observed in MCTS were maintained in cells grown under 2D conditions when these cells were derived from MCTS, indicating a potential long-term change in phenotype.

These changes provided an *in vitro* model that better reflects clinical disease that can be exploited for drug screening.

To exploit these differences, the MCTS model was used to compare the activity of 304 repurposed drugs, most that are FDA approved for indication other than cancer, in four established ovarian cancer cell lines grown in 2D and 3D. I identified a number of compounds with selective activity in MCTS that can target subpopulations of resistant cells with stem-cell like properties. In a primary drug screen between 2D and 3D cultures designed to directly compare response to 10 μM of the 304 clinically approved drugs revealed fifteen compounds with preferential activity against cells grown as MCTS as measured by CellTiter-Glo. Drug hits were classified to have 3D specific activity based on >75% viability reduction in MCTS but <50% viability reduction in 2D cultures. Secondary screening using a range 0 to 25 μM drug identified licofelone and glafenine to have the most robust and selective activity in MCTS across a genetically diverse panel of ovarian cancer cell lines. Both are anti-inflammatory drugs that had not previously been considered for the treatment of cancer. Since an increasing number of drugs are removed from the market because of unacceptable toxicity, subsequent experiments focused on licofelone. Licofelone has demonstrated a better safety profile as compared to glafenine, *i.e.*, reported increased risk of anaphylaxis and kidney failure. Importantly, treatment of MCTS using IC_{50} concentration of licofelone significantly reduced expression of stem cell markers by up to 90%, as measured using qRT-PCR and immunofluorescent (IF) immunohistochemistry in cells grown as MCTS. Consequently, Ki67 positive cells were increased from vehicle treatments (30%) in response to licofelone (57%) treatment in MCTS. Combination treatment in MCTS showed significant synergy (Combination Index <1) for sequential additions of licofelone followed by paclitaxel, or vice-versa. Likewise, licofelone addition reversed stemness (gene expression, colony forming and spheroid forming assays) in ovarian cancer cells that were induced by paclitaxel treatment in MCTS.

By performing drug screens using a MCTS model, I identified two previously unappreciated candidate anti-cancer drugs that had preferential activity on ovarian cancer cells with stem cell-like properties. When used in combination with paclitaxel, licofelone showed significant synergy and reversed the drug resistant phenotypes of MCTS.

Acknowledgements

To my graduate thesis advisor and mentor Dr. Andrew (Andy) Godwin, thank you for all of your support and guidance throughout my graduate career. The insight you provided has made me a better and more complete scientist. The high standards for which you demand research be performed has pushed me to constantly better myself and my work. It has been an honor to learn from you and work for you in the laboratory. The vast knowledge based you have bestowed upon me, and everyone you have taught, is appreciated and has driven me to better my project in every way. Applying what you have taught me will be valuable and better my career, no matter where it takes me. It has been my pleasure to work for you and to meet your challenges over the past years.

To my committee members: Dr. Fariba Behbod, Dr. Soumen Paul, Dr. Benyi Li, Dr. Adam Krieg, thank you for all of your wonderful insight. You have not only helped shaped my project, you have helped shaped me as a scientist and as a person. I have appreciated every recommendation and challenge you have provided me. The extent of guidance and broad knowledge base has supported me in more ways that I could have ever expected. Dr. Behbod's knowledge of cancer stem cells and functional assays was instrumental into guiding my project. Dr. Paul contributed vast knowledge of stem cells and how to identify and detect them as well as measure stem cell function. Dr. Li has been an incredible resource for clinical knowledge and drug development. Dr. Krieg's expertise in cellular hypoxia was instrumental into several methods and the development of my project. To Dr. David Albertini, a member of my comprehensive exam committee member, thank you for the insight into the development stages of my project. Without any of your guidance and breadth of knowledge, I would be less of a scientist than I am today without any of your support and knowledge.

To all of the members of the Godwin Laboratory that I have worked with throughout my entire career, thank you for teaching me, helping with any issue that I may have asked, and for

always providing insightful discussion. Specifically, to my other mentor, Dr. Harsh Pathak, thank you for your guidance and unwavering patience when I inevitably needed help to solve problems, run new protocols, or bounce new ideas off of you. You have taught me an incredible amount and were critical in both developing and executing my project. To our administrative assistant, Susan Ard, thank you for always being there for the lab and keeping us functioning day-to-day. We could not do any of this without your constant support. To specific members of the Godwin laboratory, Drs. Stephen Hyter, Rebecca Water, Jennifer Crow and Ziyang Pessetto, thank you for the guidance in my project. Whether it be Stephen's assistance with animal models and hypoxia, Rebecca constantly being there to talk to and discuss ideas, Jennifer for being a fellow graduate student and laying the foundation and support before me, and Ziyang's knowledge of drug screening and development; all of your support has helped me with this project and with progressing through my career. There are many other members of the Godwin Laboratory that have contributed my project and graduate career to specifically thank, but all of you have made my time during graduate school both more enjoyable and insightful.

To other supporting members at the University of Kansas Medical Center from core facilities, faculty members, staff, the Cancer Center, the Department of Pathology, and other graduate students; thank you for making KUMC a wonderful and supportive place to attend graduate school. Specific members that have supported my project include the Genomics Sequencing Facility (and Clark Bloomer) and the Department of Biostatistics (and Dr. Devin Koestler and graduate student Stefan Graw).

Also, thank you to all of my different funding sources that have made this project, any research I have participated in, and my graduate education possible. Firstly, thank you to The University of Kansas Self Graduate Fellowship (and the late Self Family Madison "Al" and Lila) not only for full graduate funding for four years but for the extensive training and development program. This was a unique and intuitive program that provided me training outside of

traditional research in areas such as communication, entrepreneurship, leadership, public policy, business development, and many other areas. This program, and the interaction with the Self Fellowship staff other astute Self Fellows, has made me a better, more rounded scientist and person. I would also like to thank other funding mechanisms that supported this research including the University of Kansas Cancer Center, the Kansas Bioscience Authority, the NIH (RO1 CA140323), and the OVERRUN Ovarian Cancer Foundation.

Finally, to my family (my mother Gayla Hirst, my father Jim Hirst, and my sister Lisa Hirst) words cannot describe how thankful I am for all of your support throughout my academic career. I would not have been able to do any of this without you. Whether it be from simple words of encouragement, financial assistance, and to you helping me recover and stay in graduate school following two major surgeries you have been there for me whenever I needed it most. You have been my inspiration and my biggest supporters. I will be forever grateful for everything you have helped me achieve. You have always shown support and interest in my career as a scientist and have encouraged my curiosity that pioneered a career in research.

Table of Contents

Chapter 1: Introduction	1
Ovarian Cancer Epidemiology	2
Ovarian Cancer Symptoms, Diagnosis, and Staging	3
Identification and Characteristics of Ovarian Cancer Subtypes	5
Ovarian Cancer Dissemination	12
Ovarian Cancer Risk Factors	13
Ovarian Cancer Therapy	18
Development of Paclitaxel for the Treatment of Ovarian Cancer	23
Drug resistance in Ovarian Cancer	26
Failures of drug development in ovarian cancer	32
Chapter 2: Exploiting Three-Dimensional Cell Culture to Better Replicate Clinical Drug Resistance in Ovarian Cancer Cell Lines	44
Introduction	45
3D Culture History.....	45
3D Culture Methods.....	46
MCTS as a Model for Resistant Disease	46
Using 3D Culture to Enhance Drug Development.....	48
Methods	50
Cell Culture.....	50
Spheroid Formation	50
<i>In Vivo</i> Tumorigenicity	51
Secondary Spheroid Assays.....	51
Cell Cycle Analysis	52
Immunofluorescence	52
Western Blot Analysis	53
RT-PCR and RNASeq Analysis	54
Colony Forming Assay	55
Results	56
Formation of Multicellular Tumor Spheroids in Established Ovarian Cancer Cell Lines Using Agarose Coated Plates	56

MCTS Formation Induced Multiple Cellular Changes Associated with Solid Tumors and Drug Resistance	57
MCTS Displayed Intermediate Gene Expression Between 2D Cultures and Tumor Xenografts.....	59
Properties Specific to Tumorigenic Cell Lines	59
Reduced Sensitivity of Ovarian MCTS to Paclitaxel Compared to 2D Cultures.....	60
Paclitaxel Treatment Reduced Proliferation and Increased Stemness.....	61
Paclitaxel Resistance and Stem-Like Phenotype was Maintained in 2D Cultures of MCTS Derived Cells	61
Discussion	77
Conclusion.....	79
Chapter 3: 3D Culture Drug Screening Identified Unappreciated Anti-inflammatory Drug Candidates that Reverse Stemness and Synergize with Paclitaxel	81
Introduction	82
Cancer Stem Cells.....	82
Stemness in Ovarian Cancer	86
Cancer Stem Cells and Drug Resistance	88
Targeting Cancer Stem Cells	89
Using 3D Cell Culture to Target Stem-like Cells	90
Methods	91
Transcriptome Sequencing and IPA Pathway Analysis	91
Drug Screening.....	91
Cell Viability Assays	92
Drug Combination Assays	92
Results	94
Cell Viability in Response to Drug Treatment in Ovarian MCTS	94
MCTS Drug Screening Identified Unappreciated Drug Candidates.....	95
The Top 3D Specific Drugs, Licofelone and Glafenine, Reduced Stemness in Ovarian MCTS	97
Pretreatment of Ovarian MCTS with Licofelone Synergistically Enhanced Paclitaxel Activity	99
Pretreatment of Ovarian MCTS with Paclitaxel Synergistically Enhanced Licofelone Activity	100
Licofelone Combination Enhanced Apoptosis and Reduced Stemness Induced by Paclitaxel.....	101
Discussion	121
Conclusion.....	125
Chapter 4: Summary, Discussion, and Future Directions.....	127
Summary.....	127

Discussion and Future Directions.....	129
Perspective	134
References	136

List of Tables

Table 1: FIGO Stages of Ovarian Cancer.....	4
Table 2: Subtypes of Ovarian Cancer	11
Table 3: Clinical Trials for the Treatment of Ovarian Cancer	36
Table 4: Qualitative Comparison of 3D Culture Methods.....	65
Table 5: 3D Hits from Primary Screening	106

List of Figures

Figure 1: Risk Factors for the Development of Ovarian Cancer.....	18
Figure 2: Disease Recurrence and Drug Resistance in Clinical Ovarian Cancer.....	22
Figure 3: Structure and Chiral Centers of Taxol.	26
Figure 4: Mechanisms of Chemotherapy Resistance in Ovarian Cancer.....	32
Figure 5: Drug Development History for Ovarian Cancer	35
Figure 6: Laboratory Models of Cancer.....	48
Figure 7: 3D Culture for the Formation of Ovarian Cancer Spheroids	51
Figure 8: Properties to Develop 3D Culture Models to Form Ovarian MCTS.....	64
Figure 9: Formation of MCTS Across Ovarian Cancer Cell Lines.....	66
Figure 10: MCTS Formation Reduced Cell Proliferation in Ovarian Cancer Cell Lines.....	67
Figure 11: Cellular Hypoxia in Ovarian Cancer Cell Lines was Increased in MCTS.....	68
Figure 12: Increased Stemness in Ovarian MCTS Compared to 2D Cultures.....	69
Figure 13: MCTS Displayed Intermediate Gene Expression Between 2D Cultures and Tumor Xenografts.	70
Figure 14: <i>In Vivo</i> Tumorigenic Assay of HIO Cell Lines.	71
Figure 15: HIO Cell Spheroids Demonstrated Low Levels of a Hypoxia Marker and High Levels of a Cell Proliferation Marker Compared to Ovarian MCTS.	72
Figure 16: Paclitaxel Resistance in MCTS Compared to 2D Cultures.	73
Figure 17: Paclitaxel Reduced Proliferation and Increased Stemness in MCTS.....	74
Figure 18: MCTS Derived Cells Maintained Paclitaxel Resistance in 2D Cultures.....	75
Figure 19: Stem Cell Gene Expression was maintained in 2D Cultures in Cells Derived from MCTS.	76
Figure 20: Drug Resistant Phenotypes of MCTS.....	80
Figure 21: Models of Cancer Stem Cell Progression.....	86
Figure 22: Cancer Stem Cells Repopulate the Tumor Following Chemotherapy.	90
Figure 23: CellTiter-Glo Measured Cell Viability and Drug Response in MCTS.....	103
Figure 24: Pathway Analysis of Transcriptome Sequences Identified Inflammation and Metabolism Upregulated in MCTS.....	104
Figure 25: 3D Cell Culture Screening Identified Drug Hits Unappreciated by 2D Cultures.....	105
Figure 26: 3D Drug Screening in Non-tumorigenic Cell Lines did not Identify 3D Specific Hits.	107
Figure 27: Secondary Screening Validated 3D Specific Hits Across Multiple Cell Lines.....	108
Figure 28: Dosage Response Validation of 2D Only Hit and 2D and 3D Hit.	109
Figure 29: Dosage Response Showed 3D Specific Activity of Both Licofelone and Glafenine.	110
Figure 30: The 3D Specific Drugs Licofelone and Glafenine Reduced Stem-like Gene Expression in Ovarian MCTS.....	111
Figure 31: Glafenine and Licofelone Treatment Increased Cell Proliferation in Ovarian MCTS.	112
Figure 32: Licofelone Showed Enhanced Activity in Paclitaxel Resistant Cells.....	113
Figure 33: Licofelone had More Activity Against Ovarian Cancer Cell-Derived MCTS than the COX inhibitor Celecoxib.	114

Figure 34: Pretreatment of Ovarian Cancer Cell-Derived MCTS with Licofelone Synergistically Enhanced Paclitaxel Activity.	115
Figure 35: Pretreatment of Ovarian Cancer Cell-Derived MCTS with Paclitaxel Synergistically Enhanced Licofelone Activity	116
Figure 36: Licofelone Blocked the Induction of Stem-like Gene Expression by Paclitaxel.	117
Figure 37: Combination of Licofelone and Paclitaxel Increased Apoptosis in Ovarian Cancer Cell-Derived MCTS.	118
Figure 38: Licofelone Decreased Stem-like Function of Paclitaxel Treated MCTS.	119
Figure 39: Licofelone Reduced the Secondary Spheroids Forming Ability of Paclitaxel Treated MCTS.	120
Figure 40: Summary of 3D Specific Drug Activity in Ovarian MCTS.	126

Chapter 1: Introduction

Ovarian Cancer Epidemiology

Ovarian cancer is the 9th leading cause of cancer and the 5th most common cause of cancer death in American women (Siegel, Naishadham et al. 2013), with an estimated 21,000 new cases and 15,000 deaths in 2015 (Howlader, Noone et al. 2015). The disease primarily affects perimenopausal and postmenopausal women in the sixth decade of life, with a mean incidence of 63 years of age and over 88% of all cases occur in women 45 years of age and older. Overall, both the incidence and 5-year survival of ovarian cancer have decreased since the 1970s (Howlader, Noone et al. 2015) though the rate of these declines has fallen behind many other cancers. For example, the overall 5-year survival for 2005 to 2011 in the United States was 45.6 %, which was lower than both breast (over 90%) and cervical cancer (almost 70%).

Ovarian cancer is more common in developed parts of the world, specifically both North America and Europe (*WHO* 2014, Ferlay, Soerjomataram et al. 2015) and the incidence rate is higher in Caucasian women (12.5%) compared to other races (Singh, Ryerson et al. 2014, ACS 2015, Howlader, Noone et al. 2015). While women of African American descent are more susceptible to die of ovarian cancer than Caucasian women (Chan, Zhang et al. 2008), a recent report of women in Cook County, IL show the relationship may be in fact tied to socio-economic status and disease diagnosis (Brewer, Peterson et al. 2015). Socio-economic factors also drive treatment decisions such as surgery that can affect survival rates between races in stage-adjusted studies (Terplan, Smith et al. 2009). Genetic links between incidence and race are not well known but there are higher rates of mutations of *BRCA2* mutations in Ashkenazi Jewish women (Berman, Costalas et al. 1996). Some studies suggest lower BRCA mutations among in eastern Asia (Khoo, Chan et al. 2002, Kim, Nam et al. 2005) and higher rates than western women in Turkey and Pakistan (Liede, Malik et al. 2002, Yazici, Glendon et al. 2002). Deep sequencing techniques suggest the rates might not be as low as previously predicted (Sakamoto, Hirotsu et al. 2016).

Ovarian Cancer Symptoms, Diagnosis, and Staging

Ovarian cancer is often termed a “silent killer” due to the vague nature of symptoms present in early stage disease. The primary symptoms of ovarian cancer at the time of diagnosis are loss of appetite, early satiety, pelvic or abdominal pain and swelling, urinary frequency and sometimes vaginal bleeding. However, these symptoms are often being ignored or misdiagnosed as other maladies, such as gastrointestinal issues or menopause, by both patients and primary physicians. Unfortunately, many of these symptoms develop after the disease has already spread to distal organs. Therefore, there is an urgent need for increased screening and improved early detection methods to circumvent the reliance on symptomatic presentation for initial diagnosis.

However, screening and detection methods for ovarian cancer have been controversial. Women with a family history of ovarian cancer (especially 1° relatives) and/or germline mutations in *BRCA1* or *BRCA2* can undergo preventative surgeries such as tubal ligations and more commonly bilateral salpingo-oophorectomy. However, in women without a family history of ovarian cancer or germline mutations in *BRCA* and other less penetrant gene mutations, the early detection of ovarian cancer is problematic for both sensitive and accurate methods as well as improving the overall survival of women with ovarian cancer. The two primary forms of screening for ovarian cancer are transvaginal sonography (TVS) and serum CA-125 testing; however, diagnosis is only confirmed by biopsy during cytoreductive surgery. CA-125 screening is used to detect ovarian cancer in asymptomatic women by testing for elevated serum levels of the antigen above the, where normal levels of the protein are 0-35 units/ml in normal patients (Jacobs, Skates et al. 1999). However, the rate of false positives for both procedures is problematic leading to unnecessary confirmation surgeries in some patients. Overall, it has been shown that yearly CA-125 and TVS screenings have no significant change in survival when compared to standard care (Buys, Partridge et al. 2011). Fortunately, recent

improvements in methodology using a multimodal screening protocol improved the positive predictive value (Menon, Gentry-Maharaj et al. 2009) and survival by 8% from years 0-7 and by 28% from years 7 to 14 (Jacobs, Menon et al. 2016). In addition, levels of CA-125 have a positive predictive value for response to chemotherapy in terms of decreased progression free survival (PFS) and overall survival (OS) (Tian, Markman et al. 2009).

Ovarian cancers are diagnosed and staged after cytoreductive surgery that removes any visible mass within the peritoneal cavity. The International Federation of Gynecology and Obstetrics (FIGO) have established guidelines for the staging of ovarian cancer summarized in **Table 1**.

Table 1: FIGO Stages of Ovarian Cancer

FIGO Staging of Ovarian Cancer		
Stage	Disease Localization	5-year survival
I	Ovaries only	~90%
IA	One ovary, capsule intact, no tumor on surface, negative washing	
IB	Tumor involves both ovaries	
IC	Tumor limited to 1 or both ovaries	
1C1	Surgical spill	
1C2	Capsule rupture before surgery or surface tumor	
1C3	Malignant cells in the ascites or peritoneal washing	
II	Pelvic extension or primary peritoneal cancer	
II2A	Extension/ metastases to the uterus and/or fallopian tubes	
IIB	Extension to other pelvic or intraperitoneal tissues	
III	One or Both ovaries, cytological or histological confirmed spread to the peritoneum outside of the pelvis or lymph nodes	30-40%
IIIA	Positive retroperitoneal lymph nodes and /or microscopic metastasis beyond the pelvis nodes	
IIIA1	Positive nodes only: (i) Metastasis < 10mm, (ii) Metastasis >10mm	
IIIA2	Microscopic, extrapelvic (above the brim) peritoneal involvement ± positive retroperitoneal lymph nodes	
IIIB	Metastases less than 2 cm and negative nodes	
IIIC	Metastases greater than 2 cm and or positive inguinal nodes	

IV	Distant Metastases including plural effusion and parenchymal liver metastases	<20%
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)	

Identification and Characteristics of Ovarian Cancer Subtypes

Ovarian cancer is a generic term used to classify cancers involving the ovaries though they can arise from many different cell types within the Müllerian compartment. The embryologic origin of the Müllerian system is critical to understanding the theories surrounding the origin of ovarian, peritoneal, and tubal cancers. Ovarian surface epithelium (OSE) is derived from the coelomic epithelium in early development. The coelomic epithelium is derived from the mesoderm, and consists of the epithelial lining of the intraembryonic body cavity or coelom, and overlies the intraembryonic body cavity (which will become the peritoneum), including the area that will develop into the gonadal structures. During fetal development, near the area that will form the gonadal structures, the coelomic epithelium invaginates to give rise to the Müllerian ducts, which will ultimately differentiate to become the fallopian tubes, uterus, cervix, and upper vagina). Therefore, while the reproductive organs and peritoneum originate from distinct pathways, the Müllerian epithelia, OSE, and peritoneal (coelomic) epithelium have a close developmental relationship.

Traditionally, epithelial ovarian, tubal, and peritoneal cancers have been viewed as separate entities with disparate origins, pathogenesis, clinical features, and outcomes. Additionally, previous classification systems for ovarian cancer have proposed two primary histologic groups, type I and type II cancers that encompass the standard histologic subtypes. The type I and type II classification is generally used to broadly classify ovarian neoplasms for research purposes based on their unique clinical and molecular genetic features. The classification was not meant to be used for clinical purposes. Type I tumors include low-grade

serous and low-grade endometrioid cancers, as well as mucinous, clear cell, and transitional cell carcinomas. Tumors in this category typically develop from atypical proliferative borderline tumors, benign cystic lesions, or endometriosis. Transitional cell tumors and mucinous tumors do not typically have Mullerian features, but may develop from cortical inclusion cysts and Walthard cell nests. However, there is an uncommon subtype of mucinous tumors which does demonstrate Müllerian (endocervical) characteristics. Generally, type I tumors are more indolent, present at an earlier stage, are confined to the ovary, and are often large. When type I tumors, specifically clear cell and mucinous cancers, are not detected early, they usually have a worse prognosis than type II cancers due to low response to chemotherapy (Koshiyama, Matsumura et al. 2014). Type II tumors are highly aggressive and account for the majority of high-grade serous and high-grade endometrioid tumors. Type II tumors are typically invasive tumors that develop *de novo* from the OSE of tubal surface. While within each subtype different pathways can be activated and genes mutated, the frequency of *TP53* or *BRCA1/2* mutations is low in Type I cancers while Type II cancers have been found to have high percentages mutations in these genes (Kurman and Shih 2010). Generally Type I tumors are more genetically stable than Type II tumors, but recurrent gene mutations found within each subtype of Type I tumors can include mutations in *KRAS*, *BRAF*, *PTEN*, *PI3KA*, and *CTNNB1* (Cho and Shih 2009). However, recent molecular profiling data suggest that these groupings no longer accurately reflect our knowledge surrounding the various origins of these genetically diverse tumors.

Epithelial ovarian cancers (EOC) were for years believed to arise primarily from the ovarian surface epithelium. In fact, Godwin and colleagues were some of the first investigators to establish ovarian surface epithelial cultures from rat and human ovaries and model incessant ovulation *in vitro* as a mechanism for transformation and tumorigenesis (Godwin, Testa et al. 1992, Godwin, Testa et al. 1993, Perez, Hamaguchi et al. 1993, Testa, Getts et al. 1994, Auersperg, Maines-Bandiera et al. 1995, Godwin, Miller et al. 1995, Dyck, Hamilton et al. 1996,

Salazar, Godwin et al. 1996, Abdollahi, Godwin et al. 1997, Abdollahi, Getts et al. 1999, Kruk, Godwin et al. 1999, Roberts, Williams et al. 2002, Yang, Smith et al. 2002, Roland, Yang et al. 2003). Epithelial ovarian cancers, serous, endometrioid, mucinous, and clear cell, account for more than 85% of all ovarian tumors. Other subtypes are much less common and arise from germ, epidermoid, stromal and border cells. Since EOCs are the most common and deadly form of ovarian cancer, I will refer to EOC as ovarian cancer for the remainder of this thesis and only discuss ovarian cancers of epithelial origin (Seidman, Horkayne-Szakaly et al. 2004, Braicu, Sehouli et al. 2011).

Typically, EOC is classified into five different histological subtypes: high-grade serous (HGS), low-grade serous (LGS), endometrioid, clear cell and mucinous. Each subtype behaves as a discrete disease with differences in presentation, progression, mutation profile and response to chemotherapy (**Table 2**) (Vaughan, Coward et al. 2011). Two distinct patterns of ovarian cancer have been observed for low-grade and high-grade tumors based on the extent of nuclear atypia and mitosis (Malpica, Deavers et al. 2004). Low grade tumors are slower growing, more genetically stable and do not respond to chemotherapy as well as the faster growing, genomically unstable high-grade tumors (Iwabuchi, Sakamoto et al. 1995, Groen, Gershenson et al. 2015, Oswald and Gourley 2015). High-grade serous carcinomas are the most common ovarian cancer subtype (over 70%) followed by endometrioid, clear cell and low-grade serous (Kurman 2013). Each subtype has distinct histological protein expression patterns, mutations and even epigenetic signatures. Further classification based on molecular profiles may provide insights into improving therapy selection (Tothill, Tinker et al. 2008, Bentink, Haibe-Kains et al. 2012).

High-grade serous tumors show a broad range of histological phenotypes with papillary, micropapillary, glandular, cribriform and trabecular structures involving columnar cells with pink cytoplasm (Burks, Sherman et al. 1996, Seidman and Kurman 1996). HGS tumors account for

both the majority of ovarian cancer diagnoses and deaths (Malpica, Deavers et al. 2004, Kurman 2013). The majority of HGS tumors are diagnosed at late stages and are associated with genomic instability (Braicu, Sehouli et al. 2011) since almost all (>95%) high-grade serous cancers have *TP53* mutations and over half have homologous DNA repair pathway deficiencies mainly represented by defects in *BRCA1*, *BRCA2*, or related proteins (Jazaeri, Yee et al. 2002, Turner, Tutt et al. 2004, Ahmed, Etemadmoghadam et al. 2010, Bell and Network 2011). The deficiencies in DNA repair pathways associate with widespread copy number alterations (Gorringe, Jacobs et al. 2007) and make HGS cancer initially sensitive to platinum based chemotherapy (and PARP inhibitors) but develop therapy resistance which will be discussed later in this chapter. However, half of all HGS tumors have functional BRCA pathways and pathologic mechanisms of these tumors still need to be elucidated (Bell and Network 2011).

The origins of HGS tumors are controversial since data suggest many may not arise from ovarian surface epithelial cells but instead originate from the fallopian tube epithelium (Kindelberger, Lee et al. 2007, Lee, Miron et al. 2007). In genetic mouse models, conditional inactivation of commonly mutated ovarian cancer genes (*BRCA1*, *TP53* and *RB1*) in ovarian surface epithelium cells leads to the formation of leiomyosarcomas and not HGSC following implantation into the mouse bursal sack (Akbari, Donenberg et al. 2014). Due to the aggressive nature of HGS tumors and the presence of early genomic instability, it is hypothesized that HGS ovarian tumors are instead metastatic lesions from the fallopian tube epithelial cells. Microdissection of the fallopian tube epithelium from patients with a disposition to ovarian cancer showed lesions with *BRCA* and *TP53* alterations that resemble HGS tumors (Piek, van Diest et al. 2001, Callahan, Crum et al. 2007, Crum, Drapkin et al. 2007, Gross, Kurman et al. 2010). Along with genetic alterations, fallopian lesions from *BRCA* patients showed gene expression profiles that mimicked HGS cancers (Tone, Begley et al. 2008). Immortalization of human fallopian tube secretory epithelial cells (using hTERT and SV40 large T antigen) were

transformed *in vivo* and *in vitro* by oncogenic *RAS* or *MYC* (Karst, Levanon et al. 2011). In contrast to ovarian surface epithelial cells, the inactivation of *Brca*, *Tp53* or *Pten* in *Pax8* over expressing mouse fallopian tubal secretory cells led to the development of HGSC (Perets, Wyant et al. 2013). To reduce the risk of HGS ovarian cancer in women *BRCA* mutation carriers it is beneficial to undergo a salpingo-oophorectomy (removal of both the ovaries along with the fallopian tubes) instead of just an oophorectomy (removal of only the ovaries) (Rebbeck, Lynch et al. 2002, Olivier, van Beurden et al. 2004).

Low-grade serous tumors are thought to be borderline tumors formed step-wise from the ovarian surface (Singer, Stöhr et al. 2005). LGS tumors are more common in younger patients but are typically slower growing than the high grade counterpart and have more frequent mutations in *KRAS* and *BRAF* along with lower *TP53* mutations (Hunter, Anglesio et al. 2015). Along with having functional p53, LGS tumors have a more stable genome with less rearrangements, mutations and tumor heterogeneity (Tone, McConechy et al. 2014). However, due to more competent DNA repair pathways, LGS tumors do not respond to chemotherapy as well as HGS tumors.

Endometrioid tumors account for about 20% of all ovarian cancers. Endometrioid tumors have a smooth outer surface with solid, cystic areas inside while the pathological phenotype involves high amounts of proliferative cells that resemble squamous or endometrioid differentiations with secretory cell features. Tumors contain cystic spaces lined by gastrointestinal-type mucinous epithelium with stratification and may form filiform papillae with at least minimal stromal support; nuclei are slightly larger than cystadenomas; mitotic activity; goblet cells and sometimes Paneth cells are present, but stromal invasion is absent (Chiesa, Deavers et al. 2010). The 5-year survival rate for endometrioid tumors is between 40 and 63%, mostly due to early stage presentation of the disease; however, there is no survival difference when matched serous patients are based on age and stage of diagnosis (Zwart, Geisler et al.

1998). Likewise with serous tumors, endometrioid tumors can be both high and low-grade with similar growth patterns distinguishing the two (Mangili, Bergamini et al. 2012). High-grade endometrioid tumors are very similar to HGS tumors in terms of genome stability and response to chemotherapy (Schwartz, Kardia et al. 2002). 15–20% of women diagnosed with endometrioid tumors also have concurrent endometriosis and the origin of endometrioid tumors is from the endometrial tissue, possibly from back flow during menstruation that implants onto the ovarian surface epithelium (Sampson 1925, Vercellini, Scarfone et al. 2000, Keita, AinMelk et al. 2011, Wang, Mang et al. 2015). Mutation profiles of endometrioid tumors reveal frequent mutations in WNT- β -catenin pathways (Schwartz, Wu et al. 2003, McConechy, Ding et al. 2014), *ARID1A* mutations (which helped link the origin to endometriosis) (Wiegand, Shah et al. 2010), and *PTEN* is altered in endometrioid tumors more than any other subtype (Obata, Morland et al. 1998).

Mucinous ovarian cancer tumors are primarily unilateral, can be very large (Riopel, Ronnett et al. 1999) and are diagnosed at early stages (most are stage I or II) while invasive disease accounts for less than 10% of all cases (Seidman, Kurman et al. 2003, Leitao, Boyd et al. 2004). The pathological definition of mucinous ovarian cancer dictates intracytoplasmic mucin is mandatory, although many mucinous tumors lack obvious apical mucin in large parts of tumor, thereby imparting an endometrioid appearance. Mucinous tumors are often heterogeneous that can be endocervical-like or intestinal-like cells with gastric superficial/foveolar and pyloric cells, enterochromaffin cells, argyrophil cells, and Paneth cells. Mucinous disease is mostly thought to originate from the gastrointestinal tract (Zaino, Brady et al. 2011) though molecular mechanisms of the disease are still not fully elucidated besides *KRAS* mutations identified in almost all mucinous tumors (Vereczkey, Serester et al. 2011) and *HER2* amplifications (Lin, Kuo et al. 2011). While the overall survival for mucinous disease is high due to the majority of cases being diagnosed at early stage, invasive disease has a worse clinical

outcome (Hess, A'Hern et al. 2004) and low response to chemotherapy due to the high expression of genes involved in drug resistance including ABC transporters (Wamunyokoli, Bonome et al. 2006).

Ovarian clear cell carcinoma accounts for approximately 5% of all ovarian cancer patients, is primarily diagnosed at early stages and is more common in Asian women (Sugiyama, Kamura et al. 2000, Tung, Goodman et al. 2003, Seidman, Horkayne-Szakaly et al. 2004). Clear cell carcinomas are primarily large, unilateral tumors that display only papillary, tubulocystic and solid architectures with hobnail cells containing clear cytoplasm. In terms of molecular mechanisms, clear cell carcinomas (CCC) are complex at the genomic level and can have mutations in *ARID1A*, *PIK3CA*, *KRAS* and *PTEN* (Tan, Iravani et al. 2011, Zannoni, Improta et al. 2014), with *PIK3CA* mutated in ~33% of patients (Campbell, Russell et al. 2004). While the pathogenesis of CCC is unknown, gene expression studies indicate clear cell ovarian cancer does not cluster with other ovarian cancers and more closely resembles lung cancers, endometriosis and renal cell carcinoma (Schwartz, Kardia et al. 2002, Zorn, Bonome et al. 2005, Domcke, Sinha et al. 2013, Kandalaf, Gown et al. 2014). Clinically, CCC cancer has a low response to chemotherapy, especially at later FIGO stages and drug response correlated to *CD44* isoform expression (Sancho-Torres, Mesonero et al. 2000).

Table 2: Subtypes of Ovarian Cancer

Sub Type	Frequency	Mutations	Clinical Prognosis
High-grade serous	~65%	<i>TP53, BRCA1, BRCA2, CDK12</i>	Often diagnosed at late stage and chromosomally unstable.
Low-grade serous	~5%	<i>BRAF, KRAS, NRAS, ERBB2</i>	Often diagnosed in younger patients, less aggressive, genomically stable.
Endometrioid	~20%	<i>PTEN, CTNNB1, PPP2R1α, MMR deficient</i>	Favorable prognosis and response to chemotherapy.
Clear-cell carcinoma	~5%	<i>PIK3CA, KRAS, PTEN, ARID1A</i>	Low response to chemotherapy and intermediate prognosis.
Mucinous	~5%	<i>KRAS, HER-2 amplification</i>	Low response to chemotherapy.

Ovarian Cancer Dissemination

Dissemination of ovarian cancer occurs differently from traditional models of cancer metastasis. Most notably, ovarian cancer disseminates primarily through the peritoneal cavity and is rarely found in patient blood. Secondly, ovarian cancer forms multicellular aggregates, or spheroids, in the peritoneal cavity that can be isolated from patient ascites fluid and play a potentially critical role in late-stage EOC growth, progression, spread and disease recurrence (Allen, Porter et al. 1987, Shield, Ackland MI Fau - Ahmed et al. 2009). A majority of patients present with ascites fluid during initial diagnosis and almost all patients will develop ascites by late-stage disease. Ascites fluid containing tumor cells and spheroids is known as malignant ascites and is associated with poor prognosis. Spheroid formation is thought to occur after single cells shed from the primary tumor, aggregate and form spheroids which can resist anoikis and survive within the peritoneal fluid. Spheroid formation has been associated with decreased patient survival, increased metastasis and drug resistance in ovarian cancer (Sodek, Ringuette et al. 2009, Lee, Mhawech-Fauceglia et al. 2013). Tumor spheroids have the ability to adhere to and invade mesothelial cells in the peritoneal cavity to form secondary lesions (Burlison, Casey et al. 2004). Secondary lesions on different peritoneal organs can cause comorbidities such as pleural effusion, bowel obstruction, thrombocytosis, thromboembolism and lymphedema. Complications from these comorbidities are often the cause of patient mortality. Buildup of ascites fluid can be up to several liters and must be managed to prevent complications through paracentesis to remove excess fluid. However, ascites fluid returns with differing frequency and multiple rounds of paracentesis can lead to many complications including sepsis. Malignant ascites fluid also contains many soluble factors that can promote tumor growth, spheroid formation, inflammation and angiogenesis. Therapy specifically targeted towards tumors cells in the malignant ascites showed a delay in time to next paracentesis (Heiss, Murawa et al. 2010).

Ovarian Cancer Risk Factors

Ovarian cancer risk is causally linked to both lifestyle and genetics. Firstly, hereditary ovarian cancer (or familial ovarian cancer) accounts for approximately 5-15% of all cases (Ziogas, Gildea et al. 2000) and are often diagnosed at an earlier age than sporadic disease. Furthermore, hereditary ovarian cancer tends to be of the high-grade serous variety (Bewtra, Watson et al. 1992). Therefore, patients with a primary relative with ovarian cancer have an increased risk of developing the disease. Specifically, there is a 2.5% risk of ovarian cancer if a sister has had the disease and a 9% risk if the mother has had ovarian cancer (Ziogas, Gildea et al. 2000). Familial breast and ovarian cancer were first observed in Lynch syndrome (a disease associated with familial cancer due to inherited mutations in DNA repair machinery) in the 1970s (Lynch and Krush 1971, Lynch, Guirgis et al. 1974). Patients with familial breast and ovarian cancer led to the identification of *BRCA1* (Miki, Swensen et al. 1994) and *BRCA2* mutations (Wooster, Neuhausen et al. 1994, Wooster, Bignell et al. 1995). *BRCA1* and *BRCA2* are mutated in the germ line of approximately 9-13% patients with hereditary ovarian cancer (Ford, Easton et al. 1994, Easton, Ford et al. 1995, Rubin, Blackwood et al. 1998). The lifetime risks of developing ovarian cancer for the women ranges from 40-60% for *BRCA1* mutation carriers and 10-20% for *BRCA2* mutation carries (Antoniou, Pharoah et al. 2003, King, Marks et al. 2003, Sogaard, Kjaer et al. 2006). Most subtypes have been linked to *BRCA1* or *BRCA2* germ line mutations but the development of HGS disease is the most common in these women carriers (Castilla, Couch et al. 1994). *BRCA1* and *BRCA2* mutations are more common in Ashkenazi Jewish women (Berman, Costalas et al. 1996, Tonin, Weber et al. 1996, Struewing, Hartge et al. 1997) as well as women from the Bahamas (Akbari, Donenberg et al. 2014). Despite having a higher risk for developing ovarian cancer, *BRCA1/2* carriers have a better clinical outcome in terms of survival, with *BRCA2* carriers having a more favorable outcome than *BRCA1* carriers (Bolton, Chenevix-Trench et al. 2012). This phenomenon is thought to be

due to *BRCA2* carriers responding better to platinum based chemotherapy (Liu, Yang et al. 2012). However, the survival benefit decreases when examined over 10 years in HGS instead of 5 years (Candido-dos-Reis, Song et al. 2015). Over time, this could be possible due to secondary intragenic mutations in *BRCA1* and *BRCA2* that restore the wild-type reading frame (conversion back to a functional BRCA) and losing favorable responses to chemotherapy (Gorodnova, Sokolenko et al. 2015). Location of the alteration in *BRCA1* or *BRCA2* may vary the risk of breast and ovarian cancer (Rebbeck, Mitra et al. 2015), including the presence of single nucleotide polymorphisms (SNPs) such as *BRCA2* SNPs in rs10088218, rs2665390, rs717852, rs9303542 and *BRCA1* SNPS in rs10088218 and rs2665390, indicating further elucidation of risks in carriers (Ramus, Antoniou et al. 2012).

However, mutations in either *BRCA1* or *BRCA2* do not result in a 100% rate of development of ovarian cancer. Further screening of these patients might be necessary to better predict the development of ovarian cancer. Concurrent mutations in 1p36 (*WNT4*), 4q26 (*SYNPO2*), 9q34.2 (*ABO*), and 17q11.2 (*ATAD5*) increased risk of all EOC subtypes while 1q34.3 (*RSPO1*) and 6p22.1 (*GPX6*) mutations increased the risk of serous ovarian cancer in *BRCA* carriers (Kuchenbaecker, Ramus et al. 2015). *BRCA1* carriers can have reduced risk with concurrent mutations in *CASP8* D302H polymorphism (Engel, Versmold et al. 2010). *PALB2*, a *BRCA2* interacting protein, has increased promoter hypermethylation which results in decreased *BRCA2* function and increased risk of ovarian cancer (Potapova, Hoffman et al. 2008).

Genetic risk factors outside of *BRCA1* or *BRCA2* mutations are not as well defined but often take place in genes involved in genomic integrity, most commonly mismatch repair (MMR). SNPs in the *TERT* locus (rs2242652 and rs10069690) were associated with decreased telomere length and increased breast and ovarian cancer risk in *BRCA* mutation carriers (Bojesen, Pooley et al. 2013). A study sequencing twelve genes for germline mutations in

patients with ovarian cancer revealed *BARD1*, *BRIP1*, *CHECK2*, *MRE11A*, *MSH6*, *NMN*, *PALB2*, *RAD51C*, or *TP53* were mutated in 24% of the 360 patients enrolled (Walsh, Casadei et al. 2011). Genes within the Fanconi anemia pathway are also associated with developing ovarian cancer, including: *RAD51C*, *RAD51D*, and *BRIP1* (Pennington and Swisher 2012, Song, Dicks et al. 2015). Other MMR genes associated with Lynch syndrome and ovarian cancer risk *MLH1*, *PMS2*, *MSH2*, and *MSH6* (Malandier, Rambaek et al. 2006, Ketabi, Bartuma et al. 2011, Crispens 2012). Mutations outside of DNA repair pathways such as *KRAS* variants have also been associated with sporadic and familial ovarian cancer without *BRCA1/2* mutations (Ratner, Lu et al. 2010).

Environment and lifestyle also play a risk for developing both hereditary and sporadic ovarian cancer (**Figure 1**) by either increasing or decreasing the lifetime risk of developing ovarian cancer. Like many cancers, age is a risk factor for ovarian cancer with most cases being diagnosed after the age of 60 and the disease being extremely rare in patients under 40 years of age (Gwinn, Lee et al. 1990). As previously discussed, surgical procedures such as tubal ligation, salpingectomy and unilateral or bilateral oophorectomy have varying degrees of success for the development of ovarian cancer by removal of the organs from which the cancer develops (Rice, Hankinson et al. 2014, Gaitskell, Green et al. 2016). The effects can change for each subtype given the nature of development from different tissues, hence why bilateral oophorectomy has a stronger influence on the development of HGS disease, since it is believed to develop from the fallopian tubes. Lifestyle factors which influence complete cycling during menstruation have some of the strongest effects on the risk of developing ovarian cancer. This hypothesis is attributed to incessant ovulation, in which the release of eggs from the ovary, the fusion on the fallopian tube and the rebuilding of the uterine wall all contribute to pathogenesis of ovarian cancer (Fathalla 1971, Godwin, Testa et al. 1992). One of the most common factors which can alter complete cycling is the use of oral contraceptives (Gwinn, Lee et al. 1990). The

increase in use of oral contraceptives could be attributed to the decrease in ovarian cancer in the last decade. The longer use of oral contraceptives has been shown to correlate to lower risk of developing ovarian cancer (Beral, Doll et al. 2008, Havrilesky, Moorman et al. 2013). The risk is reduced in both *BRCA* wild type and mutant carriers (Moorman, Havrilesky et al. 2013) (Bassuk and Manson 2015). The risk of developing each subtype is decreased following oral contraceptive use, with the exception of clear cell carcinoma (Wentzensen, Poole et al. 2016). However, the associated side effects make it a poor treatment for prevention alone (Havrilesky, Gierisch et al. 2013). Another factor that can influence menstrual cycles and the risk of ovarian cancer is child birth (Hankinson, Colditz et al. 1995), in specific the age at first birth and the number of births. In fact, it was discovered the risk of ovarian cancer decreases by approximately 10% for each 5-year increment in age at first birth (Adami, Hsieh et al. 1994). Also, the number of births for a given women has additive decrease in the risk of ovarian cancer, decreasing by about 8% for each birth (Tsilidis, Allen et al. 2011). However, the age of each woman at the onset of menopause has a weak association at best (Schildkraut, Cooper et al. 2001, Tung, Goodman et al. 2003).

Other lifestyle factors can influence the risk of ovarian cancer, such as hormone replacement therapy, breast feeding, obesity and inflammation. Hormone replacement therapy increases the risk of developing ovarian cancer, depending on the therapy. For instance, the use of estrogen increases the risk of developing ovarian cancer by 22%, while the combination of estrogen and progesterone only has about a 10% chance of developing ovarian cancer (Morch, Lokkegaard et al. 2009, Pearce, Chung et al. 2009, Hildebrand, Gapstur et al. 2010). A meta-analysis showed a similar risk for developing both HGS and endometrioid ovarian cancer in menopausal women (Beral, Gaijskell et al. 2015). Conversely, hormone replacement given for menopause symptoms may improve survival of ovarian cancer patients (Eeles, Morden et al. 2015). Another reproductive factor is breastfeeding, in *BRCA1* mutant carriers breastfeeding

lead to a reduced the risk of developing ovarian cancer (Gwinn, Lee et al. 1990, Tung, Goodman et al. 2003). Meta-analysis also suggests the duration of lifetime breastfeeding is additive in reducing the risk of developing ovarian cancer (Luan, Wu et al. 2013). Like many other cancers, cigarette smoking and alcohol consumption have at least some association with increasing the risk of developing ovarian cancer. Specifically, smoking is associated with an increased risk of developing clear cell and endometrial ovarian cancer but not serous (Beral, Gaitskell et al. 2012). Smoking increased the risk of mucinous ovarian cancer, but cessation returns can reduce the risk over time (Jordan, Whiteman et al. 2006) while heavy smoking (>10 packs per day) more than doubles the risk of developing ovarian cancer (Gram, Lukanova et al. 2012). Alcohol consumption increased the risk of ovarian cancer, but seems to have an effect only in heavy drinkers. Consumption of more than 20 drinks per week is associated with increased risk (Gwinn, Webster et al. 1986) while with moderate use the risk is less pronounced or significant (Genkinger, Hunter et al. 2006, Rota, Pasquali et al. 2012). Obesity is associated with less common subtypes of ovarian cancer and not HGS (Olsen, Nagle et al. 2013) and the lifetime risk decreases with recreation physical activity (Cannioto and Moysich 2015). Finally, inflammation increases the risk of developing ovarian cancer (Ness and Cottreau 1999) while the use of aspirin was shown to reduce risk of developing ovarian cancer from between 20-34% (Trabert, Ness et al. 2014). The use of other non-steroidal anti-inflammatory drugs (NSAIDs) showed a reduction in risk but was not significant.

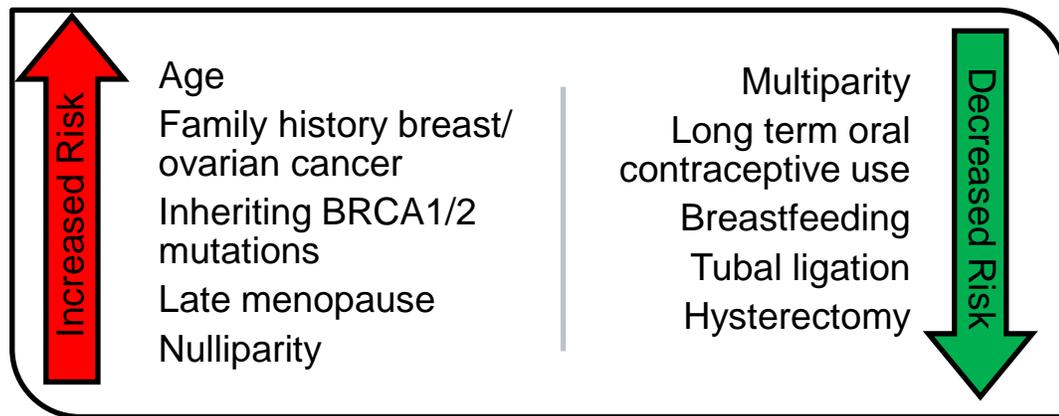


Figure 1: Risk Factors for the Development of Ovarian Cancer

Ovarian Cancer Therapy

The current standard of care for advanced ovarian cancer is cytoreductive surgery followed by a combination of platinum-based chemotherapy and paclitaxel, which has resulted in a 5-year survival rate of ~44%. The approval of platinum-based chemotherapy over alkylating agents, such as meflephan or cyclophosphamide, was based on studies with the combination of cisplatin and cyclophosphamide as well as cisplatin alone (Omura, Blessing et al. 1986, Tate Thigpen, Blessing et al. 2004). These drugs showed much higher activity than other early chemotherapeutics such as 5-fluorouracil or dactinomycin (Park, Blom et al. 1980). While both the platinum-based carboplatin and cisplatin show similar responses in terms of efficacy, carboplatin was approved over cisplatin due to reductions in side effects in carboplatin treated patients (du Bois, Luck et al. 2003). Oxaloplatin, another derivative of cisplatin, has less efficacy but can be used if patients develop allergies to other platinum-based chemotherapy (Fracasso, Blessing et al. 2003). Paclitaxel was approved following trials that showed efficacy as single agent infusion every three weeks in both platinum-sensitive and platinum-resistant patients (Thigpen, Blessing et al. 1994) and an increase in both median survival (38 months vs 24 months) and PFS (18 months vs 13 months) in combination with cisplatin when compared to

cisplatin and cyclophosphamide (McGuire, Hoskins et al. 1996). Combination of carboplatin and paclitaxel showed significant improvements in both OS and PFS (Parmar, Ledermann et al. 2003), and is now the standard of care for the majority of ovarian cancer patients. The development of paclitaxel from lab bench to the clinic will be discussed in the next section. Frontline carboplatin and paclitaxel chemotherapy for ovarian cancer following cytoreductive surgery shows good initial response and many patients show minimal to no evidence of residual tumor. The presence of any residual tumor following primary therapy is the most prognostic factor of patient response (Bristow, Tomacruz et al. 2002). However, the majority of patients will ultimately still experience disease recurrence. Upon recurrence, patients continue to receive the same chemotherapy until the tumor is classified as drug-resistant. Tumors are considered to be drug-resistant when the disease reoccurs in 6 months or less. Recurrence between 6 and 12 months is considered partially-resistant, and patients will often continue to receive frontline chemotherapy (**Figure 2**). Duration of previous platinum treatment is highly predictive of response to secondary platinum treatment (Markman, Markman et al. 2004). The standard of care following front line chemotherapy and the diagnosis of drug resistant disease is observation until disease recurrence. Observation includes physical exam every 3 months and CA125 serum screening. Any evidence of physical changes or elevated CA125 is followed by CT or other imaging to confirm disease recurrence (Salani, Backes et al. 2011). However, following CA125 to start treatment earlier than detected by imaging doesn't improve patient survival (Markman, Glass et al. 2003). The majority of ovarian cancer deaths are due to the development of resistant disease and most resistant patients with succumb to the disease within one year of the diagnosis (Griffiths, Zee et al. 2011). Developing new drugs for the treatment of resistant disease has been one of the primary goals in ovarian cancer research. Mechanisms associated with drug resistance and treatments to improve chemotherapy response will be discussed later in this chapter.

Other therapies outside of the frontline therapy for ovarian cancer have shown some survival benefit. Since optimal cytoreductive surgery is the highest prognostic factor for patient survival (Bristow, Tomacruz et al. 2002), neoadjuvant chemotherapy before surgery helps reduce tumor burden to levels where complete resection is possible (Vergote, Trope et al. 2010, Kehoe, Hook et al. 2015). However, secondary cytoreductive surgery has no survival benefit to patients but does help to predict PFS and OS based on disease burden (Rose, Nerenstone et al. 2004, Rose, Java et al. 2016). Since ovarian cancer dissemination is primarily through the peritoneal cavity, intraperitoneal (IP) chemotherapy has been evaluated to improve drug delivery to the location of disease. IP paclitaxel in combination with platinum-based chemotherapy showed improved OS (65.6 months vs 49.7 months) when compared to intravenous (IV) paclitaxel and platinum; however, only 42% of patients enrolled in the IP arm completed the trial due to side effects or complications with the peritoneal port used to administer the chemotherapy (Omura, Blessing et al. 1986, Armstrong, Bundy et al. 2006). More data supports the use of IP paclitaxel (Rothenberg, Liu et al. 2003), but many physicians and patients are not comfortable administering the drug in this route. Further development to improve the administration and reduce the side effects of IP paclitaxel are needed to increase the frequency of use in the clinic. Repurposing paclitaxel into microparticles can circumvent the use of chremophor, increase the intraperitoneal bioavailability of paclitaxel (Roby, Niu et al. 2008), and reduce the side effects (Williamson, Johnson et al. 2015). Consequently, IP cisplatin has been more controversial in terms of improvement in survival rates in ovarian cancer patients and was associated with high rates of hypertension, nausea, and vomiting (Alberts, Liu et al. 1996, Walker, Brady et al. 2016).

Targeted therapies for ovarian cancer have been difficult to develop due to the high degree of heterogeneity between patients and within tumors. Anti-angiogenic compounds have had the most success in ovarian cancer development. Bevacizumab, a monoclonal antibody target

against the angiogenesis promoting factor VEGF-A, was approved in Europe for the front line therapy of ovarian cancer based on GOG-218 (Burger, Brady et al. 2011) and ICON-7 (Perren, Swart et al. 2011). However, approval for front line therapy in the United States has not been implemented since improvements in PFS or OS have not been observed (Oza, Cook et al. 2015) and bevacizumab is associated with a wide range of GI side effects (Cannistra, Matulonis et al. 2007). However, bevacizumab is approved in both Europe and the United States for the treatment of chemotherapy resistant disease based on the AURELIA trial (Pujade-Lauraine, Hilpert et al. 2014, Poveda, Selle et al. 2015). Dose dense bevacizumab improves PFS in ovarian cancer patients and is a promising therapy for further development (Chan, Brady et al. 2016). Another angiogenesis inhibitor approved for ovarian cancer therapy is the VEGF tyrosine kinase inhibitor cediranib. Cediranib has some single agent activity in chemotherapy sensitive and resistant chemotherapy, it can be used in combination with platinum-based chemotherapy, or used as maintenance in platinum-sensitive disease (Matulonis, Berlin et al. 2009, Liu, Barry et al. 2014, Ledermann, Embleton et al. 2016). Finally, the most promising recent breakthrough approved for the treatment of ovarian cancer is poly ADP ribose polymerase (PARP) inhibitors. PARP regulates genomic integrity, DNA repair and cell death mechanisms (Herceg and Wang 2001). Therefore, PARP inhibitors can induce DNA damage and cell death. Specifically, in *BRCA* deficient, or other homologous repair deficient cells, PARP inhibitors induce the error prone DNA repair pathway non-homologous end joining (Fong, Boss et al. 2009). Therefore, PARP inhibitors were investigated for efficacy in ovarian cancer due to the high number of patients with *BRCA* and/or homologous recombination (HR) deficient tumors (Audeh, Carmichael et al. 2010). Olaparib (an oral PARP inhibitor) is effective as a maintenance therapy after platinum therapy response in patients with platinum-sensitive HGSC compared with placebo (Ledermann, Harter et al. 2012, Oza, Cibula et al. 2015) and has been given accelerated approval for maintenance therapy in Europe. However, accelerated approval for maintenance therapy in the United States has yet to be granted based on only changes in

PFS. Olaparib was recently granted accelerated approval for the use in patients that received at least three rounds of chemotherapy (Domchek, Aghajanian et al. 2016). Trials are ongoing for the treatment of both platinum sensitive and resistant disease in which olaparib has shown increased function in combination with platinum-based chemotherapy (Ledermann, Harter et al. 2012). Second generation PARP inhibitors, such as niraparib, are not as dependent on loss of *BRCA* function in ovarian tumor cells (Mirza, Monk et al. 2016), which could be the result of a novel mechanism of trapping PARP proteins to the DNA damage site (Murai, Huang et al. 2012). Despite these advances, there is still urgent need for better understanding of chemotherapy resistance and the development of new drugs for ovarian cancer.

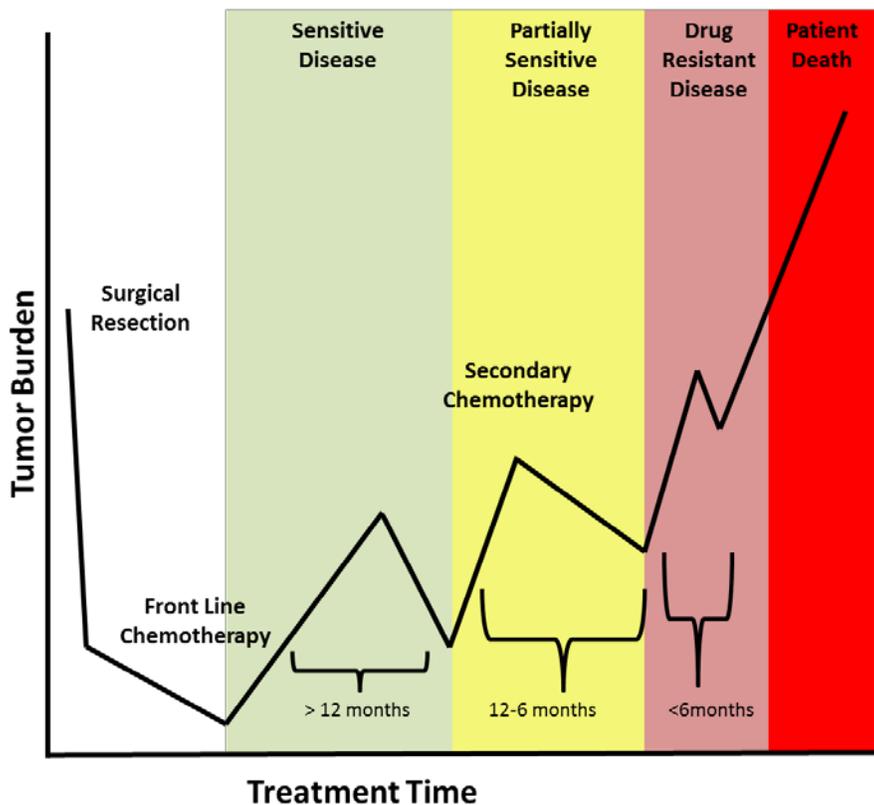


Figure 2: Disease Recurrence and Drug Resistance in Clinical Ovarian Cancer

Development of Paclitaxel for the Treatment of Ovarian Cancer

In 1992, paclitaxel was the last drug approved for the primary treatment of ovarian cancer. To reach clinical approval paclitaxel (Taxol) had to go through extensive discovery studies. Paclitaxel was founded in a natural products drug screening program through the Cancer Chemotherapy National Service Center (CCNSC) of the National Cancer Institute (NCI). The focus on plant extracts was based on screening in both sarcoma and leukemia (L1210 and P388) cell lines that discovered podophyllotoxin from *Podophyllum pelatum* and vincristine and vinblastine from *Catharanthus roseus* (Carter and Livingston 1976). Hits from CCNSC screening were subjected to bioassay-guided fractionation and animal tumor model testing for identification and validation (Driscoll 1984, Venditti, Wesley et al. 1984). *Taxus brevifolia* (Pacific Yew) bark was collected by the USDA in 1962 to be included in screening and activity of *Taxus* extracts were confirmed through Kenacid Blue (KB) cytotoxicity assays in 1964. Recollection, fractionation and structural confirmation showed the active fraction of *Taxus* bark was a diterpene compound named Taxol (Wani, Taylor et al. 1971). Taxol showed the most activity against melanoma cell lines (B16) in 1975 and was accepted for preclinical development in 1977. The most promising discovery to promote the development of Taxol was the elucidation of the cell mechanism. Unlike alkylating agents that promoted break down of tubulin, Taxol promoted polymerization and stabilization of tubulin, leading to cellular catastrophe (Carter and Livingston 1976, Kumar 1981, Parness and Horwitz 1981). Not only did Taxol cause mitotic arrests, it was also shown to inhibit migration of fibroblasts following tubulin stabilization (Schiff and Horwitz 1980). It also promotes stabilization of tubulin and has been found to have different binding sites compared to other agents in the N-terminal 31 amino acids of beta-tubulin (Rao, Krauss et al. 1994).

Phase I approval for Taxol started in 1983 but factors such as limited supply, solubility and toxicity delayed development for clinical use. The poor solubility of Taxol and high doses

needed compared to Vinca alkaloids led to the use of chremophor (a solubilizing agent linked to allergic reactions) at twice the normal dosage (Lassus, Scott et al. 1985, Davignon and Craddock 1987). However, the results from a Phase II trial in ovarian cancer brought Taxol back to leading edge of clinical development. Briefly, 47 patients with drug-refractory ovarian cancer who had one or more measureable lesions were selected for the treatment with a 24-hour infusion with doses from 10 to 250 mg/m² of Taxol (based on adverse reactions) every 22 days. Of the 47 patients, 45 were eligible for toxicity evaluation and 40 were eligible for response evaluation at the end of the study. Twelve patients responded to Taxol (30%) for periods of 3 to 15 month progression free survival (McGuire, Rowinsky et al. 1989). The ovarian cancer trial and subsequent success in breast cancer (Seidman, Reichman et al. 1992), melanoma (Einzig, Hochster et al. 1991) and head and neck cancer (Forastiere, Neuberg et al. 1993) created an increasing demand for Taxol isolation that *Taxus* bark could not sustain.

The isolation of bark from the Pacific Yew was an extensive process could only be completed in the spring or summer when sap was being produced. Likewise, the process of scraping off bark from the tree was often damaging to the tree, and not a renewable resource. To obtain 1.3 kg of drug between 2,000 and 15,000 pounds of bark were needed. Even after obtaining sufficient bark, the purification process was extremely time consuming-requiring multiple rounds of extraction, chromatography, and crystallization were needed to produce Taxol drug. To produce synthetic Taxol a precursor was needed since the compound contains 11 chiral centers, as well as 2,048 diastereomeric isomers, making *de novo* synthesis extremely problematic (**Figure 3**). The first synthetic method to produce Taxol was using semisynthetic conversion of baccatin III derivatives, isolated from the needles instead of the bark (Denis, Green et al. 1988, Cragg, Schepartz et al. 1993). The needles can be harvested year round and required minimal damage to the tree itself, making this a renewable supply of Taxol. Later on synthetic methods were developed to mass produce the drug in a sustainable fashion

(Danishefsky, Masters et al. 1996, Mukaiyama, Shiina et al. 1999, Kusama, Hara et al. 2000, Fuse, Miyamoto et al. 2006).

Trials continued with Taxol in ovarian cancer to improve administration (Weiss, Donehower et al. 1990) and evaluate granulocyte-colony stimulating factor (G-CSF) to treat adverse symptoms of the delivery (Sarosy, Kohn et al. 1992). These continued to show success in reducing disease progression so two conformation Phase II trials were performed. In 1992, data in 34 patients with metastatic ovarian cancer receiving dosages of Taxol from 180 to 350 mg/m² showed 1 patient with complete response, 5 patients with partial response and a median survival of 27 months (Einzig, Wiernik et al. 1992). In 1994, a second Phase II trial used Taxol as a salvage therapy in patients with recurrent, persistent or progressive ovarian cancer with an overall response rate of 34% (Thigpen, Blessing et al. 1994).

Following these salvage trials Taxol, now under the trade name paclitaxel, was taken to Phase III trials in combination with carboplatin. In the GOG-111 trial IV patients with advanced ovarian cancer with a residual mass > 1cm were treated with either paclitaxel (125 mg/m² over 24 hours) combined with cisplatin (75mg/m²) or cyclophosphamide (750 mg/m²) and cisplatin. Overall 410 patients were enrolled and 386 completed the trial. The overall response rate for the paclitaxel arm was 73% compared to 60% in the cyclophosphamide arm. While a similar complete response was observed, the paclitaxel treatment had a longer PFS (18 months vs 13) and OS (38 months vs 24) when compared to cyclophosphamide (McGuire, Hoskins et al. 1996). In a conformation Phase II trial in Europe and Canada, patients with advanced ovarian cancer were treated with the same regimen but with three hour infusion of paclitaxel instead of 24 hours. Overall 680 patients were enrolled and the overall response rate was increased in the paclitaxel arm (59%) when compared to cyclophosphamide (45%). Similar increases in PFS (15.5 months vs 11.5) and OS (35.6 months vs 25.8) were also observed. Despite the

promising results for the development of paclitaxel, there is still an urgent need for further drug development in ovarian cancer.

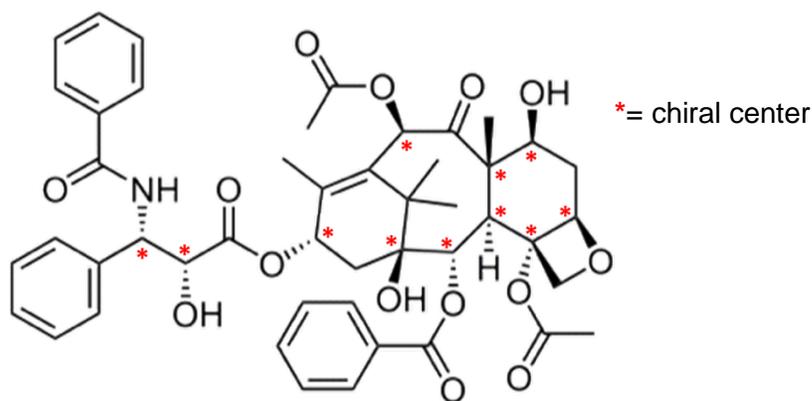


Figure 3: Structure and Chiral Centers of Taxol.

Drug resistance in Ovarian Cancer

While front line chemotherapy produces promising responses in most ovarian cancer patients, the majority of patients will develop recurrent and drug resistant disease. Drug resistance in ovarian cancer is one of the primary reasons for patient mortality and better understanding of the mechanisms is urgently needed. Drug resistance in ovarian cancer is multifaceted as it can be influenced by genetic changes, physical restriction of drug entry into cells, cell environmental changes, metabolism, hypoxia, inflammation and many other phenotypes (**Figure 4**). Resistance can arise by the evolution of intrinsically resistant cells following selection pressure of chemotherapy (Cooke, Ng et al. 2010, Gerlinger and Swanton 2010) or by phenotypic and cellular changes induced by drugs themselves leading to the promotion of compensatory or survival mechanism (Friedman 2016). Whether drug resistant ovarian cancers are clonally selected or induced as a response to chemotherapy, a better understanding of different mechanisms associated with drug resistance is pertinent to developing new therapies to improve the survival of ovarian cancer patients.

Specific cell alterations that prevent the binding of drugs to their target can drive resistance to paclitaxel and platinum drugs. Changes that alter the expression of tubulin or microtubule dynamics promote drug resistance (Mozzetti, Ferlini et al. 2005, McGrail, Khambhati et al. 2015). Mutations in tubulin can block paclitaxel binding and prevent tubulin stabilization in many cancers (Monzo, Rosell et al. 1999, Wang, O'Brate et al. 2005, Hari, Loganzo et al. 2006), but mutations are not as common in ovarian cancers (Lamendola, Duan et al. 2003, Mesquita, Veiga et al. 2005). Another mechanism that prevents drug-target interaction involves physical or biochemical restriction of drugs entering into the cell or nucleus where the target is located. Paclitaxel penetration into solid tumor xenografts is determined by tumor density and blocks drug induced apoptosis (Kuh, Jang et al. 1999). The specific entry or efflux of platinum drugs and paclitaxel is driven by the expression of drug efflux pumps. Over expression of drug transporters such as *ABCB1* (also known as MDR1) promotes both paclitaxel and cisplatin or carboplatin resistance in ovarian cancer (Johnatty, Beesley et al. 2008, Eyre, Harvey et al. 2014, Sun, Jiao et al. 2015, Wang, Liu et al. 2015, Vaidyanathan, Sawers et al. 2016). Likewise, nuclear efflux of carboplatin or paclitaxel is driven by *ABCC2* overexpression and nuclear membrane localization which promotes increased drug resistance in ovarian cancer (Surowiak, Materna et al. 2006). Inversely, protein stability and efflux is decreased in certain *ABCC2* variants and enhances patient survival (Tian, Ambrosone et al. 2012). The generation of stable, cisplatin resistant cell lines led to the identification of some of the first mechanisms of drug resistance such as glutathione synthesis (GSH). GSH expression was increased in cisplatin-resistant derived ovarian cancer cell lines and multidrug resistance was also associated with GSH expression (Godwin, Meister et al. 1992, Hamaguchi, Godwin et al. 1993). Glutathione is involved in the suppression of oxidative stress (Anderson 1998) and expression leads to the formation of GSH-drug conjugates that are exported from the cell (Colvin, Friedman et al. 1993). While the glutathione analog prodrug canfosfamide, or trade name TELCYTA, showed some survival improvement at 18 months in Phase II trials

(Kavanagh, Gershenson et al. 2005), Phase III trials showed no benefit of the drug on either PFS or OS (Vergote, Finkler et al. 2009).

The more adept a tumor is at repairing genomic alterations, the less effective carboplatin and paclitaxel become since cisplatin cross links DNA and paclitaxel stabilizes tubulin preventing chromosome separation during anaphase. The ability of ovarian cancer cells to repair DNA damage, specifically interstrand crosslinks, is enhanced in platinum resistant ovarian cancer cell lines suggesting a correlation to DNA repair and drug resistance (Johnson, Swiggard et al. 1994). Whole genome analysis of ovarian cancer patients across different levels of drug sensitivity revealed many alterations associated with drug resistance such as: DNA alterations in the coding region for other tumor suppressors such as *RB1*, *NF1*, *RAD51B* and *PTEN*. Alternatively, *CCNE1* amplification, which alters cell cycle progression and promotes *BRAC* expression or the restoration of *BRCA1* or *BRCA2* function through reversions of mutations can reduce the risk of ovarian cancer (Patch, Christie et al. 2015). This study highlights specific DNA repair mechanisms associated with genomic stability and drug resistance in ovarian cancer. As previously discussed, patients with mutant *BRCA1* or *BRCA2* have better survival than patients without, primarily due to better response to chemotherapy (Bolton, Chenevix-Trench et al. 2012) and the reversion of mutant *BRCA2* to wild type promotes cisplatin resistance (Sakai, Swisher et al. 2008). *BRCA1* mutations also enhance sensitivity to both radiation and paclitaxel in ovarian cancer cells (Zhou, Smith et al. 2003), which can also be restored by secondary mutations (Swisher, Sakai et al. 2008). The expression of different DNA polymerase (DNA pol) subunits can affect the ability of ovarian cancer cells to respond to platinum based chemotherapy. Specifically, DNA pol η (eta) can bypass GG DNA adducts caused by either oxalplatin or cisplatin and maintain replication fork stability during replication (Vaisman, Masutani et al. 2000). Also, the increase of pol η expression in ovarian cancer cells

increases cisplatin resistance and the expression of the pol η regulating miR-93 restores sensitivity to cisplatin (Srivastava, Han et al. 2015).

Many cellular changes that do not affect drug delivery or DNA integrity can also confer drug resistance in ovarian cancer. These changes can be related to cell growth, metabolism, inflammation, hypoxia, cell survival and the tumor microenvironment (Helleman, Smid et al. 2010). Chemotherapy treatment induces a phenotypical change in cell growth and morphology known as the epithelial to mesenchymal transition (EMT), measured by the loss of expression of epithelial markers such as E-cadherin and the increase of mesenchymal markers such as *SNAIL*, *SLUG*, and *TWIST* (Kajiyama, Shibata et al. 2007, Haslehurst, Koti et al. 2012). EMT can be promoted through several pathways following drug treatment or by neighboring cells in the tumor associated stroma. Interestingly, the promotion of drug resistance via cross talk between pro-tumorigenic stromal cells and drug sensitive cells can happen through the secretion of inflammatory markers such as POSTN, LOX, and FAP (Ryner, Guan et al. 2015) or through the release of extracellular vesicles, or specifically exosomes, carrying regulatory biomolecules such as miR21 (Au Yeung, Co et al. 2016, Crow, Atay et al. 2017). Targeting pathways that promote EMT such as PI3K can block EMT during drug response (Du, Wu et al. 2013). Additionally, EMT is a dynamic cellular process that can be reversed through the mesenchymal to epithelial transitions (MET). Promotion of MET by epimorphin, which induced the expression of E-cadherin and reduced the expression of snail and slug, increased the sensitivity of ovarian cancer cells to carboplatin (Yew, Crow et al. 2013). Another factor from the tumor environment that promotes drug resistance is cellular hypoxia. As solid tumors grow away from blood vessels, their oxygen supply is limited and cells become hypoxic. Hypoxia exposure in breast and ovarian cancer cell lines promotes a MDR phenotype that is maintained following *in vivo* transplant, depending on the cell line and amount of time exposed to hypoxia (Milane, Duan et al. 2011). Hypoxia mediated paclitaxel resistance occurs through the

stabilization and expression of the hypoxic regulator HIF1- α , and induced cell cycle arrest in ovarian cancer A2780 cells (Huang, Ao et al. 2010). HIF1- α also regulates cell mechanisms such as metabolism and cell death pathways (Favaro, Nardo et al. 2008). Hypoxia resistant metabolism drives spheroid formation, engraftment, metastasis and drug resistance in ovarian cancer cell lines (Liao, Qian et al. 2014). Targeted knockdown of *HIF1A* enhanced the efficacy of doxorubicin chemotherapy in ovarian cancer cells (Wang, Saad et al. 2008). Metabolism changes can be driven by hypoxia (Ai, Lu et al. 2016) as well as mitochondrial mechanisms such as oxidative stress and reactive oxygen species (Sherman-Baust, Becker et al. 2011, Chen, Huang et al. 2015). Intrinsic inflammation which is common in malignant ascites (Lane, Matte et al. 2015) and the associated pro-inflammatory microenvironment can predict drug resistance before chemotherapy treatment (Koti, Siu et al. 2015). Induced inflammation from immune responses or cellular debris following apoptosis can also trigger chemotherapy resistance through TLR-4 signaling (Kelly, Alvero et al. 2006). Cytotoxic drugs like paclitaxel and platinum based chemotherapy can induce cell survival mechanism such as autophagy (Zhang, Wang et al. 2015), anti-apoptosis pathways to confer chemotherapy resistance (Strobel, Tai et al. 1998, Marchion, Cottrill et al. 2011), and cell cycle regulators (Han, Yu et al. 2009). All of these mechanisms can occur independently and be induced by different factors, there is a clear need for a better understanding the cellular development of each and how they may be connected.

Finally, cancer stem cells or stem-like cells (cancer cells with stem cell properties) have been shown to be resistant to chemotherapy in ovarian cancer. The origin and pathways of cancer stem cells will be discussed in Chapter 3, for now the focus is on the role of cancer stem cells in drug resistance. Stemness, for this purpose of this document is defined as the expression of stem cell-associated genes, is increased following primary therapy in both *in vivo* models and ovarian cancer patients (Steg, Bevis et al. 2012, Dobbin, Katre et al. 2014).

Upregulation of *CD44*, *ALDH1A*, or *CD133* have all been shown to induce drug resistance in ovarian cancer (Baba, Convery et al. 2009, Deng, Yang et al. 2010, Cioffi, D'Alterio et al. 2015, Gao, Foster et al. 2015). One of the common characteristics of cancer stem cells is their genomic and phenotypic plasticity which allows them to adapt to harsh environments or evolve in response to different stimuli such as chemotherapy (Quintana, Shackleton et al. 2010, Pisco and Huang 2015). This adaptation ability allows cancer stem cells to regulate or be induced by a number of previously discussed mechanisms of drug resistance, connecting multiple pathways at one through a common cell phenotype. Cellular hypoxia can induce stemness in ovarian cancer cells via the expression of many different stem cell genes *CD133*, *OCT4*, *SOX2*, and *NANOG* (Liang, Ma et al. 2012, Wu, Du et al. 2014, Ramadoss, Sen et al. 2016) and the knock down of different stem cell genes increases the sensitivity to chemotherapy (Landen, Goodman et al. 2010, Seo, Kim et al. 2016). Ovarian cancer stem cells also can better repair platinum induced DNA adducts (Srivastava, Han et al. 2015) have increased expression of drug transporters (Kobayashi, Seino et al. 2011) and can promote EMT and inflammation (Yin, Chen et al. 2010). Interestingly, a long term (28 day) analysis of ovarian cancer cells as they recover from chemotherapy showed a multistep process which started with the induction of genomically unstable cells due to polyploidy followed by EMT, inflammation, and stemness (Rohnalter, Roth et al. 2015). These data support stemness as a common up and downstream mediator of several drug resistance pathways in ovarian cancer, making it a promising area of development for drug development.

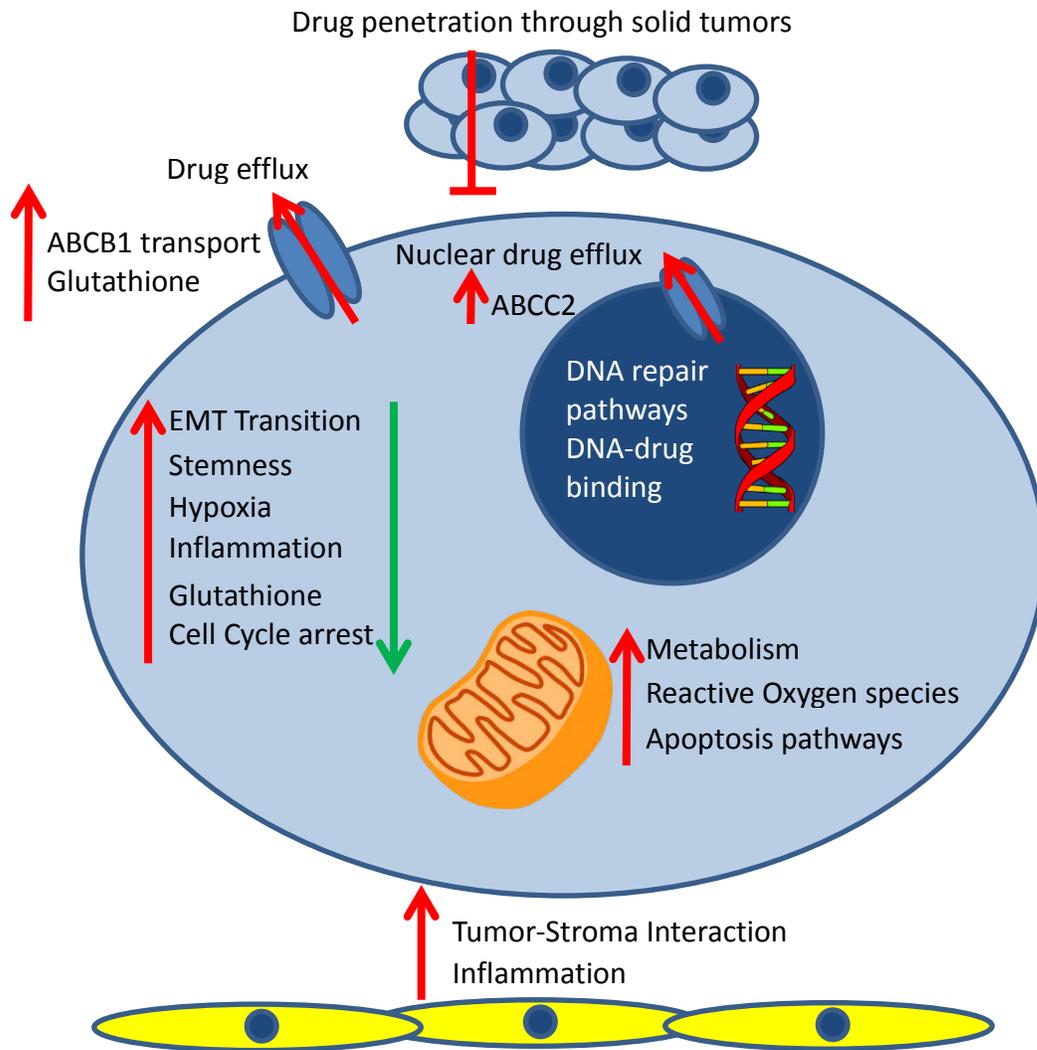


Figure 4: Mechanisms of Chemotherapy Resistance in Ovarian Cancer

Failures of drug development in ovarian cancer

While there has been some success in drug development for ovarian cancer, the relative survival rate has been stagnant since the 1990s (**Figure 5**). Many approaches have been tried to improve survival in ovarian cancer patients. Some have improved PFS but not OS which leads to an urgent need for the development of new drugs. One of the most common approaches has been to increase the amount of chemotherapy given via increased dosage or duration of chemotherapy. However, based on the previously discussed mechanisms

associated with chemotherapy resistance, adding more chemotherapy to patients may be counterintuitive. For instance, maintenance therapy adding more cycles of chemotherapy for three cycles, six cycles, and 24 weeks showed no improvement in PFS or OS (Bell, Brady et al. 2006, Pecorelli, Favalli et al. 2009, Mannel, Brady et al. 2011). Maintenance therapy for 12 months did show some increase in PFS, but no effect on OS (Markman, Liu et al. 2003, Markman, Liu et al. 2009). Likewise, therapies that increased paclitaxel dosage and dose dense infusions of paclitaxel showed change in PFS but not OS, and were associated with higher degrees of toxicity (Omura, Brady et al. 2003, Katsumata, Yasuda et al. 2009, Katsumata, Yasuda et al. 2013). The GOG-182 and ICON5 trials addressed whether the issues of adding more chemotherapy having limited effects were due to specific resistance to platinum-based chemotherapy and paclitaxel. These trials added a third cytotoxic agent to the front line therapy of platinum-based chemotherapy and paclitaxel. These cytotoxic agents were gemcitabine (a nucleoside analog), doxorubicin (a DNA intercalating agent), and topotecan (a topoisomerase inhibitor). None of the third cytotoxic agents improved the activity of front line chemotherapy in ovarian cancer patients (Bookman, Brady et al. 2009). These data support a multi-drug resistance phenotype that requires additional activities besides more chemotherapy or cytotoxic drugs to improve the survival of ovarian cancer patients.

While some targeted therapies have had success in ovarian cancer as previously discussed, the majority of clinical trials with targeted therapies demonstrate limited success. This was most notably observed in the GOG-170 trials where 12 different agents, many of which had been approved for other cancers, were tested in refractory ovarian cancer patients and only 1 showed improved in patient survival. Bevacizumab, an angiogenesis inhibitor, showed improvement in clinical response when combined with carboplatin in platinum-sensitive ovarian cancer (Burger, Sill et al. 2007). The other 11 drugs showed minimal to no clinical response, the drugs were: the SRC kinase inhibitor dasatinib (Schilder, Brady et al. 2012), an EGFR

inhibitor gefitinib (Schilder, Sill et al. 2005, Posadas, Liel et al. 2007), a pan-kinase inhibitor sorafenib (Matei, Sill et al. 2011), a dual HER2/EGFR inhibitor lapatanib (Garcia, Sill et al. 2012, Leslie, Sill et al. 2012), an mTOR inhibitor temsirolimus (Behbakht, Sill et al. 2011), a protein kinase C beta inhibitor enzastaurin (Usha, Sill et al. 2011), a synthetic progesterone inhibitor mifepristone (Rocereto, Brady et al. 2010), a pan-kinase inhibitor motesanib (Schilder, Sill et al. 2013), a uPAR inhibitor urokinase-derived peptide (A6) (Gold, Brady et al. 2012), an IL-12 plasmid EGEN-001(Alvarez, Sill et al. 2014) and the monoclonal antibody against HGF rilotumumab (Martin, Sill et al. 2014). Likewise, the GOG-146 trials (C, D, F, H, J, K, L, N, O, P, Q) evaluated single agents in recurrent ovarian cancer and the majority of trials failed due to lack of efficacy (Markman, Blessing et al. 2000, Plaxe, Blessing et al. 2002, Armstrong, Blessing et al. 2003, Hoffman, Blessing et al. 2003, Miller, Blessing et al. 2003, Miller, Blessing et al. 2005, Secord, Blessing et al. 2008, Aghajanian, Blessing et al. 2009, Schilder, Blessing et al. 2010, Herzog, Sill et al. 2011), while two trials failed due to low accrual or toxicity (McGuire, Blessing et al. 2000, Covens, Blessing et al. 2006). Many of these drugs showed promising preclinical activity yet had little to no effects in patients (typically less than 10% clinical response as defined by improvements in PFS or OS when compared to the standard of care). For instance, dasatanib showed strong inhibition of ovarian cancer cells lines through SRC kinase signaling, and synergy with paclitaxel and carboplatin (Teoh, Ayeni et al. 2011, Xiao, Xu et al. 2015). Gefitinib (or the trade name Iressa) was shown to regulate DNA damage responses and synergize with carboplatin in ovarian cancer cell lines (Smith, Gaikwad et al. 2008, Ohta, Ohmichi et al. 2012). Many other promising drugs have failed clinical trials in ovarian cancer. Overall, 101 clinical trials were reviewed here for the treatment of ovarian cancer. These were classified as failure based on limited to no clinical response, success based on modest to significant clinical improvement in survival or toxicity profile, or toxic based on trials ended early due to clinical side effects. Of these 101 trials, 58.4% were classified as failure, 34.6% were classified as a success, and 7% were classified as toxic (**Table 3**). These high failure rates

suggest limitations of pre-clinical development that need to be improved in order to identify new drugs for ovarian cancer. The focus of my thesis will be to apply this knowledge of chemotherapy resistance in clinical models to improve the *in vitro* representation of ovarian cancer cell lines to drug-resistant disease that can be exploited for drug screening and development.

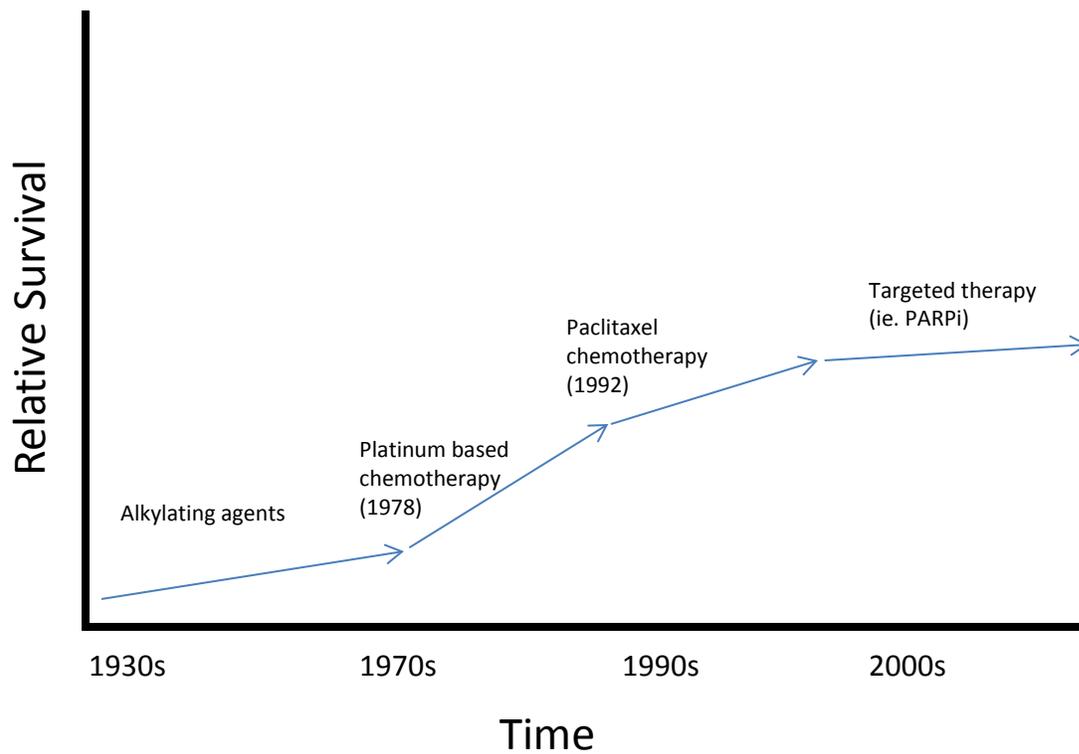


Figure 5: Drug Development History for Ovarian Cancer

Table 3: Clinical Trials for the Treatment of Ovarian Cancer

Trial	Treatments	Outcome	Reference
	<ul style="list-style-type: none"> • Doxorubicin and cyclophosphamide • Doxorubicin, cyclophosphamide and carboplatin 	Complete response rate was increased from 26% to 51% with the addition of cisplatin	(Omura, Blessing et al. 1986)
	<ul style="list-style-type: none"> • IV cisplatin and paclitaxel • IP cisplatin and paclitaxel and IV paclitaxel with bevacizumab 	Bevacizumab does not provide an increase in PFS and adds additional risk to bowel obstruction.	(Konner, Grabon et al. 2011)
	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • IV carboplatin and paclitaxel followed by six cycles of paclitaxel 	Maintenance therapy with paclitaxel does not prolong PFS or OS.	(Pecorelli, Favalli et al. 2009)
	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • IV carboplatin and paclitaxel with epirubicin 	No improvement in survival or time to treatment failure in patients.	(du Bois, Luck et al. 2003)
	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • IV carboplatin and paclitaxel with topotecan 	No improvement in OS or PFS.	(Pfisterer, Weber et al. 2006)
	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • IV carboplatin and paclitaxel with epidoxorubicin 	No change in 3-year survival (79% and 78.7%) with the addition of epidoxorubicin.	(Bolis, Danese et al. 2006)
	<ul style="list-style-type: none"> • IV cetuximab dose escalation to skin rash 	Cetuximab showed minimal activity in patients with recurrent ovarian cancer, patients with elevated serological markers had a higher cancer of early progression.	(Schilder, Pathak et al. 2009)
AGO OVAR 2.5	<ul style="list-style-type: none"> • Carboplatin • Carboplatin and gemcitabine 	Addition of gemcitabine improved PFS but not OS.	(Pfisterer, Plante et al. 2006)
AGO OVAR3	<ul style="list-style-type: none"> • IV cisplatin and paclitaxel • IV carboplatin and paclitaxel 	No significant difference in PFS or overall survival between treatment arms. Better tolerability to carboplatin.	(du Bois, Luck et al. 2003)
AGO-OVAR 12	<ul style="list-style-type: none"> • Carboplatin and paclitaxel • Carboplatin and paclitaxel with nintedanib 	Improvement in PFS, but more gastrointestinal toxicities with nintedanib.	(du Bois, Kristensen et al. 2016)
AURELIA	<ul style="list-style-type: none"> • Paclitaxel with or without bevacizumab • Pegylated liposomal doxorubicin with or without bevacizumab • Topotecan with or without bevacizumab 	Paclitaxel plus bevacizumab was the best combination — increased response rate, PFS and overall survival.	(Pujade-Lauraine, Hilpert et al. 2014, Poveda, Selle et al. 2015)
CHORUS	<ul style="list-style-type: none"> • Primary surgery followed by six cycles of chemotherapy • Three cycles of primary chemotherapy, then surgery, followed by three more cycles of completion chemotherapy 	Chemotherapy before surgery is an acceptable standard of care.	(Kehoe, Hook et al. 2015)
EORTC	<ul style="list-style-type: none"> • Primary debulking surgery followed by platinum-based chemotherapy • Neoadjuvant platinum-based chemotherapy followed by debulking surgery 	Neoadjuvant chemotherapy was similar to primary chemotherapy as complete debulking was the strongest predictor of survival.	(Vergote, Trope et al. 2010)

Trial	Treatments	Outcome	Reference
GOG-170D	<ul style="list-style-type: none"> • IV bevacizumab 	Bevacizumab showed a 21% response rate with 40.3% of patients with progression over 6 months in recurrent or persistent ovarian cancer patients.	(Burger, Sill et al. 2007)
GOG-111	<ul style="list-style-type: none"> • IV cisplatin and cyclophosphamide • IV cisplatin and paclitaxel 	Increase in PFS and overall survival with cisplatin and paclitaxel treatment with an increase in median survival (38 months vs 24 months) and PFS (18 vs 13 months).	(McGuire, Hoskins et al. 1996)
GOG-218	<ul style="list-style-type: none"> • Carboplatin, paclitaxel and bevacizumab with bevacizumab maintenance • Carboplatin, paclitaxel and bevacizumab with placebo maintenance • Carboplatin, paclitaxel and placebo with placebo maintenance 	Increase of PFS by 3.8 months with the addition of bevacizumab.	(Burger, Brady et al. 2011)
GOG-0170M	<ul style="list-style-type: none"> • Oral dasatinib 	Dasatinib as a single agent had no effect of recurrent ovarian cancer patients.	(Schilder, Brady et al. 2012)
GOG-1	<ul style="list-style-type: none"> • Surgical resection only <ul style="list-style-type: none"> • Melphalan • Radiation 	All patients appeared to benefit from chemotherapy following surgical resection compared to radiation or no treatment.	(Hreshchshyn, Park et al. 1980)
GOG-114 and SWOG-9227	<ul style="list-style-type: none"> • IV paclitaxel followed by IV cisplatin • IV high dose carboplatin followed by IV paclitaxel then IP paclitaxel 	11 month improvement in OS in the high dose carboplatin and IP paclitaxel treatment, along with increased toxicity.	(Markman, Bundy et al. 2001)
GOG-126D	<ul style="list-style-type: none"> • IV pyrazoloacridine 	Pyrazoloacridine had modest activity in patients with platinum resistant cancer, with 1 complete responder in 24 patients.	(Plaxe, Blessing et al. 2002)
GOG-126E	<ul style="list-style-type: none"> • Oral valspador and IV paclitaxel 	Combination of paclitaxel and valspador showed minimal effects in patients with paclitaxel-resistance cancer.	(Fracasso, Brady et al. 2001)
GOG-126G	<ul style="list-style-type: none"> • IV Ledoxantrone 	Ledoxantrone has minimal activity against platinum resistant disease.	(Hoffman, Blessing et al. 2000)
GOG-126I	<ul style="list-style-type: none"> • IV 9-aminocamptothecin 	9-aminocamptothecin showed limited activity in platinum-resistant ovarian cancer.	(Miller, Blessing et al. 2005)
GOG-126J	<ul style="list-style-type: none"> • Docetaxel 	Docetaxel is active in 4% of paclitaxel-resistant patients with significant hematological toxicity.	(Rose, Blessing et al. 2003)
GOG-126L	<ul style="list-style-type: none"> • Cisplatin plus gemcitabine 	Gemecetbine combination with cisplatin showed modest activity with an overall response rate of 16%.	(Brewer, Blessing et al. 2006)
GOG-126N	<ul style="list-style-type: none"> • IV paclitaxel weekly 	Weekly paclitaxel showed a response rate of 20.9% in platinum and paclitaxel-resistant patients.	(Markman, Blessing et al. 2006)

Trial	Treatments	Outcome	Reference
GOG-126O	<ul style="list-style-type: none"> • IV 3-AP before carboplatin for four days, every 21 days 	Platinum sensitivity based on DNA damage was restored with 3-AP combination but the trial was halted before survival was measured due to methemoglobinemia.	(Kunos, Radivoyevitch et al. 2012)
GOG-126R	<ul style="list-style-type: none"> • IV nanoparticle, albumin-bound (nab) paclitaxel 	Nab-paclitaxel showed promising response with median PFS of 4.5 months and OS of 17.4 months in recurrent ovarian cancer.	(Coleman, Brady et al. 2011)
GOG-126T	<ul style="list-style-type: none"> • IV belinostat with carboplatin on a three day cycle for six cycles 	Belinostat addition to carboplatin showed little activity (ORR of 7.4%) in patients with platinum-resistant ovarian cancer.	(Dizon, Blessing et al. 2012)
GOG-132	<ul style="list-style-type: none"> • IV Cisplatin • IV Paclitaxel • IV Cisplatin and paclitaxel 	Cisplatin alone showed better PFS than paclitaxel alone. Combination therapy showed similar OS but better toxicity profile.	(Muggia, Brady et al. 2000)
GOG-132	<ul style="list-style-type: none"> • IV Oxaliplatin 	Minimal activity with partial response (4.3%) and stable disease (39.1%) in platinum resistant ovarian cancer.	(Fracasso, Blessing et al. 2003)
GOG-133 and SWOG-8790	<ul style="list-style-type: none"> • IP α-interferon • Observation 	Trial was closed early but indicated possible improvement in recurrence and survival.	(Alberts, Hannigan et al. 2006)
GOG-134 and SWOG-9238	<ul style="list-style-type: none"> • IV paclitaxel 135 mg/m² • IV paclitaxel 117mg/m² • IV paclitaxel 250 mg/m² 	Dosage response for response rate but no survival benefit and higher toxicity at higher dosages.	(Omura, Brady et al. 2003)
GOG-146C	<ul style="list-style-type: none"> • 30 min infusional topotecan for 5 days 	Topotecan showed a 33% response rate in patients with platinum sensitive ovarian cancer, but with a significant hematological response.	(McGuire, Blessing et al. 2000)
GOG-146D	<ul style="list-style-type: none"> • IV pyrazoloacridine 	Pyrazoloacridine exhibited partial response in platinum sensitive patients.	(Plaxe, Blessing et al. 2002)
GOG-146F	<ul style="list-style-type: none"> • 24-h infusional topotecan 	Response rates are much lower than longer administrations of topotecan in platinum sensitive patients.	(Markman, Blessing et al. 2000)
GOG-146H	<ul style="list-style-type: none"> • IV byrostatin 	Inactive as a single agent in recurrent or persistent platinum-sensitive cancer.	(Armstrong, Blessing et al. 2003)
GOG-146J	<ul style="list-style-type: none"> • IV dolastatin-10 	Minimal activity in recurrent platinum-sensitive ovarian cancer.	(Hoffman, Blessing et al. 2003)
GOG-146K	<ul style="list-style-type: none"> • IV topotecan for 3 days 	Well tolerated but less response than 5 day infusion.	(Miller, Blessing et al. 2003)
GOG-146L	<ul style="list-style-type: none"> • IV capecitabine 	Trial closed after first stage of accrual due to low response rate in patients with platinum-sensitive cancer.	(Miller, Blessing et al. 2005)
GOG-146M	<ul style="list-style-type: none"> • IV tirapazamine followed by IV cisplatin 	Combination of tirapazamine followed by cisplatin showed robust response (55%) and an increase in OS from 10.9 months to 26.4 months. However, tirapazamine was associated with significant toxicity.	(Covens, Blessing et al. 2006)

Trial	Treatments	Outcome	Reference
GOG-146N	<ul style="list-style-type: none"> • IV bortezomib 	Bortezomib as a single agent in recurrent, platinum-sensitive disease had an objective response rate of 3.8%.	(Aghajanian, Blessing et al. 2009)
GOG-146O	<ul style="list-style-type: none"> • IV irifulven 	Irofulven had modest activity in patients with recurrent ovarian cancer with 12.7% partial responders and 54.6% stable disease.	(Schilder, Blessing et al. 2010)
GOG-146P	<ul style="list-style-type: none"> • IV Cetuximab and carboplatin 	Modest activity of cetuximab in combination with carboplatin in recurrent ovarian cancer patients.	(Secord, Blessing et al. 2008)
GOG-146Q	<ul style="list-style-type: none"> • IV topotecan every 5 days • IV topotecan every 7 days 	Topotecan showed limited activity in both the every 5 day (27%) and every 7 days (12%) in patients with recurrent ovarian cancer.	(Herzog, Sill et al. 2011)
GOG-152	<ul style="list-style-type: none"> • Chemotherapy alone • Secondary cytoreductive surgery following chemotherapy 	No survival benefit to secondary surgery, but residual disease does predict PFS and OS.	(Rose, Nerenstone et al. 2004, Rose, Java et al. 2016)
GOG-157	<ul style="list-style-type: none"> • Three cycles of adjuvant carboplatin and paclitaxel • Six cycles of adjuvant carboplatin and paclitaxel 	No change in recurrence rate between three or six cycles.	(Bell, Brady et al. 2006)
GOG-158	<ul style="list-style-type: none"> • IV cisplatin and paclitaxel • IV carboplatin and paclitaxel 	Carboplatin and paclitaxel were not inferior to cisplatin and paclitaxel. Less toxicity in the carboplatin arm	(Ozols, Bundy et al. 2003)
GOG-160	<ul style="list-style-type: none"> • IV trastuzumab 	Low rate of response in recurrent or refractory ovarian cancer.	(Bookman, Darcy et al. 2003)
GOG-162	<ul style="list-style-type: none"> • IV cisplatin and IV paclitaxel for 24 hours • IV cisplatin and IV paclitaxel for 96 hours 	No significant change in response for prolonged infusion of paclitaxel for 96 hours.	(Spriggs, Brady et al. 2007)
GOG-170C	<ul style="list-style-type: none"> • oral gefitinib 	Gefitinib had limited clinical activity across recurrent ovarian cancer patients. Prescreening for patients with mutant EGFR may improve activity.	(Posadas, Liel et al. 2007) (Schilder, Sill et al. 2005)
GOG-170F	<ul style="list-style-type: none"> • Oral sorafenib 	Sorafenib had modest activity in recurrent but was associated with substantial toxicity.	(Matei, Sill et al. 2011)
GOG-170G and GOG-229D	<ul style="list-style-type: none"> • Oral Lapatanib 	Lapatanib has minimal activity in recurrent ovarian cancer patients but may have benefits to patients with mutant EGFR (E690K).	(Garcia, Sill et al. 2012) (Leslie, Sill et al. 2012)
GOG-170I	<ul style="list-style-type: none"> • IV Temsirolimus 	Temsirolimus showed modest activity below the PFS threshold for advancement in recurrent ovarian cancer patients. Could be considered for selection based on cyclin D 1.	(Behbakht, Sill et al. 2011)
GOG-170J	<ul style="list-style-type: none"> • Oral enzastaurin 	Enzastaurin had no effect across recurrent ovarian cancer patients.	(Usha, Sill et al. 2011)
GOG-170K	<ul style="list-style-type: none"> • Oral mifepristone 	In patients with recurrent ovarian cancer mifepristone had a response rate of 4.5%.	(Rocereto, Brady et al. 2010)

Trial	Treatments	Outcome	Reference
GOG-170L	<ul style="list-style-type: none"> • Oral Motesanib 	High central nervous system toxicity in recurrent ovarian cancer patients.	(Schilder, Sill et al. 2013)
GOG-170N	<ul style="list-style-type: none"> • Twice daily subcutaneous urokinase-derived peptide (A6) 	No responses were observed in patients with persistent or recurrent ovarian cancer.	(Gold, Brady et al. 2012)
GOG-170P	<ul style="list-style-type: none"> • IV rilotumumab every 14 days 	Limited activity of rilotumumab in recurrent ovarian cancer patients (3.2% complete response and 6.5% 6-month PFS).	(Martin, Sill et al. 2014)
GOG-170Q	<ul style="list-style-type: none"> • IP EGEN-001, an IL-12 plasmid, weekly 	EGEN-001 showed limited activity with 35% stable disease and 30% had a PFS greater than 6 months.	(Alvarez, Sill et al. 2014)
GOG-172	<ul style="list-style-type: none"> • IV cisplatin and paclitaxel • IP cisplatin and paclitaxel and IV paclitaxel 	Increase in OS in the IP paclitaxel arm (65.6 months vs 49.7 months). However, only 42% of IP patients completed the study due to side effects and complications.	(Omura, Blessing et al. 1986) (Armstrong, Bundy et al. 2006)
GOG-175	<ul style="list-style-type: none"> • IV carboplatin and IV paclitaxel for 3 courses followed by weekly paclitaxel • Observation 	Maintenance paclitaxel for 24 weeks showed no increase in recurrence-free interval.	(Mannel, Brady et al. 2011)
GOG-182 and ICON5	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • carboplatin, paclitaxel, and gemcitabine • Carboplatin, paclitaxel, and doxorubicin • carboplatin plus topotecan, then carboplatin plus paclitaxel • carboplatin plus gemcitabine, then carboplatin plus paclitaxel 	No improvement in PFS or OS following the addition of a third cytotoxic agent.	(Bookman, Brady et al. 2009)
GOG-186C	<ul style="list-style-type: none"> • IV Paclitaxel pliglumex 	PPX showed modest activity as a second or third line agent in recurrent ovarian cancer with a response rate of 58% with either partial response or stable disease.	(Sabbatini, Sill et al. 2008)
GOG-186D	<ul style="list-style-type: none"> • IV karenitecin 	12% response rate in recurrent disease.	(Kavanagh, Sill et al. 2008)
GOG-186F	<ul style="list-style-type: none"> • IV docetaxel followed by trabectedin with filgrastim, pegfilgrastim or saragramostim every 3 weeks 	Combination is well tolerated and showed an increase in PFS (4.5 months) and OS (16.9 months) compared to single agent taxane in recurrent ovarian cancer.	(Monk, Sill et al. 2011)
GOG-198	<ul style="list-style-type: none"> • Oral thalidomide • Oral tamoxifen 	There was no difference in response between thalidomide and tamoxifen in delaying the recurrence of ovarian cancer.	(Hurteau, Brady et al. 2010)
GOG-239	<ul style="list-style-type: none"> • Oral selumetinib twice daily until progression 	Selumetinib treatment in recurrent low-grade carcinoma showed 15% objective response, 65% stable disease, and 1.9% complete response.	(Farley, Brady et al. 2013)
GOG-252	<ul style="list-style-type: none"> • IP cisplatin, and IV and IP paclitaxel and bevacizumab • IP carboplatin, and IV weekly paclitaxel and bevacizumab • IV carboplatin, and IV weekly paclitaxel and bevacizumab 	No difference in PFS between study arms and higher rates of hypertension, nausea and vomiting in the IP cisplatin group than in the other study arms	(Walker, Brady et al. 2016)

Trial	Treatments	Outcome	Reference
GOG-262	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel every 21 days with or without bevacizumab • IV carboplatin every 21 days and IV weekly paclitaxel with or without dose-dense bevacizumab 	Increase in PFS in patients receiving dose-dense treatment versus those receiving dosing every 21 days who were not given bevacizumab.	(Chan, Brady et al. 2016)
GOG-26C	<ul style="list-style-type: none"> • IV Cisplatin 	Overall response rate of 20% showed the first evidence of cisplatin against ovarian cancer.	(Tate Thigpen, Blessing et al. 2004)
GOG-26C	<ul style="list-style-type: none"> • IV Taxol every 3 weeks 	The overall response rate was 37% for patients with either platinum sensitive or resistant ovarian cancer.	(Thigpen, Blessing et al. 1994)
GOG-3	<ul style="list-style-type: none"> • Melphalan • Melphalan plus 5-fluorouracil • Melphalan and 5-fluorouracil plus dactinomycin • fluorouracil and dactinomycin plus cytoxan 	Melphalan alone was not improved by the addition of 5-fluorouracil or dactinomycin.	(Park, Blom et al. 1980)
GOG-95 and SWOG-9047	<ul style="list-style-type: none"> • Adjuvant IP radioactive chromic phosphate • Adjuvant cyclophosphamide and cisplatin 	No significant differences in survival and lower recurrence in cisplatin treated patients.	(Young, Brady et al. 2003)
GOG-9919	<ul style="list-style-type: none"> • Dose dense carboplatin/ paclitaxel for six cycles 	While the overall response rate was high (58%) severe neuropathy and thrombocytopenia limited further development.	(Tiersten, Sill et al. 2010)
ICON4/AGO-OVAR-2.2	<ul style="list-style-type: none"> • Carboplatin • Carboplatin and paclitaxel 	Superior PFS and OS in patients with platinum sensitive ovarian cancer with the addition of paclitaxel	(Parmar, Ledermann et al. 2003)
ICON6	<ul style="list-style-type: none"> • Platinum-based chemotherapy and cediranib with cediranib maintenance therapy • Platinum-based chemotherapy 	Significant improvement in PFS.	(Ledermann, Embleton et al. 2016)
ICON7	<ul style="list-style-type: none"> • Carboplatin, paclitaxel and bevacizumab with bevacizumab maintenance • Carboplatin and paclitaxel 	Increase in PFS with the addition of bevacizumab. But not OS benefit based on long term follow up.	(Perren, Swart et al. 2011) (Oza, Cook et al. 2015)
JGOG 3016	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel every 21 days • IV carboplatin every 21 days and dose-dense weekly paclitaxel 	28.0 month PFS for dose-dense paclitaxel compared to 17.2 month PFS in the standard of care. Dose dense paclitaxel showed higher patient drop out due to side effects.	(Katsumata, Yasuda et al. 2009, Katsumata, Yasuda et al. 2013)
NCT01847274	<ul style="list-style-type: none"> • Oral niraparib maintenance therapy for 28 days 	21.0 month PFS in BRCA germline mutant patients compared to 5.5 months is placebo. Survival increased was lower with treatment in non-gBRCA mutants (9.5 months vs 3.9 months).	(Mirza, Monk et al. 2016)
MITO-2	<ul style="list-style-type: none"> • Carboplatin and paclitaxel • Carboplatin and pegylated liposomal doxorubicin 	No difference in PFS or overall survival.	(Pignata, Scambia et al. 2011)
MITO-7	<ul style="list-style-type: none"> • Weekly carboplatin and weekly paclitaxel • Carboplatin and paclitaxel every 3 weeks 	No Difference in PFS or OS survival.	(Pignata, Scambia et al. 2014)

Trial	Treatments	Outcome	Reference
MITO-11	<ul style="list-style-type: none"> • IV paclitaxel • IV paclitaxel plus oral pazopanib daily 	In patients with recurrent or refractory ovarian cancer pazopanib prolonged PFS to 6.35 months compared to 3.49 months.	(Pignata, Lorusso et al. 2015)
NCT00753545	<ul style="list-style-type: none"> • Platinum-based chemotherapy • Platinum-based chemotherapy with olaparib maintenance therapy 	Significant increase in PFS with olaparib maintenance.	(Ledermann, Harter et al. 2012)
NCT00866697	<ul style="list-style-type: none"> • IV platinum and taxane chemotherapy followed by placebo maintenance • IV platinum and taxane chemotherapy followed by pazopanib maintenance 	Improved PFS with the addition of pazopanib but no difference in overall survival between groups	(du Bois, Floquet et al. 2014)
NCT01081951	<ul style="list-style-type: none"> • Carboplatin and paclitaxel • Carboplatin and paclitaxel with olaparib maintenance therapy 	Increase in PFS with olaparib maintenance therapy.	(Oza, Cibula et al. 2015)
NCT01116648	<ul style="list-style-type: none"> • Olaparib • Olaparib and cediranib 	Increase in PFS when cediranib is added to olaparib.	(Liu, Barry et al. 2014)
OCEANS	<ul style="list-style-type: none"> • Carboplatin and gemcitabine • Carboplatin, gemcitabine and bevacizumab with bevacizumab maintenance therapy 	Increased PFS with the addition of bevacizumab.	(Aghajanian, Blank et al. 2012)
S0009	<ul style="list-style-type: none"> • Neoadjuvant IV paclitaxel and carboplatin 	Neoadjuvant therapy of cisplatin and paclitaxel in bulky disease had a PFS of 29 months and an OS of 34 months.	(Tiersten, Liu et al. 2009)
S0200	<ul style="list-style-type: none"> • IV carboplatin and pegylated liposomal doxorubicin on a 4 week schedule 	Combination showed an improvement in PFS but not OS after long term follow up.	(Alberts, Liu et al. 2008, Markman, Moon et al. 2010)
S0904	<ul style="list-style-type: none"> • IV docetaxel • IV docetaxel plus oral vandetanib 	Combination of docetaxel with vandetanib in recurrent ovarian cancer patients did not prolong PFS compared to docetaxel alone.	(Coleman, Moon et al. 2014)
S9618	<ul style="list-style-type: none"> • Treatment regimen of carboplatin and paclitaxel based on CA-125 serum levels 	No improvement in 2-year survival based on historical control.	(Markman, Glass et al. 2003)
S9619	<ul style="list-style-type: none"> • Combined IV cisplatin and paclitaxel with IP paclitaxel over 21 day cycles for 6 cycles 	In 68 women with optimally debulked ovarian cancer the combination of IV and IP chemotherapy showed a 91% 2-year survival rate and a median survival of 51 months.	(Rothenberg, Liu et al. 2003)
S9912	<ul style="list-style-type: none"> • IV pegylated liposomal doxorubicin with IP cisplatin and paclitaxel and IP paclitaxel 	Liposomal doxorubicin did not improve PFS (25 months) with a median survival of 51 months.	(Smith, Moon et al. 2009)
SCOTROC	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • IV carboplatin and docetaxel 	Similar PFS. Docetaxel is associated with greater neutropenia while paclitaxel is more likely to induce sensory peripheral neuropathy	(Vasey, Jayson et al. 2004)

Trial	Treatments	Outcome	Reference
SWOG-8412	<ul style="list-style-type: none"> • IV cisplatin plus IV cyclophosphamide • IV carboplatin plus cyclophosphamide 	Carboplatin plus cyclophosphamide showed a significantly better therapeutic index than cisplatin plus cyclophosphamide with similar response rates (61 and 52% respectively) and lower toxicity in the carboplatin arm.	(Alberts, Green et al. 1992)
SWOG-8501	<ul style="list-style-type: none"> • IV cisplatin plus cyclophosphamide • IP cisplatin plus cyclophosphamide 	The median survival was longer in the IP cisplatin group (49 months) than the IV cisplatin (41 months) and has fewer side effects.	(Alberts, Liu et al. 1996)
SWOG-8835	<ul style="list-style-type: none"> • IP mitoxantrone • IP floxuridine 	Floxuridine treatment showed a PFS exceeding 15% at 1 year with less toxicity in ovarian cancer patients with minimal residual disease.	(Muggia, Liu et al. 1996)
SWOG-9106	<ul style="list-style-type: none"> • CMC- Carboplatin (1500 mg/m²), mitoxantrone (75 mg/m²), and cyclophosphamide (120 mg/m²) • CTC- Cisplatin (165 mg/m²), thiotepa (600 mg/m²), and cyclophosphamide (5625mg/m²) with stem cell rescue 	CMC was the favorable treatment when compared to CTC when comparing OS (29 months to 22months) and PFS (13 months and 8 months).	(Stiff, Shpall et al. 2004)
SWOG-9324	<ul style="list-style-type: none"> • IV vinorelbine 	Vinorelbine 6-month survival rate for women with recurrent ovarian cancer was 65% with a median survival of 10.1 months.	(Rothenberg, Liu et al. 2004)
SWOG-9326	<ul style="list-style-type: none"> • Oral alteramine in four dosages over 14 days every 28 days 	Alteramine treatment in patients in complete clinical remission showed a 2-year survival rate of 75%.	(Rothenberg, Liu et al. 2001)
SWOG-9701 and GOG-178	<ul style="list-style-type: none"> • IV carboplatin and paclitaxel • IV carboplatin and paclitaxel followed by 12 months of paclitaxel 	Significant increase in PFS following 12 months of paclitaxel.	(Markman, Liu et al. 2009) (Markman, Liu et al. 2003)

Chapter 2: Exploiting Three-Dimensional Cell Culture to Better Replicate Clinical Drug Resistance in Ovarian Cancer Cell Lines

Introduction

3D Culture History

Since the early 1900s the ability of scientists to isolate and grow cells from humans in the laboratory has led to incredible and significant scientific breakthroughs. One of the most commonly used cell lines (HeLa) was developed from Henrietta Lacks' cervical cancer sample in the early 1950s (Scherer, Syverton et al. 1953). To this day, HeLa cells are still used in cell biology research. Traditionally cells were grown on flat surfaces such as glass cover slips or plastic petri dishes where they were able to attach to and propagate. However, the phenotype of these cells often did not represent the structure or function of the tissue or organ from which they were isolated since their growth was limited to a two-dimensional (2D) surface. The use of improved cell culture methods to better replicate three-dimensional (3D) biological structures have been in demand since the 1970s. It was discovered that using non-adherent cell culture conditions (also known as 3D cell culture) such as agarose coated cell culture plates led to cell aggregation and changes in morphology (Yuhas, Li et al. 1977). 3D culture of mammary epithelial cells induced morphological differentiation and induced secretory function that was maintained for up to one month *in vitro* (Emerman and Pitelka 1977). Using collagen overlay of Madin-Darby canine kidney (MDCK) and normal murine mammary gland (NMuMG) cell lines formed lumens that more resembled tissues of origin for each cell culture (Hall, Farson et al. 1982). The use of 3D culture has even led to cell-based engineering of functional organoids capable of animal implantation (Toda, Watanabe et al. 2002). The use of 3D cell culture with cancer cell lines forms solid, multicellular structures known as multicellular-tumor spheroids (MCTS) (Durand and Sutherland 1972, Sutherland and Durand 1976). The primary objective of this chapter will be to use 3D cell culture to better replicate clinical drug response in ovarian cancer cell lines that can be exploited for downstream drug discovery and development.

3D Culture Methods

The basis of 3D culture needs only two elements: a surface on which the cells cannot attach and a way of bringing cells into close proximity to enhance cell-to-cell contact. There are many different methods for 3D cell culture that include agarose coated or round-bottom non-adherent plates that bring cells together on a concave surface (Yuhas, Li et al. 1977, Friedrich, Seidel et al. 2009, Hribar, Finlay et al. 2015); a hanging drop culture method that uses small, suspended droplets in which gravity pulls the cells together at the bottom of each drop (Timmins, Harding et al. 2005, Timmins and Nielsen 2007); a magnetic levitation method that elevates cells above the culture dish within a condensed magnetic field (Lin, Chu et al. 2008, Guo, Loh et al. 2014); and a rotating cell culture method that uses centripetal force to bring cells together and prevents them from attaching to the moving surface (Granet, Laroche et al. 1998, Jessup, Frantz et al. 2000, Rhee, Zhou et al. 2001). Derivations of these methods using biological scaffolds such as solid or semi-solid extracellular matrix (ECM) can aid formation of 3D cultures (Chevallay and Herbage 2000, Carletti, Motta et al. 2011). The type of scaffold can change cell behavior and differentiation based on physical and chemical properties of the scaffold (Zheng, Yang et al. 2014, Tan, Fang et al. 2015). Recent advances in 3D printing allow for cells to be physically constructed in specific positions to generate organoids more similar to human tissue (Dai, Ma et al. 2016, Mandrycky, Wang et al. 2016, Vanderburgh, Sterling et al. 2016).

MCTS as a Model for Resistant Disease

One of the limiting factors in developing therapies for EOC is that in the majority of the studies the cell culture models don't recapitulate patient tumors. Standard 2D cell culture models have physiological limitations in modeling solid tumors. However, using non-adherent, three-dimensional (3D) cell culture of standard cell lines can induce the formation of MCTS. MCTS formation induces morphological, histological, and gene expression changes, which more accurately reflect both tumor xenografts and patient tumors compared to traditional 2D cultures (Lee, Mhawech-Fauceglia et al. 2013, Thoma, Zimmermann et al. 2014). Many of these

changes are driven by heterogeneous populations of cells that contribute to spheroid formation, including actively proliferating, quiescent, hypoxic, and inner necrotic cells (Sutherland, MacDonald et al. 1977, Freyer and Sutherland 1980). Furthermore, transition to MCTS alters extracellular matrix (ECM) remodeling, cell adhesion and metabolism which have all been associated with both drug resistance and tumor reoccurrence in EOC (Huang, Ao et al. 2010, Steg, Bevis et al. 2012, Chien, Kuang et al. 2013). MCTS formation in the patient ascites is also thought to be a critical mechanism for EOC growth and dissemination in advanced disease (Burlison, Casey et al. 2004, Shield, Ackland MI Fau - Ahmed et al.). 3D culture can provide an intermediate method between the rapid, low cost 2D culture models, and the slower, more biologically relevant *in vivo* models (**Figure 6**).

MCTS formation and growth are associated with many drug resistance mechanisms. Large MCTS, with a diameter greater than 300 μm , exhibit many morphological, signaling and growth changes. As with large solid tumors, many drugs cannot penetrate the spheroid, lowering their effective dosage (Minchinton and Tannock 2006). Lower molecular weight, hydrophobic drugs have been shown to enhance spheroid penetration (Minchinton and Tannock 2006, Fayad, Rickardson et al. 2011). However, MCTS drug resistance is not just attributed to decreased drug availability. A myxoma-virus model showed spheroid cells have intrinsic resistance to apoptosis through signaling pathways acquired upon aggregation and not reflected in 2D cultures (Correa, Komar et al. 2012). Limited nutrient and oxygen diffusion at the spheroid center creates gradients in cell proliferation, hypoxia, and necrosis across the MCTS (Sutherland and Durand 1984). Metabolic changes, cellular senescence, hypoxia, and alterations in other signaling pathways have been identified as potential critical modulators of drug and radiation therapy resistance in different MCTS (Sutherland and Durand 1976, Graham, Kobayashi et al. 1994, Desoize and Jardillier 2000). Using MCTS in frontline drug screening may identify drugs that can circumvent resistance mechanisms and inhibit tumor growth.

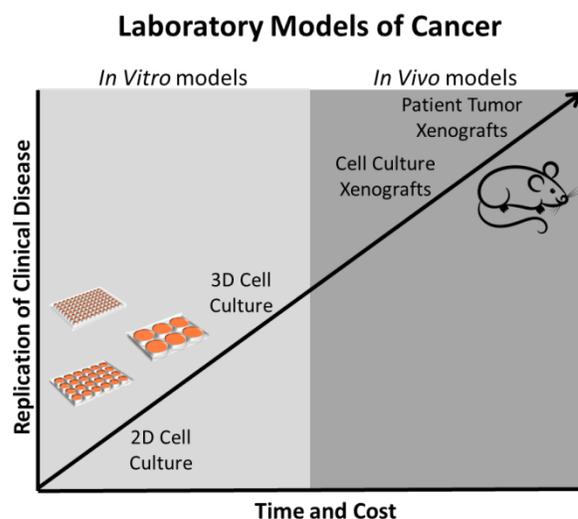


Figure 6: Laboratory Models of Cancer

Using 3D Culture to Enhance Drug Development

“The valley of death” has been used to describe the poor success rate of cancer drug development (Adams 2012). Only one out of every 1,000 drug candidates enter into a clinical trial and most of these agents fail to move into Phase III studies due to a lack of efficacy (Marchetti and Schellens 2007, Zaenker and Entschladen 2009). The use of MCTS as a model for improved drug development was proposed over 10 years ago (Abbott 2003, Kunz-Schughart, Freyer et al. 2004) and recent advances in screening methods have shown 2D drug screening favors the selection of mitotic inhibitors while 3D screening has identified inhibitors of mitochondrial function that can block hypoxia-resistant and stem-like cell mitochondrial metabolism (Fayad, Rickardson et al. 2011, Pasto, Bellio et al. 2014, Wenzel, Riefke et al. 2014, Zhang, Fryknas et al. 2014). However, the paradigm shift for using MCTS in drug screening has been slower than predicted. Methods to reliably produce MCTS and to measure spheroid viability have been major limiting factors in the routine use of 3D culture in drug screening. 3D culture systems require additional time and effort to plate and grow spheroids as compared to traditional 2D culture models (**Figure 6**). Without improvements in culturing methods and evidence that using tumor spheroid models are superior in identifying

unappreciated anti-tumor drugs compared to 2D cultures, the use of MCTS in drug screening will remain limited. While 3D drug screening has been shown to identify unappreciated drugs and predict *in vivo* efficacy in ovarian cancer cells, this model focused on the attachment of single cells to omentum cells, more representative of early stage disease (Kenny, Lal-Nag et al. 2015). The use of alternative MCTS models that represent late stage disease is needed to develop drugs for patients whose prognosis is substantially worse than those diagnosed at an early stage. The focus of this chapter will be to develop a 3D cell culture model that reflects aspects of drug resistant ovarian cancer that cannot be appreciated using traditional 2D cultures and that can be easily and reproducibly adapted for further drug screening.

Methods

Cell Culture

Ovarian cancer cell lines used in these studies were previously described in detail (Godwin, Meister et al. 1992, Yao, Godwin et al. 1995, Pathak, Zhou et al. 2015). All of the cell lines were maintained in normal growth media consisting of RPMI 1640 media supplemented with fetal bovine serum (FBS) (10% (vol/vol)), insulin (7.5 µg/mL), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere with 5% CO₂. Immortalized and non-tumorigenic human ovarian surface epithelial (HOSE) cells, immortalized via SV40 large T antigen (Capo-Chichi, Smith et al. 2002, Roland, Yang et al. 2003), were cultured in medium 199 and MCDB 105 (1:1) supplemented with FBS (15% (vol/vol)), insulin (0.25 U/mL), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL).

Spheroid Formation

Spheroids were formed using the liquid-overlay method with agarose coated 96-well plates, modified from a previous publication (Friedrich, Seidel C Fau - Ebner et al. 2009). Briefly, 1.5% agarose was dissolved in RPMI 1640 media and then used to coat the bottom of 96-well flat bottom plates with 50 µL of the agarose solution. Agarose was allowed to solidify for a minimum of 30 minutes and then 3,000 cells were plated in 50 µL using standard cell culture media. These plated cells were then allowed to form spheroids undisturbed for four days. On day 4 spheroids were visually inspected and 50 µL of fresh, standard media was supplemented to each well (**Figure 7**).

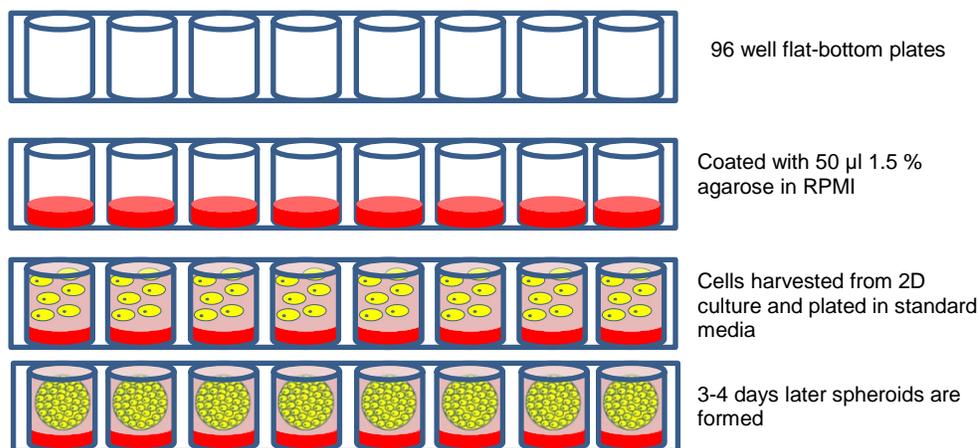


Figure 7: 3D Culture for the Formation of Ovarian Cancer Spheroids

***In Vivo* Tumorigenicity**

All procedures involving mice were approved by the University of Kansas Medical Center (KUMC) Institutional Animal Care and Use Committee (IACUC). Female 5 to 6 week-old C.B-17 severe combined immunodeficient (SCID) mice (Harlan) were used for this study. Cell lines including the tumorigenic A1847 cell line and the non-tumorigenic cell lines HIO80, HIO107, HIO114, HIO117, and HIO120 were grown to ~80% confluency in conditions previously described. Mice were randomized by weight and 1×10^6 cells were suspended in 500 µL phosphate-buffered saline and injected intraperitoneally into three mice per cell line. Health checks were performed twice a week to evaluate any signs of distress or a body weight decrease of greater than 10%. If either of the above symptoms occurred, or six months from date of injection passed, exploratory necropsies were performed to evaluate internal tumorigenesis and/or ascites formation. All tumors were collected for both RNA extraction and formalin-fixation followed by hematoxylin & eosin (H&E) stain. Images were taken using a Nikon Eclipse 80i microscope with MetaMorph 7.7 (Molecular Devices).

Secondary Spheroid Assays

For secondary assays, spheroids were removed from each well using wide bore pipette tips and pooled together from 96 wells for each single treatment. Spheroids were centrifuged at 2,000 RPM for 5 minutes at 4°C to pellet, the media was removed, and the spheroids washed with

sterile PBS. Following another spin and removal of PBS, spheroids were dissociated in a 1:1 (vol/vol) combination of trypsin and Accutase (10mL) for 30 minutes at 37°C, with mild shaking every 3 to 10 minutes. Disassociated cells were diluted in complete media and spun at 2,000 RPM for 5 minutes at 4°C, rinsed with sterile PBS, spun again, and suspended in standard cell culture media. Cells were then plated overnight on a 10 cm cell culture dish. Following, overnight seeding, viable cells were counted for subsequent assays following standard protocols.

Cell Cycle Analysis

Cells were grown as 2D cultures in 6 cm dishes (2×10^5 cells/dish) for 24 h, 48 h, or 72 h or as 3D culture spheroids as described above for 4 days and 7 days. Cells were collected from 2D cultures by trypsinization and from spheroids using trypsin/Accutase treatment as described above. Cells were immediately fixed using 70% ice-cold ethanol and stored overnight at -20°C before cell cycle analysis using propidium iodide staining (Guava Cell Cycle reagent, EMD Millipore) following the manufacturer's established protocol. The cell cycle assays were performed three independent times with two technical replicates for each sample. A Guava EasyCyte HT instrument (EMD Millipore) was used to measure the changes in cell cycle distribution of the cells.

Immunofluorescence

Sections (4 μ m) from formalin-fixed, paraffin-embedded (FFPE) cell line spheroids were made for immunofluorescence (IF) staining. After deparaffinization and rehydration, tissue sections were treated using citrate buffer (pH 6.0) for antigen retrieval. Then, sections were blocked with 1% normal goat serum for 1 h, and then incubated with Ki67 rabbit mAB (1:1,000, Cell Signaling Tech) in a moist chamber overnight at 4 °C. On the following day, the cells were washed with PBS and then incubated with secondary antibody (goat anti-rabbit IgG, DyLight 594, Thermo Scientific) for 1 h at room temperature. After several washes, the coverslips containing cells

were mounted onto glass slides in VECTASHIELD® mounting medium with DAPI (Vector Labs, Inc.). Pimonidazole staining was performed following the manufacture's protocol (Hypoxyprobe). Briefly, spheroids were incubated for 2 h with 200 µM pimonidazole from Hypoxyprobe-1 and then fixed for 30 minutes in formalin and sectioned as described above. Primary mouse antibody (Hypoxyprobe-1 Mab1; 1:100, HPI) was incubated overnight at 4°C followed by secondary antibody (FITC-Mab 4.3.11.3, HPI) for 1 h at room temperature. Fluorescence digital images were captured using a Nikon Eclipse 80i microscope attached with a Nikon Q-imaging camera adaptor. MetaMorph Image Analysis software (version 7.7.0.0) was used to acquire and analyze images.

Western Blot Analysis

All lysates were extracted using RIPA buffer supplemented with protease inhibitors (Roche Molecular Biochemicals) and phosphatase inhibitors (Fisher Scientific). 2D culture lysates were prepared by washing cells with cold PBS, scraped in lysis buffer and incubated for 10 min on ice. 3D culture lysates were collected by combining spheroids from one 96-well plate, spheroids were centrifuged at 2,000 RPM for 5 min at 4°C to pellet, washed with cold PBS, spun again, suspended in lysis buffer for 30 minutes on ice, and then briefly sonicated. Lysates were spun at 14,000 RMP for 10 min at 4°C and the supernatant was collected for quantification and western blots. Protein concentration was measured using the *DC* Protein Assay (Bio Rad) following manufacture's protocol. For each sample, 30 µg of whole-cell extract was electrophoresed on a 4-20% precast gradient polyacrylamide gel (Bio-Rad) and transferred onto nitrocellulose membranes using the Trans-Blot Turbo (Bio-Rad). After blocking with 5% skim milk (Difco), membranes were incubated overnight at 4°C with primary antibodies, HIF1-α (1:1,000, Cell Signaling) and PARP (1:500, Cell Signaling). After incubation with HRP-conjugated secondary antibody (1:1,000) at room temperature, development was carried out using chemiluminescence substrate (Pierce). Pixel densities of blot images were calculated

using Image-J software (NIH). Changes in protein levels were normalized to loading controls and expressed as fold change relative to treatment controls.

RT-PCR and RNASeq Analysis

RNA was isolated using Trizol and Phase Lock Gel Heavy tubes (5 Prime) followed by RNeasy Mini Kits (Qiagen) following manufacture's protocols. RNA quality (260:280 ratio > 1.8) and quantity was assessed using the Infinite 200Pro (Tecan). For TaqMan qRT-PCR, 1 µg of RNA of subjected to reverse transcription using random primers, 5X first strand buffer, 0.1 M DTT, 10 mM dNTPs, and SuperScript III (ThermoFisher) following manufacture's protocol. For amplification, 10 µL of TaqMan Gene Expression Master Mix (2x) (AppliedBiosystems) was combined with 2 µL of diluted cDNA (1:2), 7 µL of sterile water, and 1 µL of TaqMan primers (*PROM1*, *NANOG*, *POU5F1*, *SOX2*, *CD44*, *KLF4*, *ALDH1A*, *LOX*, *JMJD1A*, *VEGF*, *PPIA*, *BACTIN*, and *GAPDH*). Amplification was performed by initial polymerase activation for 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 10 minutes on the Bio-Rad CFX96 (BioRad). The fluorescence threshold value was calculated using the CFX96 real-time system software (BioRad). The relative change in mRNA levels was measured by the delta-delta method normalizing to the geometric mean of all three housekeeping genes (*PPIA*, *BACTIN*, and *GAPDH*).

For transcriptome sequencing, RNA was isolated from 2D cultures at 80% confluency, from 3D cultures at 7 days of spheroid growth, and from A1847 xenograft tumor tissue samples harvested at terminal necropsy. RNA was prepared for paired end sequencing on a HiSeq 2500 using a stranded library prep kit (Illumina). Initial analyses were prepared using RSEM expected gene counts. First, data was filtered to remove non/low-expressed genes. This resulted in a total of ~14,000 genes examined for differential expression between the different models of cell growth. Next, normalization factors were calculated to scale the library sizes followed by estimation of tag wise negative binomial dispersion values. Genes were then

compared that were 1.5-fold upregulated or down regulated in either 3D cultures or tumor xenografts compared to 2D cultures.

Colony Forming Assay

For soft agarose colony forming assays, 500 viable cells were counted and plated into 6-well plates in 0.6% agarose in RPMI 1640 with fetal bovine serum (10% (vol/vol)), insulin (7.5 µg/mL), penicillin (100 U/mL), and streptomycin (100 µg/mL), on top of a base of 1.4% agarose in the same media conditions. Colonies grew for 2 weeks at 37 °C in a humidified atmosphere with 5% CO₂. Colonies were stained with crystal violet and imaged using the Alpha View Imager (Alpha Innotech Corporations) for colony counting. Colony diameter was measured using 10 random images from each well using the Micromaster (Fisher Scientific) and colony diameter was measured for each colony 3 times using Micron Software (1.09, Westover Scientific). Images were taken from biological triplicates and used for quantification and statistics.

Results

Formation of Multicellular Tumor Spheroids in Established Ovarian Cancer Cell Lines Using Agarose Coated Plates

To form MCTS from ovarian cancer cell lines, 3D culture models need two basic properties: (1) to prevent cell adherence to the culture plate and (2) to bring cells together in a restricted space to increase cell-cell contact and limit spheroid size (**Figure 8A**). Ovarian cancer cell lines adhered to standard low-attachment plates in round bottom 96-well plates that would have limited the size of spheroids (**Figure 8B**). However, soft agar coated 10 cm tissue culture dishes prevented cells from attaching to the surface of the plate but do not restrict the area of growth to form consistent spheroids (**Figure 8C**). Likewise, soft agar allowed the cells to invade into the media, making spheroid recovery for secondary assays burdensome. These data support the need to develop a 96-well plate format that both prevents cell adherence to the culture plate and bring cells together in a restricted area.

In order to develop more relevant *in vitro* models for drug screening, qualitative comparisons of a variety of ovarian cancer cell line-based 3D culture methods were performed. These methods were selected based on using a 96-well plates and included agarose coated plates, hanging drop cultures, ultra-low attachment (ULA) plates, and magnetic levitation cultures. Protocols were compared for liquid handling, the use of artificial or biological reagents, cost, reproducibility, and if spheroids needed to be transferred after formation. Methods low in cost and most similar to handling 2D cultures provided the best system for directly comparing cellular differences between cultures. Based on the minimal use of artificial reagents (inert agarose), low cost, single spheroids formed per well, and minimal spheroid transfer, the agarose coated-liquid over-lay method for 3D culturing of ovarian cancer cells (**Table 4**) was selected for subsequent experimentation. The agarose coated 96-well plates were established based on a previously described method (Friedrich, Seidel C Fau - Ebner et al. 2009). Twelve

ovarian cancer cell lines were cultured under these conditions in standard growth media and spheroid formation was measured after four and seven days using light microscopy. Tight spheroids were formed after four days in the majority of cell lines (A1847, SKOV3, OVCAR3, OVCAR8, C30, PEO1, OVCAR5, OVCAR10, and OVCAR4) while three of the cell lines formed loose aggregates that dissociated during pipetting (UPN275, CP70, and A2780) (**Figure 9**). The agarose coated plates prevented cell adherence to the surface, formed on spheroid per well of consistent size, and prevented spheroids from invading the solid surface for future biological assays. This method provided an in-well 3D culture protocol that did not require transferring spheroids or specialized media that can be developed for studying ovarian cancer drug resistance.

MCTS Formation Induced Multiple Cellular Changes Associated with Solid Tumors and Drug Resistance

To evaluate the phenotypes associated with spheroid formation (MCTS), cellular changes were measured using a variety of biological assays in 2D and 3D cultured cells. The most notable changes associated with 3D culturing included reduced cell proliferation, induction of cellular hypoxia, and induction of stem-like gene expression and function.

Cell proliferation in MCTS was measured using immunofluorescence (IF) staining and cell cycle analysis of spheroid sections and 2D cultures of A1847 cells. Staining for the proliferation marker Ki67 showed a gradient of positive cells on the spheroid surface and Ki67 negative cells in the inner mass of the spheroid (**Figure 10A**). However, near ubiquitous positive staining of Ki67 was observed in 2D cultures (**Figure 10A**). Cell cycle analysis of single cells disassociated from A1847 spheroids showed an increased accumulation of cells in G1 phase (58% at day 4 and 53% at day 7) as compared to cells grown in 2D cultures (40%, 45%, and 34% at 24 h, 48 h and 72 h, respectively) (**Figure 10B**). These data indicate lower proliferation in A1847-derived MCTS.

The potential presence of cellular hypoxia was detected using pimonidazole staining in A1847 spheroid sections. Pimonidazole staining was positive in the inner spheroid mass of spheroids at day 4 (**Figure 11A**) and was not detected in any of the cells cultured in 2D (data not shown). To confirm cellular hypoxia, HIF-1 α stabilization was measured in A1847 cells in both 2D and 3D cultures using Western blot analysis. HIF-1 α is stabilized in A1847 cells when cultured under hypoxic conditions (1% oxygen) but not when cultured under atmospheric oxygen levels (**Figure 11B**). However, when cells are grown under 3D conditions, HIF-1 α is stabilized in A1847 spheroids at day 4 under atmospheric oxygen levels (**Figure 11B**). Gene expression analysis of hypoxia regulated genes (*JMJD1A*, *LOX*, *VEGF*) using qRT-PCR was performed across four ovarian cancer cell lines (A1847, OVCAR3, OVCAR4 and OVCAR8) and one non-tumorigenic ovarian surface epithelial cell line (HIO107) when grown under 2D and 3D culture conditions. Significant increases ($p < 0.05$, $n=3$, t-test) of the hypoxia-related genes were observed in the 3D cultured cells relative to the 2D cultured cells (**Figure 11C**).

In addition, expression of stem cell genes associated with drug resistance in ovarian cancer (*ALDH1A*, *CD133*, *CD44*) and traditional stem cell markers (*NANOG*, *OCT4*, *SOX2*) were measured in tumor cells from both 2D and 3D cultures. The expression of *ALDH1A*, *NANOG*, and *OCT4* was significantly increased ($p < 0.05$, $n=3$, t-test) in 3D cultures compared to 2D cultures across all four ovarian cancer cell lines (**Figure 12A**). The other stem cell markers (*CD133*, *CD44*, and *SOX2*) were significantly increased ($p < 0.05$, $n=3$, t-test) in A1847, OVCAR3, and OVCAR4 cell lines (**Figure 12A**). None of these stem cell markers were significantly increased in the HIO107 cell line spheroids, indicating the drug induction of stem-like properties may be specific to tumorigenic cells in propagated in 3D cultures. Finally, stem cell function was measured using a soft agar colony forming assay. Cells from 2D cultures and viable cells from dissociated spheroids of both A1847 and OVCAR8 cell lines were used for these assays. Cloning efficiency was increased in the A1847 and OVCAR8 spheroid-derived

cells compared to their 2D cultured cells as indicated by an increased number of colonies formed (**Figure 12B**). Taken together, these data suggest there is an increase in multiple cellular mechanisms associated with clinical drug resistance in 3D cultures when compared to 2D cultures in ovarian cancer cell lines.

MCTS Displayed Intermediate Gene Expression Between 2D Cultures and Tumor Xenografts

Similar to MCTS, a gradient of Ki67 positive staining was observed in A1847 mouse tumor xenografts and not in 2D cultures (**Figure 13A**). A comparison of expression levels for hypoxia related and stem-like genes indicated the 3D cultures of A1847 cells express an intermediate level between the 2D cultures and *in vivo* tumors (**Figure 13B-C**). Comparison of the gene expression data derived from RNA sequencing of 2D and 3D cultured cells and the mouse xenografts showed over half of the genes (148 out of 269) overexpressed by at least 1.5-fold in A1847 3D cultures compared to 2D cultures were also overexpressed in the A1847 tumor xenografts compared to 2D cultures (**Figure 13D**). These data support the hypothesis that 3D cultures might provide a better reflection of *in vivo* solid tumors than 2D cultures, while taking less time and money to develop than *in vivo* tumors.

Properties Specific to Tumorigenic Cell Lines

To test if the stem-like properties included in 3D cultures were specific to ovarian cancer cell lines, non-tumorigenic cell lines from the human ovarian surface epithelium were used. Primary HOSE cells were previously immortalized using SV40 large T antigen to derive HIO80, HIO107, HIO114, HIO118, and HIO120 (Auersperg, Maines-Bandiera et al. 1995, Dyck, Hamilton et al. 1996, Kruk, Godwin et al. 1999, Grobelny, Godwin et al. 2000, Capo-Chichi, Smith et al. 2002, Roland, Yang et al. 2003, Smith-Beckerman, Fung et al. 2005). The *in vivo* tumorigenicity of A1847 cell lines was compared to each HIO cell line implanted via Intraperitoneal (i.p.) injection. While the A1847 cell line formed solid tumors the HIO cell lines did not form detectable tumors

up to 25 weeks after implantation (**Figure 14A-D**). In 3D cultures, the HIO cell lines formed spheroids smaller than the A1847 cell lines and did not change in size from day 4 to day 7 (**Figure 15A**). Consequently, spheroids from the HIO cells were negative for pimonidazole, had almost ubiquitous expression of Ki67 (**Figure 15B**). Unlike that of ovarian MCTS, spheroids from HIO cells did not express higher levels of stem cell-related gene when compared to 2D cultures (**Figure 12A**). These data reveal that 3D culture of non-tumorigenic cell lines do not alter the phenotype in the same ways as tumorigenic cell lines, making the changes observed in ovarian cancer cell lines specific to their growth in 3D and not an artifact of the agarose plate method.

Reduced Sensitivity of Ovarian MCTS to Paclitaxel Compared to 2D Cultures

In order to determine differences in drug sensitivity between 2D and 3D cultured cells the dose response was measured for paclitaxel, one of the front-line therapy drugs for ovarian cancer. Cells were plated overnight before the addition of paclitaxel (from 3 μ M to 1.3 nM) to 2D cultured cells or for four days to 3D cultured cells in order to allow the spheroids to form. Cell viability to paclitaxel was measured 72 hours after drug treatment (**Figure 16A**). Viability measurements performed 3 days after the addition of drug, showed cells in spheroids to have a substantial decrease in drug sensitivity to paclitaxel compared to the parental 2D cultures (**Figure 16B**). The established IC_{50} values clearly show decrease in paclitaxel sensitivity in the MCTS compared to the 2D cultures (A1847, 168 nM vs 7 nM; OVCAR3, >3,000 nM vs 8 nM; OVCAR8, 97 nM vs 7 nM; OVCAR4, >3,000 nM vs < 1.3 nM). To measure drug resistance, the viability of cells surviving high dosages of paclitaxel (333 nM, 1 μ M, and 3 μ M) in MCTS compared to 2D cultures was measured using the average cell viability for each dosage. For each of the four cell lines there was a significant increase ($p < 0.05$, $n=9$, t-test) in the percent viability across the three highest dosages of paclitaxel in 3D cultures compared to 2D cultures: A1847 (50% 3D vs 16% 2D), OVCAR3 (60% 3D vs 40% 2D), OVCAR 4 (64% 3D vs 19% 2D),

and OVCAR8 (42% 3D vs 11% 2D) (**Figure 16B**). While it was previously shown that drug resistant pathways and mechanisms were upregulated in 3D cultures, these data directly support 3D cell culture as a direct model of drug resistance in ovarian cancer cell lines.

Paclitaxel Treatment Reduced Proliferation and Increased Stemness

The effect of paclitaxel treatment on expression of stem cell-related markers and proliferation was measured. In both patient tumor samples and *in vivo* PDX models there is a reported decrease in Ki67 levels and an increase in gene expression of stem cell markers following chemotherapy (Steg, Bevis et al. 2012, Dobbin, Katre et al. 2014). These phenotypes were evaluated in the MCTS model. Ki67 staining was evaluated in spheroids treated with vehicle (DMSO) or 1 μ M paclitaxel. A decrease in Ki67 positive cells (41% positive in the vehicle treated group compared to 24% positive in the paclitaxel treated group) was detected (**Figure 17A**). Additionally, ovarian MCTS showed a significant ($p < 0.05$, $n=3$, t-test) and robust increase in multiple stem cell markers by qRT-PCR following 1 μ M paclitaxel treatment in A1847 (*CD133*, *NANOG*, *OCT4*, and *SOX2*), OVCAR3 (*ALDH1A*, *CD133*, *KLF4*, and *SOX2*), OVCAR4 (*ALDH1A*, *CD133*, *SOX2*), and OVCAR8 (*CD133*, *ALDH1A*, and *KLF4*) (**Figure 17B**). These data support the hypothesis that the response to paclitaxel in ovarian MCTS mimic that of observed responses in ovarian cancer patients where the cells that survive and repopulate following chemotherapy are more stem-like as the disease progresses (Steg, Bevis et al. 2012, Dobbin, Katre et al. 2014). Modeling this phenomenon will provide a better understanding of disease progression and drug response, and potentially provide a platform for unappreciated drug screening to improve patient survival.

Paclitaxel Resistance and Stem-Like Phenotype was Maintained in 2D Cultures of MCTS Derived Cells

Drug resistance in MCTS can be associated with the lack of drug penetration to cells within the middle of the spheroids (Ong, Zhao et al. 2010). Maintaining the drug resistant phenotype in

cells isolated from MCTS and subsequently grown in 2D cultures would support cellular changes as opposed to limitations associated with physical properties of drug delivery in MCTS. To test if phenotypic changes in MCTS are contributing to the changes in paclitaxel response, MCTS were disassociated into single cells following either vehicle control or paclitaxel treatment for 3 days and viable cells were grown in 2D cultures overnight. The drug sensitivity of these MCTS-derived cells grown as a monolayer was then compared to the drug sensitivity of the parental 2D cultures (**Figure 18A**). Cells derived from untreated MCTS showed reduced sensitivity to paclitaxel compared to parental 2D cultures of both A1847 and OVCAR8 cells (**Figure 18B**, blue curves). Cells derived from paclitaxel treated MCTS showed an even greater decrease in paclitaxel sensitivity when plated into 2D cultures (**Figure 18B**, red curves). To support these changes in dosage response the IC_{50} from cells derived from untreated MCTS was measured to be ~47 nM, which was approximately 6-fold higher than the IC_{50} calculated for the parental A1847 2D cultured cells (~7 nM). Of significance, the IC_{50} for A1847 cells derived from paclitaxel treated MCTS was not observed at concentration up to 3 μ M, indicating a robust retention of paclitaxel resistance that is not just a phenomenon of 3D culture. A similar pattern of response to paclitaxel was observed for OVCAR8 cells (IC_{50} values were ~7 nM, ~18 nM, and > 3 μ M for parental, untreated MCTS-derived, and paclitaxel treated MCTS-derived cells, respectively).

In order to demonstrate that the phenotypic changes produced within MCTS are persistent, gene expression of stem cell markers was measured in cells derived from MCTS after seeding into 2D cultures from both vehicle and paclitaxel treated spheroids. The expression of stem cell markers was maintained in the 2D cultures of cells derived from vehicle treated spheroids for three days (days 9 to 12 from spheroid plating) for *CD133*, *ALDH1A*, *KLF4*, *NANOG*, and *OCT4* (**Figure 19**, blue lines). Interestingly, in cells derived from paclitaxel treated spheroids, gene expression of stem cell markers was greatly increased from day 9 to

day 12 in *CD133*, *ALDH1A*, *KLF4*, *SOX2*, *NANOG*, and *OCT4* (**Figure 19**, red lines). Taken together, these data support that the drug resistant phenotype is not just due to physical restrictions of 3D culture, but it is a stable phenotype that is transferrable to 2D cultures while maintaining stem-like gene expression patterns.

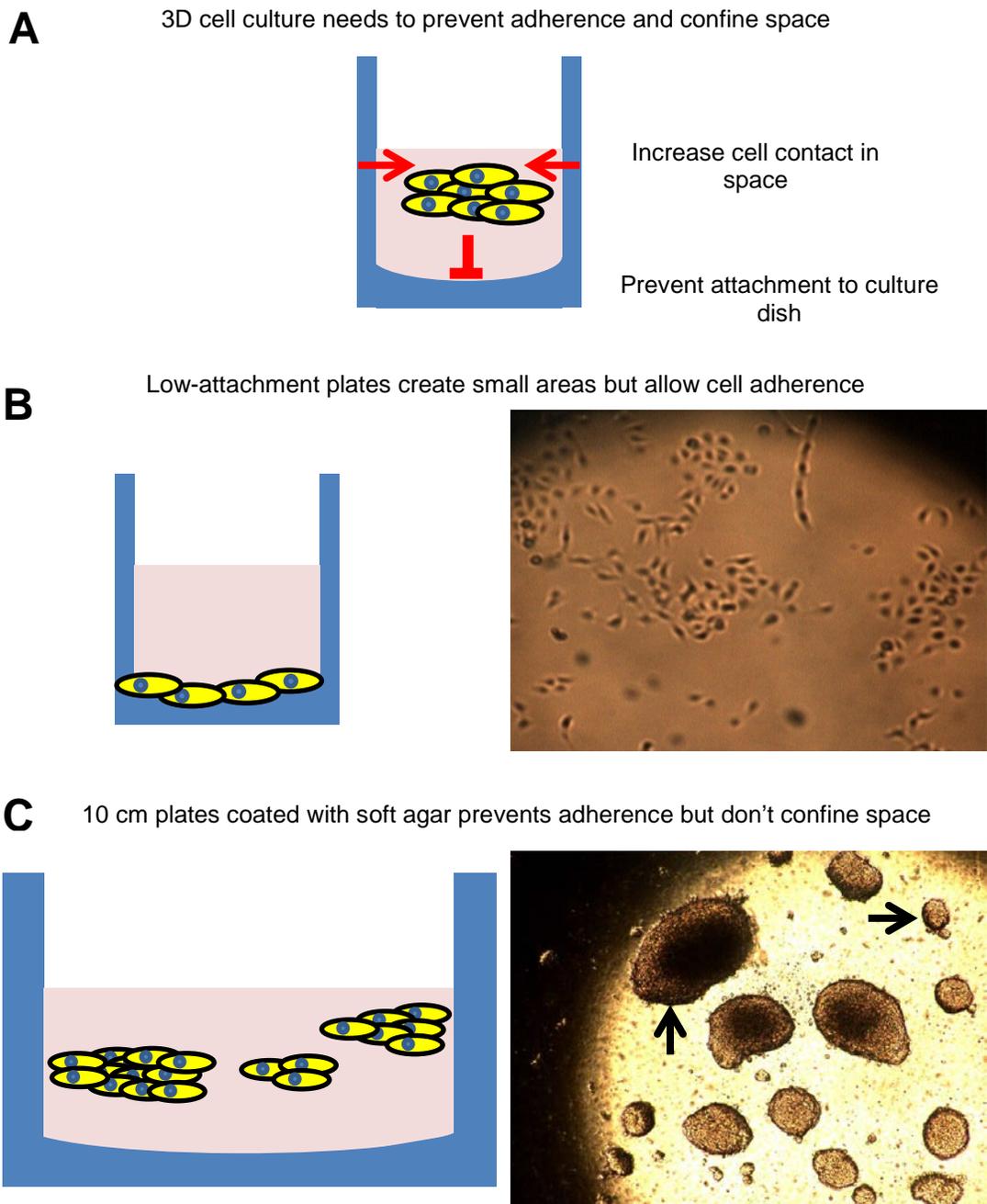


Figure 8: Properties to Develop 3D Culture Models to Form Ovarian MCTS

(A) MCTS formation needs a confined space to increase cell-cell contact and limit spheroid size as well as a culture surface that blocks cell adherence. (B) 96-well low-attachment plates provide a confined space but do not prevent adherence of ovarian cancer cell lines to the culture plate. (C) 10 cm dishes coated with soft agarose allow spheroid formation but there is not size limitation and spheroids can be large (vertical black arrow) or much smaller (horizontal black arrow).

Table 4: Qualitative Comparison of 3D Culture Methods.

Methods	Liquid Handling	Reagents Used	Cost	Reproducibility	Spheroid Transfer Before Screen
Agarose Coated Plates	Yes, used to coat plates	Yes. Inert agarose	Low	Yes	No
Hanging Drop	Yes, used to form droplets	No	Low	Low, droplets fall from plate easily.	Yes, centrifugation
ULA Plates	No	No	High	Yes	No
Magnetic Levitation	No	Yes. Magnetic and artificial	High	Yes	Yes. Remove magnets

3D cell culture methods were qualitatively compared to select a protocol for further development. Methods were compared based on differences between traditional 2D culture protocols. Features that were compared included the amount of liquid handling required to plate cells, the use of artificial reagents, the overall cost, the reproducibility of spheroid formation, and the need to transfer spheroids between plates in order to carry out the desired drug screens.

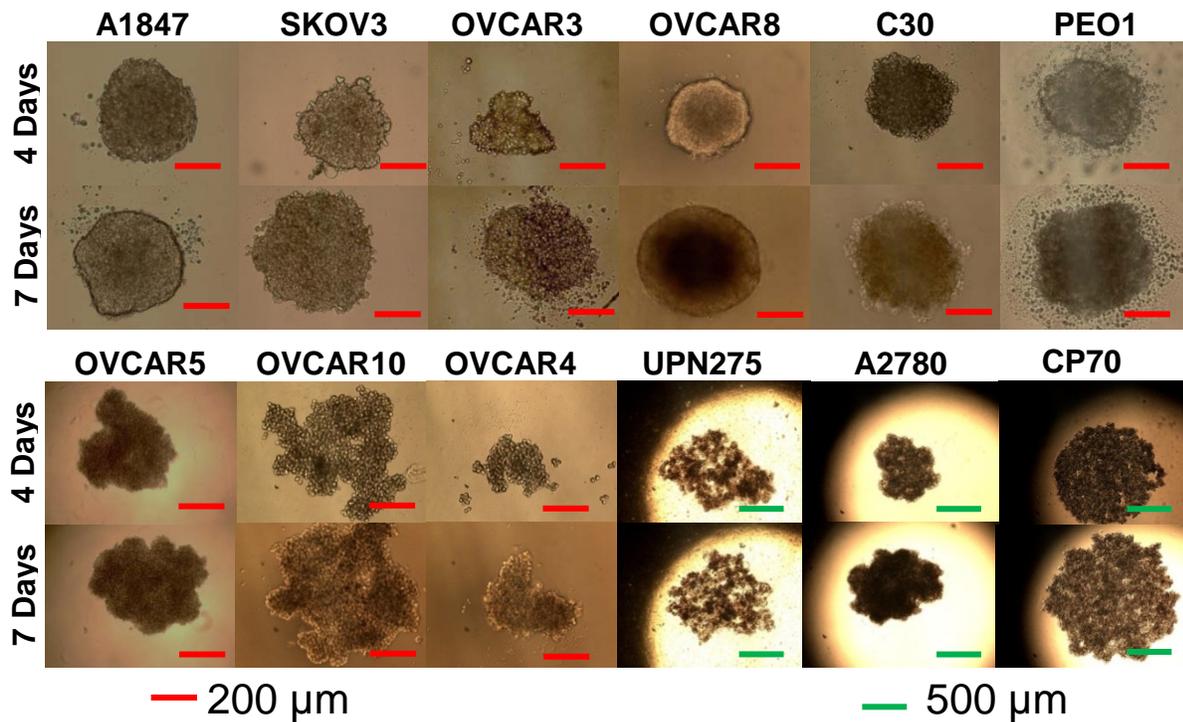


Figure 9: Formation of MCTS Across Ovarian Cancer Cell Lines.

Light microscopy images of MCTS of EOC cell lines on agarose coated 96-well plates 4 and 7 days following cell seeding. Images were taken at either 10x (green scale bars) or 40x (red scale bars). Compact spheroids formed across the majority of cell lines within 4 to 7 days.

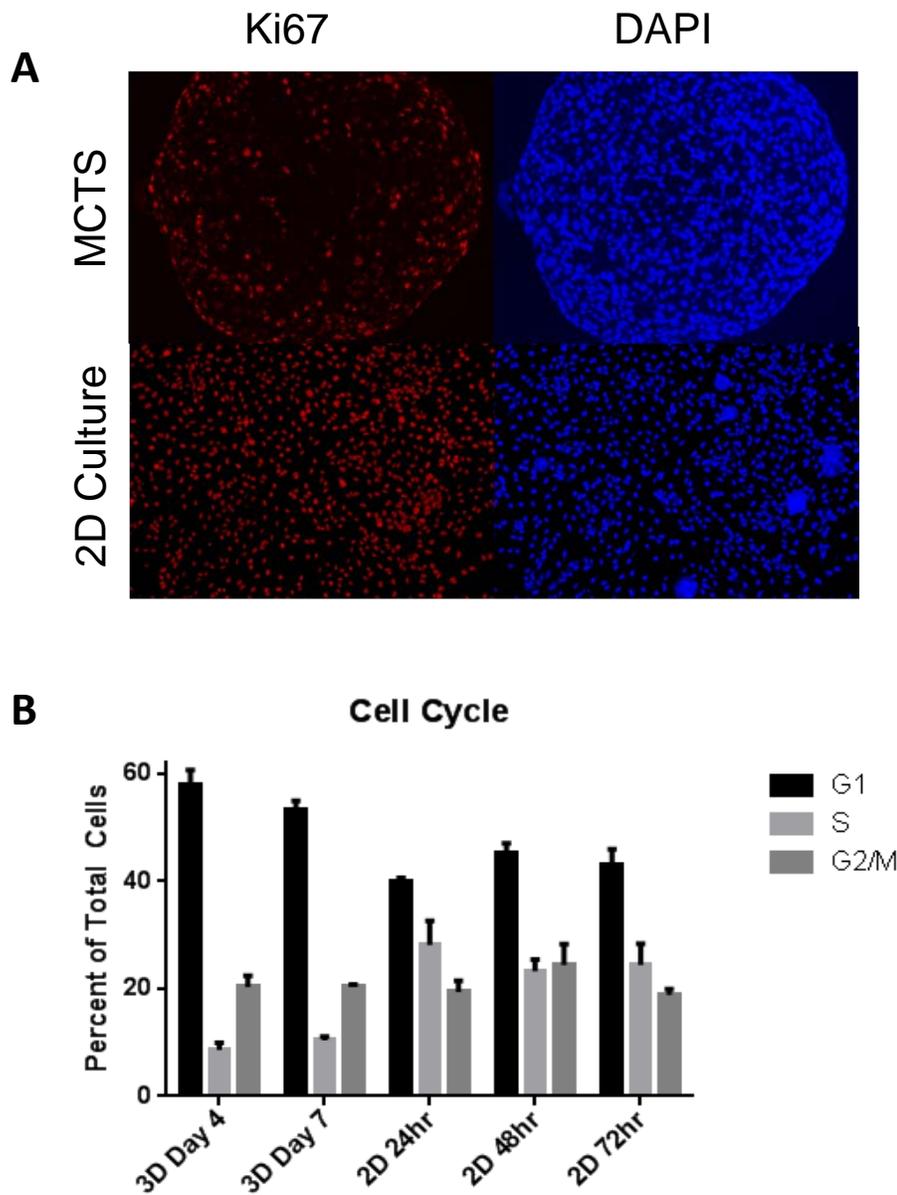


Figure 10: MCTS Formation Reduced Cell Proliferation in Ovarian Cancer Cell Lines.

(A) Immunofluorescence staining of Ki67 for A1847 cell MCTS (day 7) sections and 2D cultures (day 4) shows a decreased in proliferation in MCTS. (B) Cell cycle analysis using propidium iodine reveals an increase in G1 cell populations at both day 4 and day 7 for A1847 cells cultured as MCTS, compared to 2D cells at 24 h, 48 h and 72 h. All bar graphs are represented as mean +/- SD, n=3.

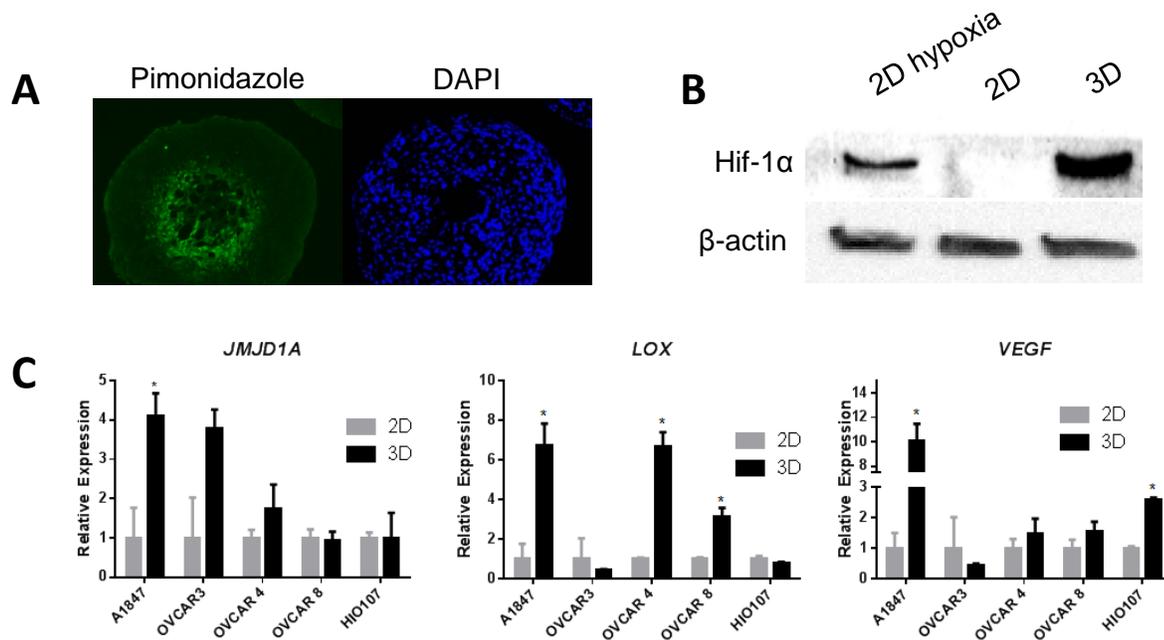


Figure 11: Cellular Hypoxia in Ovarian Cancer Cell Lines was Increased in MCTS.

(A) Piminidazole staining of A1847 cells indicates cellular hypoxia in the inner cells at day 7, A1847 MCTS. Cell nuclei were labeled with DAPI. (B) Western blot analysis shows HIF-1α protein in A1847 2D cultures under hypoxia (0.5 % oxygen) and in A1847 MCTS, but not in A1847 2D cultures under atmospheric oxygen. (C) Expression of hypoxia related genes (*JMJD1A*, *LOX*, and *VEGF*), as measured using qRT-PCR, were increased in a panel of ovarian cell line grown as MCTS compared to parenteral 2D cultures under atmospheric oxygen at day 7 of spheroid growth (n=3) (* = p<0.05, t-test). All bar graphs are represented as mean +/- SD, n=3.

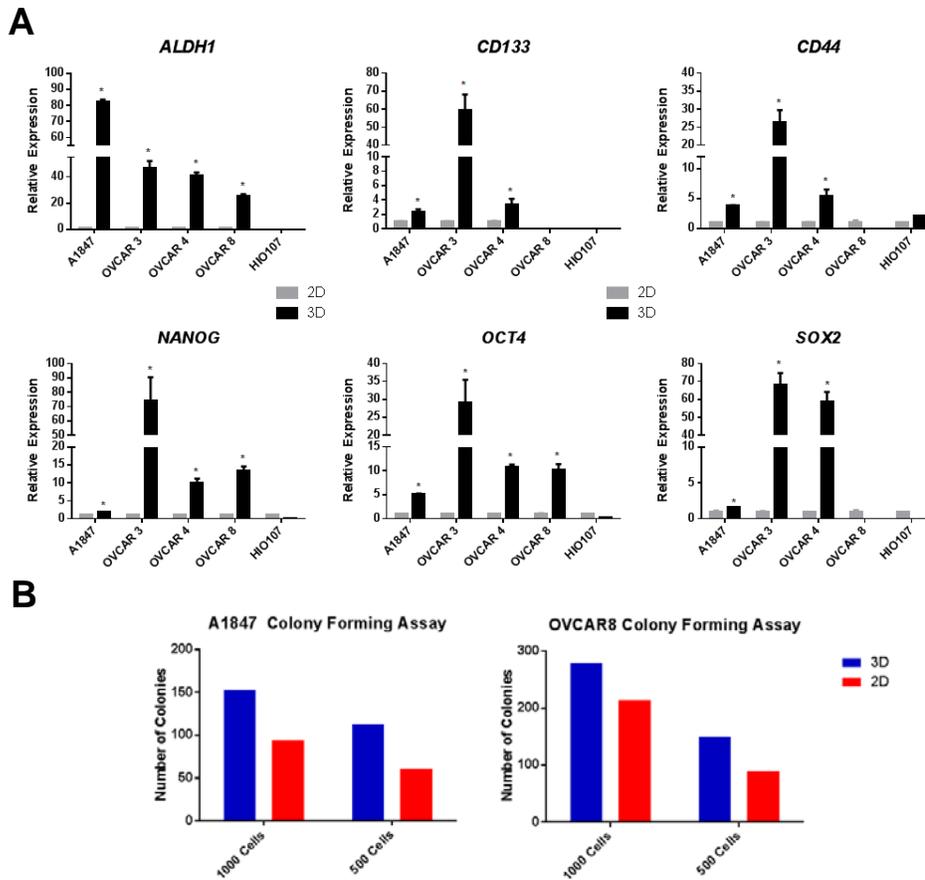


Figure 12: Increased Stemness in Ovarian MCTS Compared to 2D Cultures.

(A) Expression of stem-like gene transcripts were measured using TaqMan qRT-PCR. Several stem-like genes (*ALDH1A*, *CD133*, *CD44*, *NANOG*, *OCT4* and *SOX2*) were upregulated in a panel of ovarian cancer cell line at day 7 for MCTS compared to parental 2D cultures (*= $p < 0.05$, t-test). All bar graphs are represented as mean \pm SD, $n=3$. (B) A1847 and OVCAR8 MCTS after 7 days were separated into single cells to compare colony formation in soft agar to parental 2D cultures. Viable cells following dissociation were counted and either 1,000 or 500 cells were plated into soft agar dishes and total colonies were counted after 14 days in culture. All bar graphs are represented as mean, $n=2$.

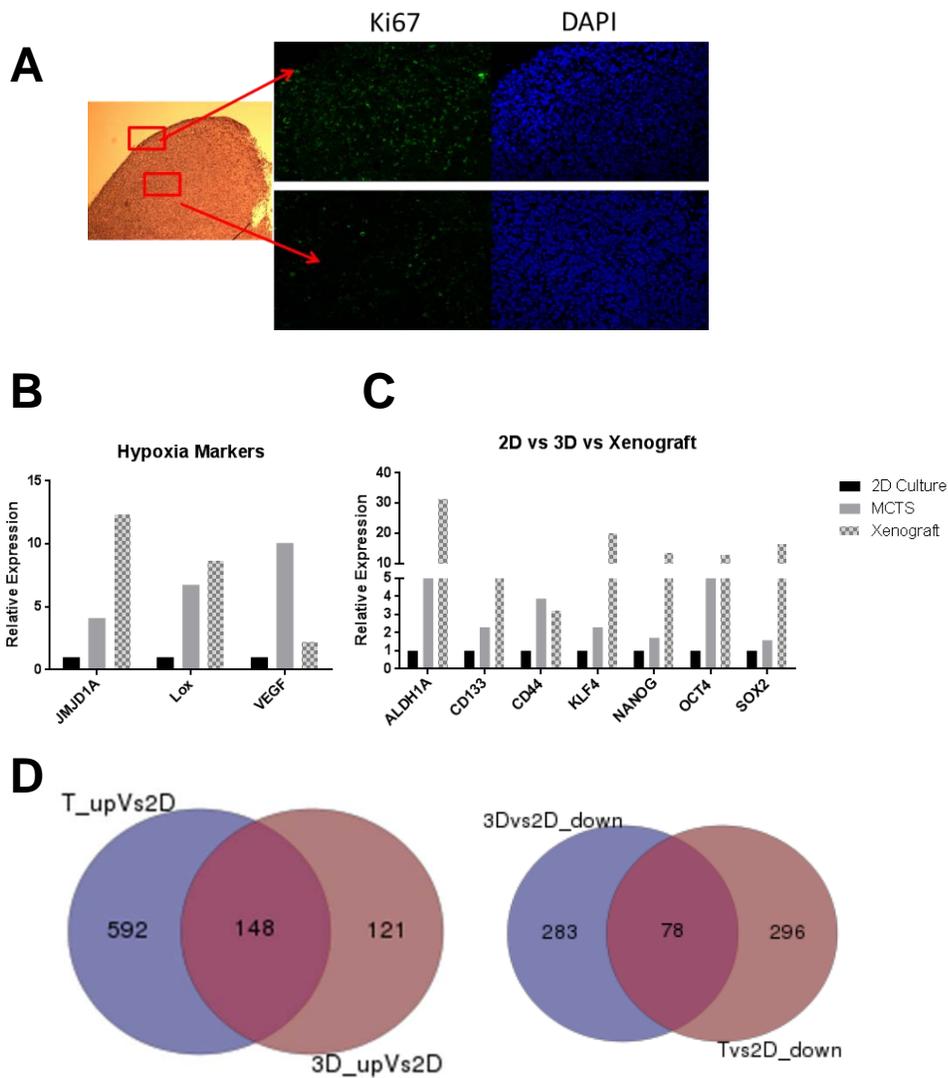


Figure 13: MCTS Displayed Intermediate Gene Expression Between 2D Cultures and Tumor Xenografts.

(A) Ki67 staining in tumor sections from A1847 xenografts reveals higher proliferation of cells on the periphery of the tumor as compared to those within the tumor mass. (B) Expression of hypoxia related genes (*JMJD1A* and *LOX*) in MCTS, as measured using qRT-PCR, were lower than levels detected in A1847 xenografts (highest) and higher than levels measured in parenteral 2D cultures (lowest). *VEGF* expression was higher in 3D cultures than both 2D and A1847 xenografts. (C) Expression of stem-like genes transcripts were measured using TaqMan qRT-PCR. The relative expression level of all of the stem-like genes (*ALDH1A*, *CD133*, *CD44*, *NANOG*, *OCT4* and *SOX2*) were again intermediates in the MCTS when compared to 2D cultures (lowest) and A1847 xenografts (highest). (D) Gene expression was measured using RNA-sequencing and analyzed for genes which are increased or decreased in A1847 MCTS and xenograft models compared to 2D cultures. Shown is the Gene Ontology (GO) analysis of the transcripts with at least a 1.5-fold increase or decrease in expression between either A1847 MCTS or ovarian tumor derived xenografts in mice when compared to A1847 2D cultured cells. All bar graphs are presented as mean +/- SD, n=3.

Mouse xenografts

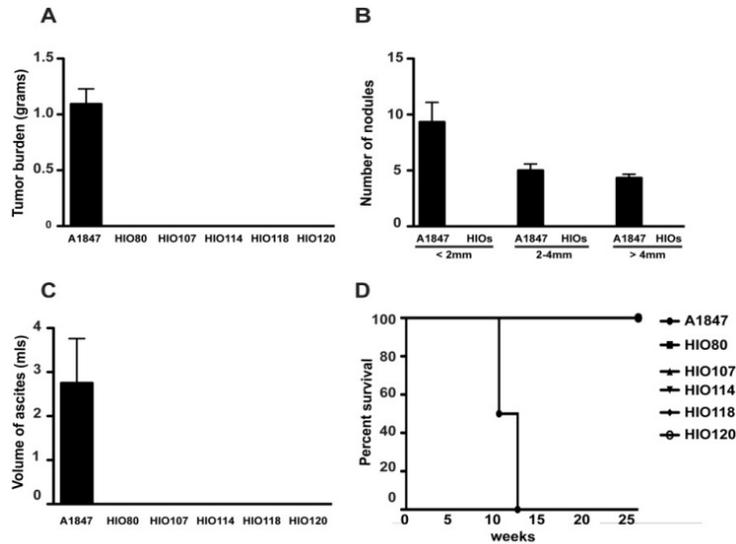


Figure 14: *In Vivo* Tumorigenic Assay of HIO Cell Lines.

(A) Weight (in grams) of any visible tumor collected during terminal necropsy of intraperitoneal xenografts of A1847, HIO80, HIO107, HIO114, HIO118, or HIO120 cell lines. No tumor was visible to collect for any of the HIO implanted cell line, while ~1 gram was collected from A1847 xenografts between 10 and 15 weeks. (B) Number of small (< 2 mm), medium (2-4 mm), or large (> 4 mm) tumor nodules counted in the peritoneal cavity during terminal necropsy of A1847 tumor xenografts. There were no visible nodules in the HIO cell line transplants. (C) Volume of ascites fluid (mL) collected from the peritoneal cavity during terminal necropsy shows ~3 mL of fluid in A1847 xenografts with no collectable fluid from HIO xenografts. (D) Survival curve for A1847 and HIO xenografts shows A1847 cell lines cause terminal disease between 10 and 13 weeks in mouse xenografts, while mice implanted with HIO cells remained healthy through 25 weeks.

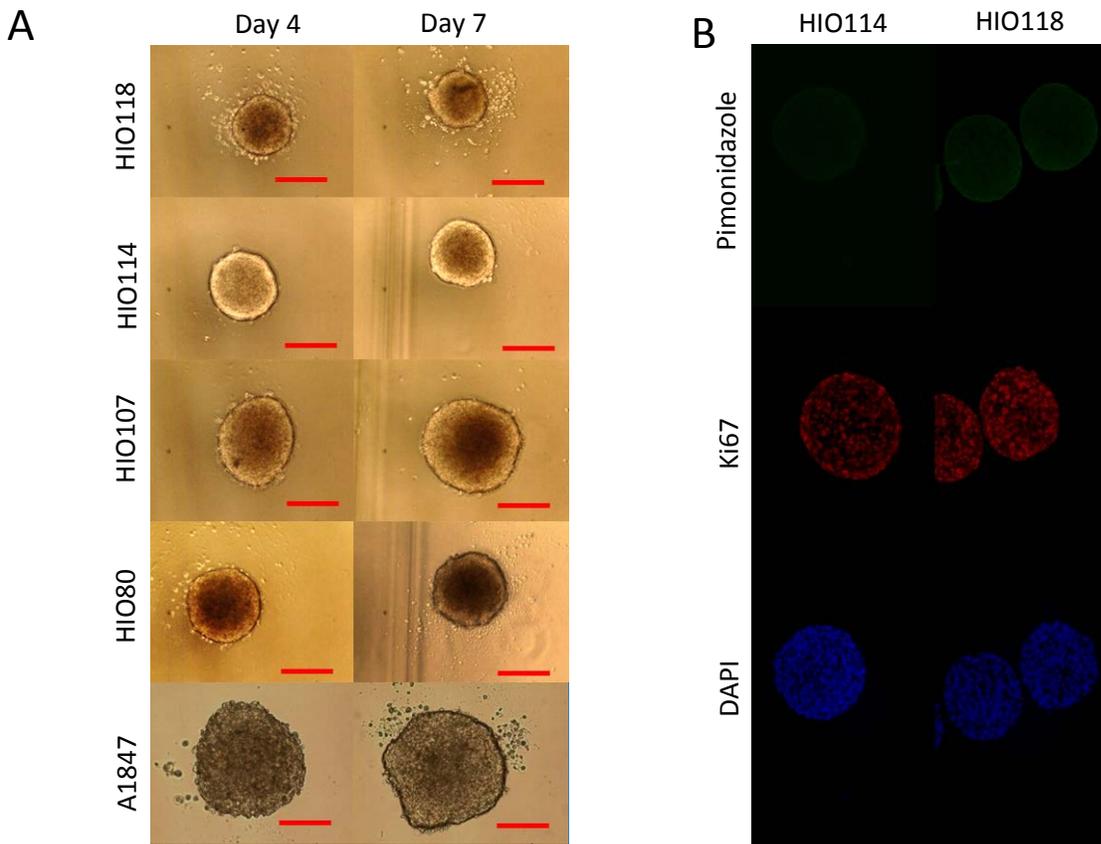


Figure 15: HIO Cell Spheroids Demonstrated Low Levels of a Hypoxia Marker and High Levels of a Cell Proliferation Marker Compared to Ovarian MCTS.

(A) Light microscopy demonstrate that 3D culture spheroids from HIO cells are smaller than A1847 spheroids and do not expand significantly in size from day 4 to day 7. (B) Immunofluorescence staining of Ki67 and pimonidazole in a cross section of HIO114 and HIO118 spheroids at day 7 show little to no pimonidazole staining and high levels of Ki67 staining. Cell nuclei are labeled with DAPI.

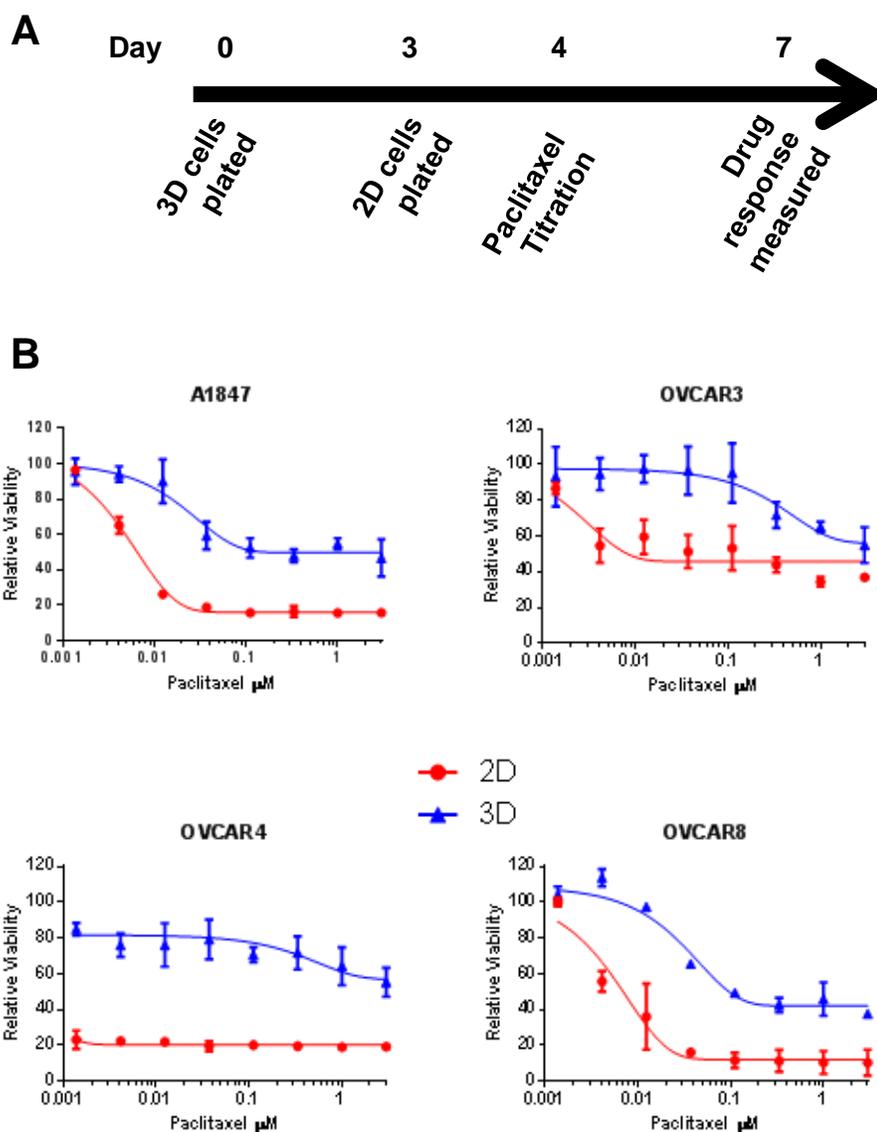


Figure 16: Paclitaxel Resistance in MCTS Compared to 2D Cultures.

(A) Ovarian cell lines were grown in both 2D and 3D cultures and treated with a serial dilution of paclitaxel for 72 h. 2D cultured cells were seeded 24 h prior to drug addition, while MCTS were cultured for 4 days prior to the addition of drug. Drug response in both 2D and 3d was measured using CellTiter-Glo and normalized to vehicle treated viability. (B) MCTS showed a robust decreased in paclitaxel sensitivity compared to 2D cultures in A1847, OVCAR3, OVCAR4, and OVCAR8 cell lines. All data are represented as mean \pm SD, $n=3$.

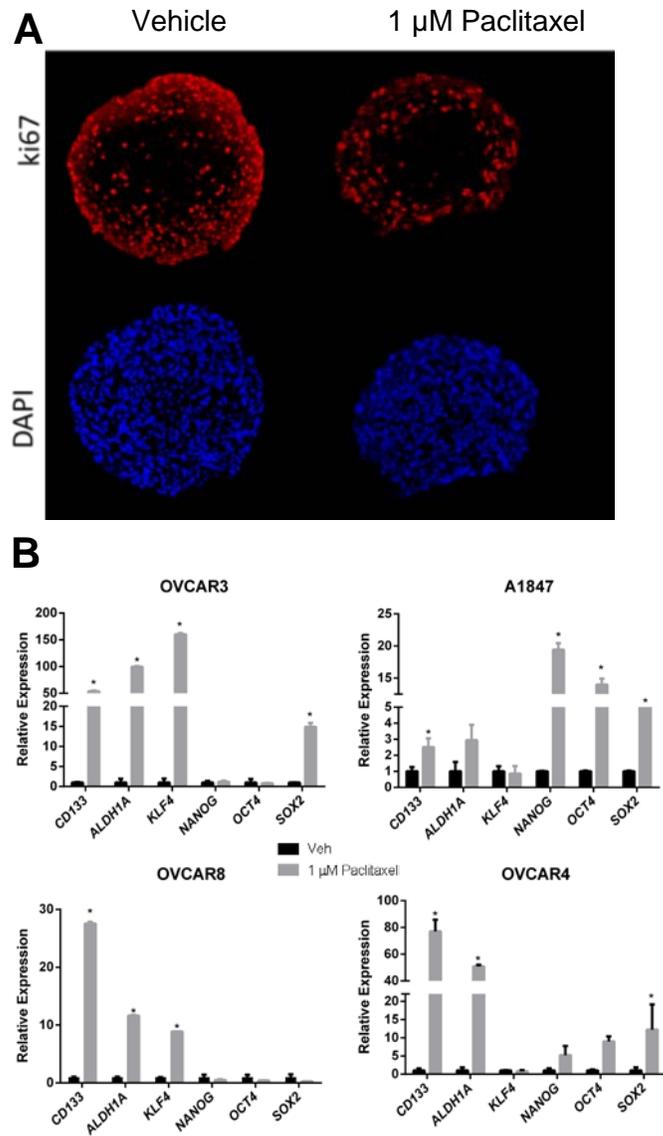


Figure 17: Paclitaxel Reduced Proliferation and Increased Stemness in MCTS.

(A) Immunofluorescence detection of Ki67 and DAPI staining of nuclei reveal a decrease in Ki67 expression following paclitaxel treatment in MCTS. (B) Expression of stem-like genes (*ALDH1A*, *CD133*, *KLF4*, *OCT4* and *SOX2*) was measured using TaqMan qRT-PCR. Gene expression was increased following 1 μ M paclitaxel treatment in MCTS compared to 2D cultures (n=3) (*= $p < 0.05$, t-test). All data are represented as mean \pm SD, n=3.

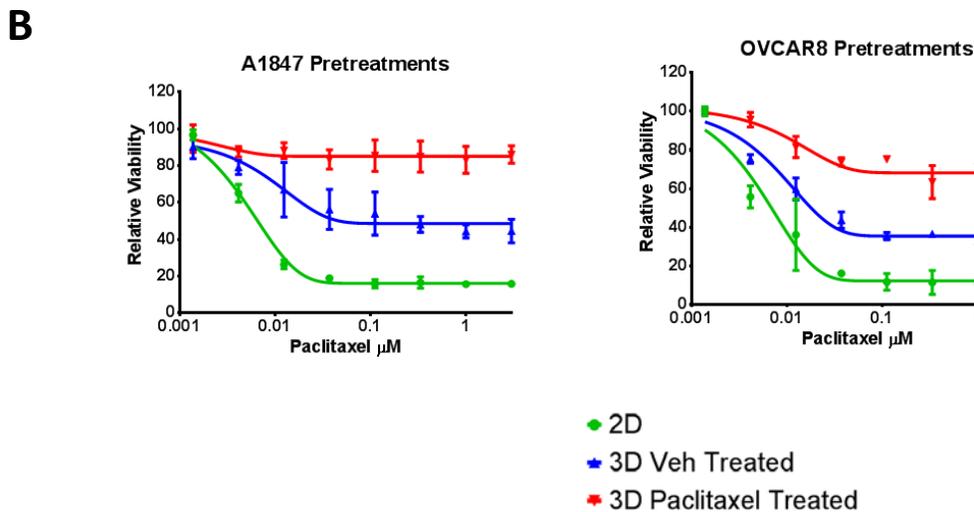
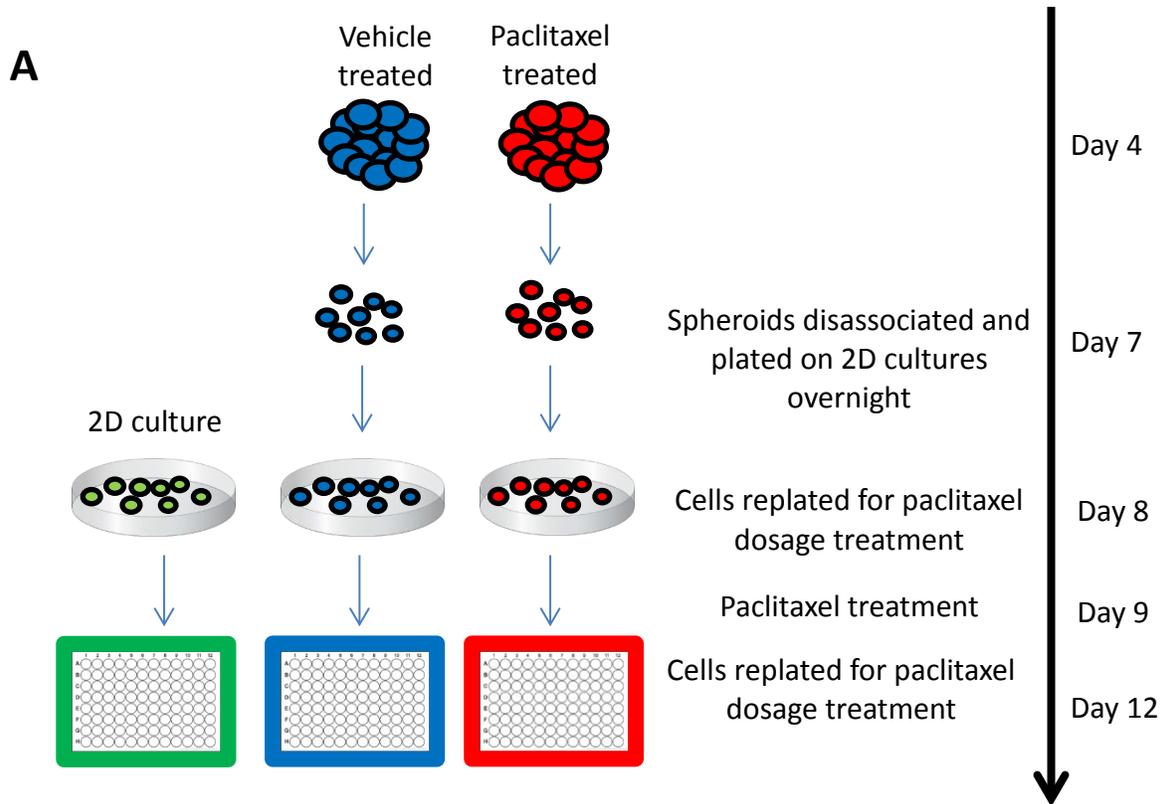


Figure 18: MCTS Derived Cells Maintained Paclitaxel Resistance in 2D Cultures.

(A) Graphical representation of methods to measure paclitaxel response in cells derived from pretreated MCTS. (B) Paclitaxel dosage response in A1847 and OVCAR8 cells from 2D cultures, vehicle (DMSO) treated MCTS derived cells, or 1 μM paclitaxel treated MCTS derived cells. MCTS derived cells in 2D culture retain paclitaxel resistance compared to 2D cells. Paclitaxel pretreatment further decreased the dosage response of A1847 and OVCAR8 MCTS derived cells. All data are represented as mean \pm SD, $n=3$.

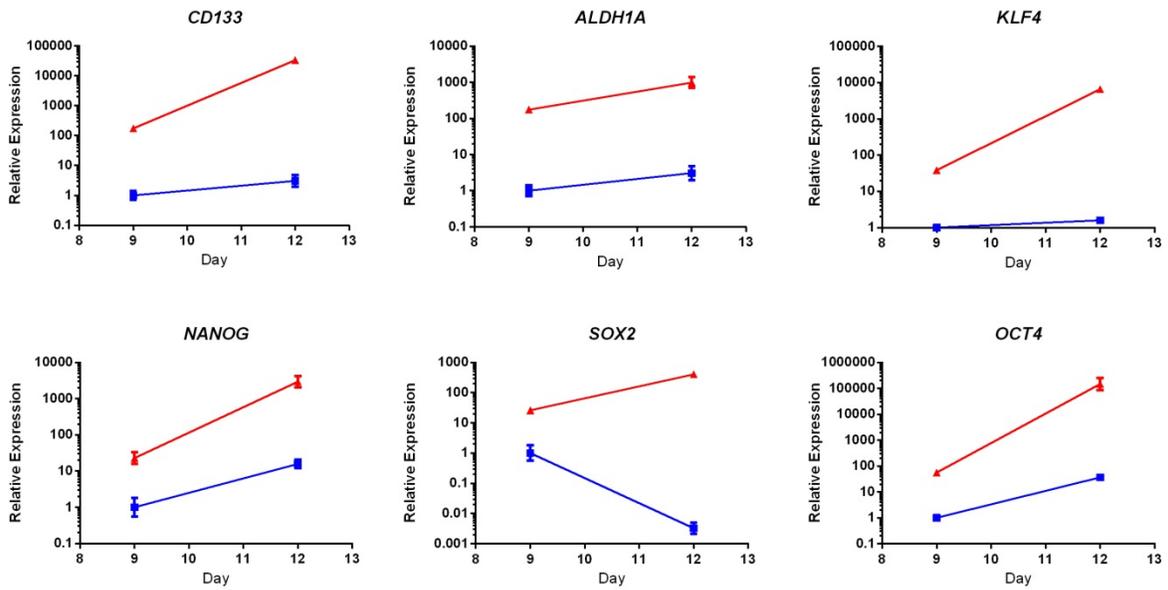


Figure 19: Stem Cell Gene Expression was maintained in 2D Cultures in Cells Derived from MCTS.

Gene expression was measured in 2D cultured cells derived from both vehicle and paclitaxel treated spheroids. The expression of gene associated with a stem cell phenotype is maintained in cells from day 8 to day 11 in vehicle treated spheroids (blue line) while expression is increased in the cells from paclitaxel treated spheroids (red line). All data are represented as mean +/- SD, n=3.

Discussion

There continues to be an important and essential need to improve *in vitro* models of ovarian cancer to support drug development to discover new therapies that improve patient survival. Furthermore, a recent study has shown discrepancies between established ovarian cell lines and patient samples with respect to mutation profiles and gene expression (Domcke, Sinha et al. 2013), suggesting these discrepancies between cell lines and clinical disease could be limiting the effectiveness of drug development. To enhance the clinical representation of ovarian cancer cell lines, a 3D culture model was developed using the liquid-overlay method on agarose coated 96-well plates. This method produced single MCTS per well across a panel of ovarian cancer cell lines, without the need for specialized media or the transfer of spheroids between wells. Since the spheroids could also be recovered through pipetting, biological assays were easy to perform and changes in various cellular properties between 2D and 3D cultures, specifically those associated with response to paclitaxel, could easily be measured. Any changes in properties between these two models would be strictly from the physical 3D growth of the cells and not from any biological agent and would better represent changes in solid tumors that are missing when cells are exclusively propagated in 2D cultures.

The majority of ovarian cancer patients will succumb to drug resistance and methods to improve patient therapy using additional chemotherapy or cytotoxic agents have not proved successful in clinical trials. Drug screening models that more accurately reflect drug resistant disease are urgently needed. Using a 3D culture model, cells took on characteristics associated with drug resistance, including reduced proliferation rates, increase in cellular hypoxia, and expression of cancer stem cell associated markers when compared to 2D cultures (Baba, Convery et al. 2009, Deng, Yang et al. 2010, Milane, Duan et al. 2011, Cioffi, D'Alterio et al. 2015, Gao, Foster et al. 2015). As shown in **Figure 20** the properties of spheroids developed using ovarian cancer cell lines showed features of drug resistance including a gradient of cell

proliferation, increased cellular hypoxia in spheroids cores, and increased stem-like phenotypes and function.

While the nature of cancer stem cells will be discussed in the next chapter, stem-like properties can be induced through the cellular mechanisms identified to be upregulated by hypoxia and senescence (Mani, Guo et al. 2008, Molina, Hayashi et al. 2010, Shuang, Wu et al. 2014, Wu, Du et al. 2014, Dosch, Ziemke et al. 2015, Inukai, Hara et al. 2015). The hypoxic and stem-like signatures were specific to ovarian cancer spheroids and not induced in the non-tumorigenic HIO cell lines. This could be due to the genomic plasticity and adaptability of cancer cells (Quintana, Shackleton et al. 2010, Pisco and Huang 2015). While the 3D culture model may be an intermediate between 2D cultures and *in vivo* tumor xenografts, the time and cost of 3D cultures are more similar to 2D cultures. Over half of the genes upregulated in 3D culture compared to 2D culture were also upregulated in tumor xenografts. However, there were 592 genes upregulated in the tumor xenografts that were not in 3D cultures. This could be due to multiple cells types such as fibroblasts, endothelial cells, and immune cells from the host interacting with the tumor. Further, the co-culture of non-tumorigenic cells could enhance the clinical representation of MCTS even further (Kwon, Smith et al. 2015). However, the scope and experimental design of this study was to specifically compare the effects of culturing ovarian cancer cells in 3D versus 2D conditions.

Supporting the notion that 3D cultured cells behave more like *in vivo* tumors than 2D cultured cells, MCTS were more resistant to paclitaxel than their 2D parental cell lines. As observed in clinical specimens following paclitaxel treatment and disease progression, stem-like markers were robustly increased and cell proliferation was reduced in ovarian MCTS (Steg, Bevis et al. 2012, Wang, Yo et al. 2012). Not only was the stemness phenotype more apparent in MCTS, the percent of cells surviving paclitaxel treatment was greatly increased compared to 2D cultures. Studying stemness can be problematic in many models due to the low number of

cells (Baba, Convery et al. 2009, Kryczek, Liu et al. 2012, Cioffi, D'Alterio et al. 2015) and a model that enhances the surviving population could allow for better screening when screening for drugs that enhance chemotherapy efficacy. These surviving cells may be a promising target for drug development since the majority of patients will develop disease recurrence following surgery and platinum-based chemotherapy. Reducing this population of “cells in waiting” could delay or prevent disease recurrence in the clinical setting and improve patient survival.

While the drug resistance mechanisms are upregulated in ovarian MCTS, the physical properties of the spheroids could be contributing to the reduced response to paclitaxel. Physical penetration of drug through solid tumors can increase resistance (Ong, Zhao et al. 2010). However, my data demonstrate that the resistant phenotype is still maintained in 2D cultures from cells derived from tumor spheroids and provide more evidence that the biological changes such as stemness and hypoxia are more likely driving resistance in the MCTS model. The cells derived from either vehicle or paclitaxel treated MCTS maintained stem-like gene expression for up to three days in 2D culture. These data support the idea that a change in growth is not just a phenomenon of the 3D culture system. Whether this is driven by epigenetic changes or just the positive feedback loop between stem cell transcription factors (Boyer, Lee et al. 2005, Ivanova, Dobrin et al. 2006) is an area for further investigation. It is likely that both epigenetics and transcription regulation contribute to the coordination of multiple drug resistance mechanisms.

Conclusion

Using 3D culture, the MCTS model enhanced stem-cell and other drug resistant properties in ovarian cancer cells that are more indicative of clinical disease than traditional cell culture. Upon treatment with paclitaxel there were significantly more surviving cells in the 3D model at high dosages than 2D. This method provides an *in vitro* model of drug resistance and an assay platform (96-well format) to perform drug screens. Implementing this drug resistant model for

high-throughput drug screening could allow identification of drugs that target resistance in ovarian cancer. The next chapter of this thesis will exploit this model in a proof of principle study using a mid-throughput drug screening library to identify unappreciated drug candidates that can target stem-like properties of ovarian cancer.

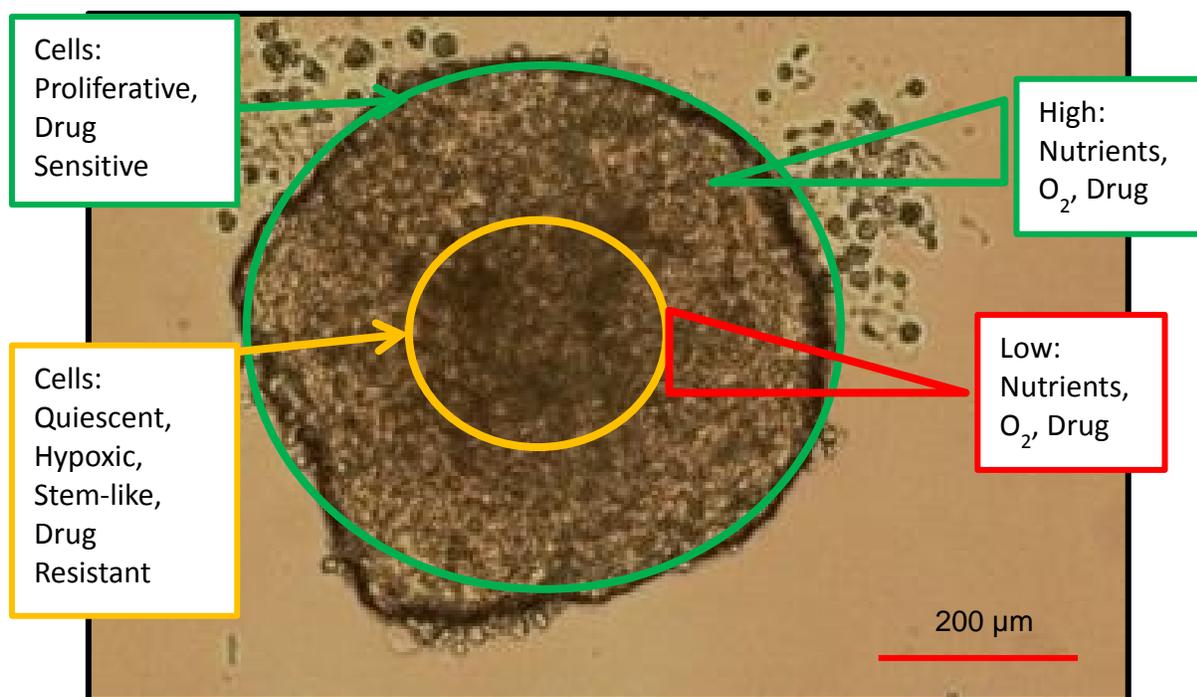


Figure 20: Drug Resistant Phenotypes of MCTS.

Graphical representation of changes induced in MCTS of ovarian cancer cell lines. Many of these changes were directly overserved in this chapter including reduced cell proliferation and hypoxia in the spheroids core (yellow circle and box) and more proliferative, drug sensitive cells on the spheroids exterior (green circle and box, left side). The spheroid environment can change from the inside with low concentrations of oxygen and other nutrients (red box, right side) and high concentrations of oxygen and nutrients on the outside (green box, right side). Red scale bar represents 200 microns.

Chapter 3: 3D Culture Drug Screening Identified Unappreciated Anti-inflammatory Drug Candidates that Reverse Stemness and Synergize with Paclitaxel

Introduction

Cancer Stem Cells

While cancer is commonly thought of a disease of cell growth, however cells within tumors need repopulate over long periods of time and often survive harsh environments. To adapt to harsh environments, such as radiation or chemotherapy, and long-term repopulate tumors some cancer cells use similar survival and adaptation mechanisms that are common in embryonic and tissue development. These cells have been termed cancer stem cells (CSCs) and have provided novel insight into cancer growth and response (Wicha, Liu et al. 2006). Understanding how these CSCs grow, adapt, and regulate different responses is critical to developing therapies that might improve patient survival.

This concept of CSCs has arisen from the striking degree of similarity noted between stem cells and cancer cells. Clinical and experimental observations have found that tumor structure, development, and behavior often mimicked that of somatic stem cells, including the fundamental abilities to self-renew and differentiate. Adult or somatic stem cells exist throughout the body after embryonic development and are present in different types of tissues and organs. These stem cells remain in a quiescent or non-dividing state for years until stimulated by the signals triggered by tissue renewal, damage, and remodeling processes. Tumors are formed from large amounts of cells; however the individual cells within a tumor can be heterogeneous. Different cells taken from the same tumor can differ in their ability to initiate tumor formation in serial transplants into mice, indicating that some cells form tumors better than others. Also, the tumors formed from these tumor initiating cells contained populations of both tumor forming and non-forming cells, showing an ability of tumor cells to self-differentiate (Greene 1952). This heterogeneity in tumor formation and differentiation resembles those properties of normal stem cells, which also have the ability to serial transplant and differentiate. Stem cells are small population of cells from normal or developmental tissue that populate/replenish tissues or

specific cell populations though the capability to differentiate into different cell types and have long-term self-renewal. Normal stem cells are typically classified based on their ability to differentiate into different cells types as either totipotent, pluripotent, or multipotent. Apart from differentiation properties, specific populations of stem cells are identified by surface markers via cell sorting. Cancer cells with these similar properties have been observed and named cancer stem cells or cancer stem-like cells.

The ability of cancer cells to differentiate into different cell types was well established in teratocarcinomas which contained structures resembling many different types of the body (Pierce and Dixon 1959, Pierce, Dixon et al. 1960, Pierce and Verney 1961). Differences in stem cells within cancer were shown to contribute growth and development differences such as colony formation and therapy response (Hamburger and Salmon 1977). More recently, it has become clear that heterogeneity within individual tumors relates to developmental stem cell pathways regulates tumor development, progression, and response to chemotherapy. These properties within individual cancer cells with clonal, long-term populating, and self-renewal capacity are the standards for identifying CSCs (Nguyen, Vanner et al. 2012). CSCs are associated with cancer initiation, development, progression, and drug resistance.

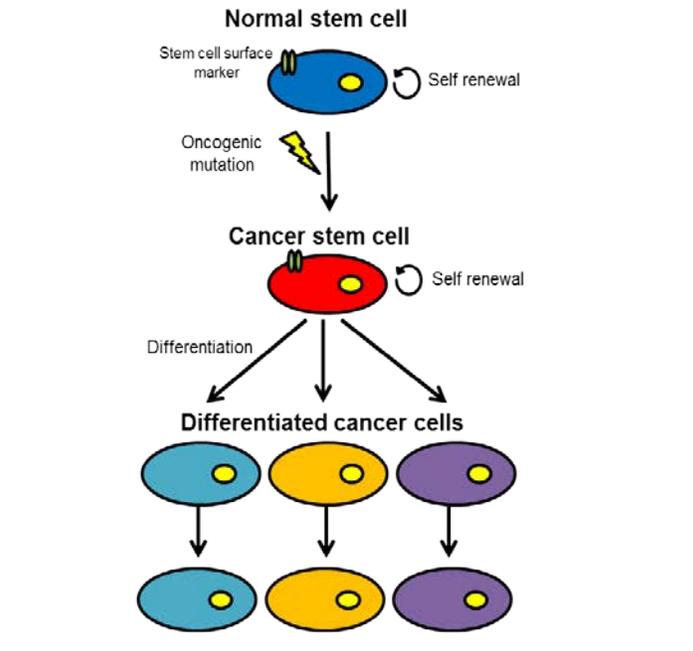
The first study to directly identify specific populations of cells with the same tumor capable of initial tumor was in acute myeloid leukemia (AML). In this study, AML cells were transplanted into severe combined immune-deficient (SCID) mice from patient samples that were either CD34⁺ or CD34⁻ (a marker of myeloid differentiation). Cells from the CD34⁺ population formed tumors while the CD34⁻ did not. Interestingly these cells were less differentiated than traditional colony forming cells isolated from AML samples, indicating a more stem-like phenotype (Lapidot, Sirard et al. 1994). The expression of surface markers traditionally used to identify hematopoietic stem cells on the surface of cancer cells (CD34⁺⁺, CD38⁻) lead to the hypothesis that tumors develop through oncogenic mutations in myeloid stem cells that differentiate and

populate tumors long term (Bonnet and Dick 1997). The standard methods for identifying CSCs is the expression of stem cell surface markers, their ability to form colonies in soft agar, and most importantly the ability to form tumors in immunocompromised mouse using limited number of implanted cells (Clarke, Dick et al. 2006, Nguyen, Vanner et al. 2012). Using these methods CSCs have also been discovered in solid tumors or breast (Marsden, Wright et al. 2009), brain (Singh, Clarke et al. 2003), prostate (Collins, Berry et al. 2005), colon (Ricci-Vitiani, Lombardi et al. 2007), and pancreas (Hermann, Huber et al. 2007). CSCs from ovarian cancer have been characterized and will be discussed in next section (Bapat, Mali et al. 2005). Single cell gene expression studies from colon cancer xenografts revealed genetic diversity similar to the normal developmental pathway of stem cells within the colon epithelium. Gene expression heterogeneity of cells with stem and non-stem like features were observed in both the normal gut epithelium and colon tumors. Single cell xenografts from stem-like cells led to heterogeneous populations in the tumor of both stem-like and non-stem like cells. (Dalerba, Kalisky et al. 2011).

The nature of cancer stem cells have been extensively studied and led to controversy as to if cancers arise sequentially from mutations in somatic progenitor stem cells or if cancer cell plasticity can induce a stem cell phenotype in cancer cells from differentiated tissue (Kreso and Dick 2014). The data presented above support the theory that normal somatic stem cells acquire oncogenic mutations, which in turn promotes expansion of these progenitor cells, thus increasing their predisposition to cancer development by promoting long-term survival and pluripotency over their normal tendency towards relative quiescence and proper differentiation. However, other data suggests there could be a back conversation of tumor cells due to selective pressure that induce the stem cell phenotype in malignant cells derived from differentiated cells (**Figure 21**). The ability of normal, differentiated cells to revert back to stem cells through pathways such as EMT (Mani, Guo et al. 2008) led to the idea that cancer cells could also de-

differentiate into cancer stem cells. The ability of cancer cells to convert into a stem cell like phenotype has been observed in many tumor models (Molina, Hayashi et al. 2010, Gupta, Fillmore et al. 2011, Magee, Piskounova et al. 2012, Shuang, Wu et al. 2014, Dosch, Ziemke et al. 2015, Fessler, Borovski et al. 2015). The mechanisms of this conversion can be related to many different tumor pathways. One of the common characteristics of cancer cells is their genomic and phenotypic plasticity which allows them to adapt to harsh environments and evolve in response to different cellular changes or stimuli such as chemotherapy (Quintana, Shackleton et al. 2010, Pisco and Huang 2015), EMT (Ye, Tam et al. 2015), interactions with the tumor microenvironment (Charles, Ozawa et al. 2010, Vermeulen, De Sousa et al. 2010), and hypoxic conditions (Liang, Ma et al. 2012, Wu, Du et al. 2014, Ramadoss, Sen et al. 2016). Specific drivers of these, such as the hypoxia response histone demethylase JARID1B, can promote tumor maintenance but are not essential for tumor initiation or progression (Roesch, Fukunaga-Kalabis et al. 2010). Cancer cells that acquire stem cell function but are not identified to be from developmental stem cells (linear CSCs) are often referred to as “stem-like” cells; however, the distinction between CSCs and stem-like cells is not well defined.

Linear Cancer Stem Cell Progression



Acquired Stemness in Cancer Cells

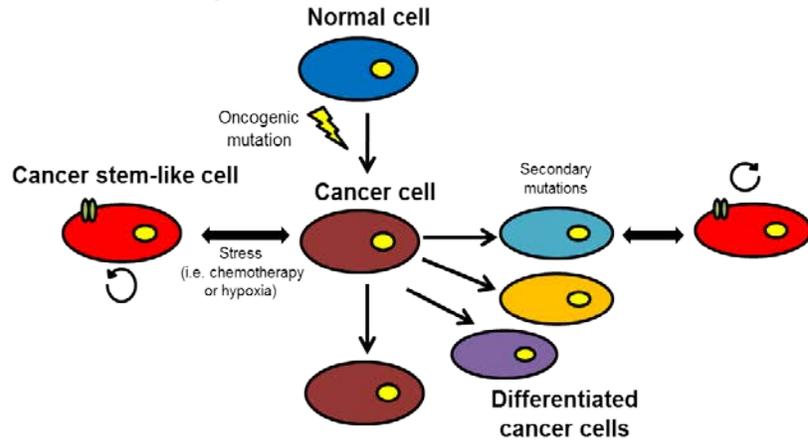


Figure 21: Models of Cancer Stem Cell Progression.

Stemness in Ovarian Cancer

Since the origins and pathogenesis are still debated, evidence for the nature of somatic stem cell transformation in ovarian cancer is not well defined. As discussed in Chapter 1, evidence is contradictory to either the ovarian surface epithelium or the of the fallopian tube epithelium being the origin of a subset of tumors, *i.e.*, HGS ovarian cancer. However both the ovarian surface epithelium and fallopian tube epithelium have stem cell niches with cells with regenerative

properties (Bowen, Walker et al. 2009, Capel 2014, Ng, Tan et al. 2014). Some evidence supports there could be a stem cell niche within the junction between the ovarian surface the fallopian tube that helps repair the damage to the ovarian surface following follicle release (Flesken-Nikitin, Hwang et al. 2013). Notch and Wnt, canonical stem cell pathways, have been shown to regulate differentiation in fallopian tube organoids and could contribute to fallopian tube repair (Kessler, Hoffmann et al. 2015). Fallopian stem-like cells (CD44⁺ and PAX8⁺) can be isolated from distal end of the tube and are capable of clonal growth and self-renewal (Paik, Janzen et al. 2012, Wang, Sacchetti et al. 2012). Since these stem cell niches are located near the areas of ovarian and fallopian surface repair and precursor lesions they could be hotspots for the development of tumors from mutations in somatic stem cells. One recent study has shown that *SOX2* is overexpressed in the fallopian tubes of patients with HGS disease and in *BRCA1/BRCA2* mutation carriers (Hellner, Miranda et al. 2016), indicating a possible stem cell precursor lesion.

Despite unclear evidence as to the pathogenesis of CSC from somatic stem cells in ovarian cancer, there is evidence of stem-like cells and stemness related genes in ovarian cancer progression and drug response. Stem-like cells from ovarian cancer were first discovered by isolating cells from patient ascites fluid and testing for sphere-forming ability and passage through SCID mice (Bapat, Mali et al. 2005). Two canonical stem cell factors, *CD133* (prominin-1) and *ALDH1A* (Aldehyde dehydrogenase-1A1), define ovarian cancer stem-like cells (Ferrandina, Bonanno et al. 2008, Ferrandina, Martinelli et al. 2009, Kryczek, Liu et al. 2012). Other stem cells markers such as *CD44*, *CD117*, EpCAM (*CD326*), and *CD24* have also been implicated as stem cell markers in ovarian cancer (Garson and Vanderhyden 2015). *CD133* expression in cell culture models, animal models, and patient tumors is associated with enhanced tumorigenicity, drug resistance, and disease recurrence (Baba, Convery et al. 2009, Curley, Therrien et al. 2009, Steg, Bevis et al. 2012, Dobbin, Katre et al. 2014). *ALDH1A*

expression has a negative correlation to progression-free survival in ovarian cancer patients and knockdown of *ALDH1A* has been shown to restore chemotherapy sensitivity *in vitro* by increasing dose response by over 40-fold in *ALDH1A* knock down cells (Landen, Goodman et al. 2010, Wang, Yo et al. 2012). Co-expression of CD133 and ALDH1A in clinical ovarian cancer samples is associated with both increased time to recurrence and decreased overall survival (Silva, Bai et al. 2011). In this same study, the expression of both CD133 and ALDH1A had additive effects in ovarian cancer cells where CD133⁺/ALDH1A⁺ were the most tumorigenic cells in SCID mice followed by CD133⁺/ALDH1A⁻, then CD133⁻/ALDH1A⁺, and finally CD133⁻/ALDH1A⁻ cells showed no tumorigenic potential at all. Single cells that were positive for both CD133 and ALDH1A had the ability for both self-renewal and differentiation into subpopulations of CD133⁺/ALDH1A⁻, CD133⁻/ALDH1A⁺, or CD133⁻/ALDH1A⁻, indicating a hierarchical order regulated by BMP2 (Choi, Ingram et al. 2015). The markers, CD133 and ALDH1A, are expressed in ovarian and fallopian stem cells, and could indicate a potential progression from somatic stem cells to ovarian cancer through acquired oncogenic mutations in fallopian stem cells, mimicking the progression first observed in myeloid leukemia (Bonnet and Dick 1997, Flesken-Nikitin, Hwang et al. 2013). But, the mechanisms have not been well established. However, it is clear stem-like characteristics contribute to drug resistance and disease recurrence in ovarian cancer. Therefore, preclinical drug screening models that better replicate these stem-like properties *in vitro* could be useful in developing more efficacious drugs for ovarian cancer.

Cancer Stem Cells and Drug Resistance

Stem cell gene expression can be used to predict both disease progression and drug response in patients across many different cancer (Ginestier, Hur et al. 2007, Gentles, Plevritis et al. 2010, Neumeister, Agarwal et al. 2010, Eppert, Takenaka et al. 2011, Merlos-Suarez, Barriga et al. 2011, Chebouti, Blassl et al. 2016). CSCs or stem-like cell populations are increased

following treatment with many different forms of chemotherapy and/or radiation when evaluated in tumor xenograft models. Further, when the tumor repopulates with CSCs chemotherapy, the resulting tumor's response to therapeutic treatment is decreased (**Figure 22**) (Bao, Wu et al. 2006, Hermann, Huber et al. 2007, Dylla, Beviglia et al. 2008). Some of these studies suggest the number of stem cells in the initial tumor reflect number of total cells that survive chemotherapy and/or radiation. It has also been shown that stem cells propagate the tumor after chemotherapy and that inhibition of stemness blocks tumor repopulation (Chen, Li et al. 2012). In support of this observation, researchers have shown that stem cell gene expression of *CD133*, *ALHD1A*, and *CD44* increased through the duration of treatment and in recurrence in ovarian cancer patient samples (Steg, Bevis et al. 2012). Likewise, the amount of stem-like cells is increased in chemotherapy resistant ovarian cancer cell lines (Hosonuma, Kobayashi et al. 2011). These data support the idea that CSCs or stem-like cancer cells contribute to drug resistance and recurrence in cancer. The mechanisms which control drug resistance were discussed in Chapter 1. Taken together, these studies suggest that acquisition of a stemness-like phenotype is a central mechanism of drug resistance. Furthermore, this change is associated with the induction of many other drug resistance phenotypes and also can be initiated or maintained in a feedback loop from exposure to hypoxia and other cellular stress.

Targeting Cancer Stem Cells

The previous data support the idea that targeting CSCs or stem-like cells may improve the response to chemotherapy in cancer. The silencing of different stem cell-related genes using targeted siRNAs, increase the sensitivity of tumor cells to chemotherapy in ovarian cancer models (Landen, Goodman et al. 2010, Seo, Kim et al. 2016). Drug screening methods have been adapted to targeting CSCs as well. For instance, salinomycin was identified from a screen of over 16,000 compounds to selectively kill breast cancer stem cells; however, the clinical development of this drug has not progressed due to high neurotoxicity (Gupta, Onder et al.

2009). A larger library screen of 31,624 small molecules identified several candidates that could target glioblastoma CSCs using stem cell enriched media (Visnyei, Onodera et al. 2011). In ovarian cancer, screening has identified mitotic inhibitors with activity against differentiated cancer cells and against stem-like cells (Mezencev, Wang et al. 2012). Drugs targeting specific cellular processes pathways associated with CSCs such as EMT, metabolism, or Wnt/ β -catenin signaling can inhibit stemness (Scheel, Eaton et al. 2011, Shank, Yang et al. 2012, Nagaraj,

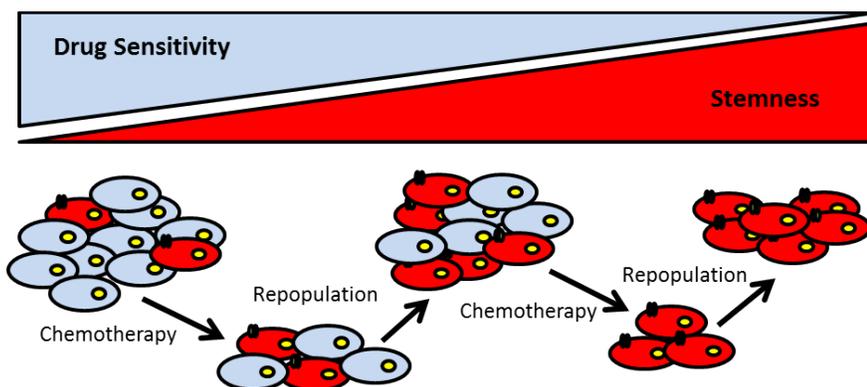


Figure 22: Cancer Stem Cells Repopulate the Tumor Following Chemotherapy.

Joseph et al. 2015). Targeting specific stem-cell proteins has been difficult; however, disulfiram (the anti-alcoholism drug) has been found to reduce ALDH1A activity and selectively inhibit CSC growth (Yip, Fombon et al. 2011, Liu, Kumar et al. 2013, Liu, Wang et al. 2016).

Using 3D Cell Culture to Target Stem-like Cells

As shown in Chapter 2, MCTS formation has been shown to induce stem cell-like properties, including expression of stem cell markers including, *CD133*, *ALDH1A*, *SOX2*, *OCT4* and *NANOG*. This same phenomenon has been observed across many different cancer cell lines (Liu, Wang et al. 2013, Liao, Qian et al. 2014, Oktem, Bilir et al. 2014, Zhang, Hua et al. 2016). The use of MCTS models for drug development could help identify unappreciated or existing drugs capable of targeting these stem-like characteristics of patient tumors associated with drug resistance that are crucial for improving survival. A drug screen against 2D cell cultures with low stem-like properties directly compared to a 3D culture drug screen with stem-like properties

could identify drugs that specifically target stem-like cells. In this chapter, I will explore using 3D cell culture drug screening to identify unappreciated drug candidates that target stem-like cells and improve the efficacy of chemotherapy in ovarian cancer.

Methods

All methods were performed as previously described in Chapter 2 unless otherwise noted.

Transcriptome Sequencing and IPA Pathway Analysis

For transcriptome sequencing (RNA-seq), RNA was isolated from 2D cultures at 80% confluency, from 3D cultures at 7 days of spheroid growth from twelve ovarian cancer cell lines (A1847, SKOV3, OVCAR3, OVCAR8, C30, PEO1, OVCAR5, OVCAR10, OVCAR4, UPN275, A2780, and CP70). RNA was prepared for paired end sequencing on a HiSeq 2500 using a stranded library prep kit (Illumina). Initial analyses were prepared using RSEM expected gene counts. First, data were filtered to remove non/low-expressed genes. This resulted in a total of ~14,000 genes examined for differential expression between the different 2D and 3D cultures. Next, normalization factors were calculated to scale the library sizes followed by estimation of tag wise negative binomial dispersion values. Genes were then compared that were 1.5-fold upregulated or down regulated in 3D cultures compared to 2D cultures. To identify pathway regulators IPA Pathway Analysis (QIAGEN) was used and the top 5 regulators based on p value were measured.

Drug Screening

Four ovarian cancer cell lines (A1847, OVCAR3, OVCAR4, and OVCAR8) and two non-tumorigenic cell lines (HIO107 and HIO118) were grown as both 2D and 3D cultures for drug screening. 2D cultures were plated overnight before drug treatment, while spheroids were grown for 4 days before the additional of drug. A custom 304 drug library was purchased from the 10 mM, FDA-Approved Library at Selleckchem. Drugs were diluted into 1 mM stock plates

in DMSO and then twice diluted in media by the Hamilton Nimbus 96 with the final dilution of 5 μ l drugs into 95 μ l cell media, for a final concentration of 10 μ M drug. After 72 h of drug exposure, cell viability was measured using CellTiter-Glo incubated 1:1 to culture media for 1 h at 37°C. Relative viability was established for the control (DMSO) treated cells and directly compared to the 2D and 3D cultures for each drug treatment. Secondary screening was performed at 25 μ M following the same plating protocol across all 3D hits and select hits from 2D culture and hits from 2D and 3D culture. For all dosage response and function assays licofelone powder was purchased from Santa Cruz Biotechnology and glafenine hydrochloride powder was purchased from Sigma Aldrich Inc.

Cell Viability Assays

When comparing cell viability assays all methods Cell-TiterGlo (Promega), picogreen (ThermoFisher), and acid phosphatase (Sigma Aldrich) were performed based on manufacture's protocol. For luciferase reporting vectors A8417 cells were previously transfected with the firefly luciferase vector pWZL-Luc. Cells were grown in standard media with Geneticin (ThermoFisher). For correlation to cell seeding number, cells were plated on 3D culture plates at 1,000, 2,000, 3,000, 4,000, 5,000 or 6,000 cells per well. Spheroids were grown for 7 days and then viability assays were performed for single spheroids. Standard curves were made using Graphpad Prism and r^2 values were calculated. Methods were also compared for ease of use for drug screening by qualitative measurements such as spheroids transfer and liquid handling.

Drug Combination Assays

Drug combination studies were performed using the combination index (CI) method described by Chou and Talalay (Chou and Talalay 1984). Spheroids from A1847 and OVCAR8 cell lines were grown for 4 days before addition of either paclitaxel (1 μ M or 250 nM) or licofelone (10 μ M or 20 μ M) and then 3 days later serial dilutions of licofelone were added on top of the paclitaxel

treated cells or serial dilutions of paclitaxel were added on top of the licofelone treated cells. Assays were performed as biological triplicate using triplicate wells within each experiment. Cell viability following 72 h of treatment from the serial dilutions was evaluated using CellTiter-Glo as described above and the viability data were then analyzed using CalcuSyn (version 2.1, BioSoft, UK) to calculate the synergy between the two drugs at each molar ratio evaluated. Drug combinations which yielded CI values less than 1 were considered to be synergistic as previously reported (Chang and Chou 2000, Chou 2008) and used in our laboratory (Pessetto, Ma et al. 2014, Pathak, Zhou et al. 2015).

Results

Cell Viability in Response to Drug Treatment in Ovarian MCTS

While spheroids size can be used for spheroid forming assays, using spheroid size might not accurately measure drug response. For example, when A1847 spheroids are treated with carboplatin from 400 μM to 12.5 μM for 72 hours, there is no discernable size between the spheroids. When exposed to the high doses (100 - 400 μM) of drug, the spheroids appeared distressed but the overall “size” did not discernably change, indicating that the diameter or volume may not be useful in determining spheroid drug response (**Figure 23A**). To more accurately measure drug response, four different cell viability assays were compared (CellTiter-Glo, Pico Green, luciferase reporting vectors, and acid phosphatase) in order to establish an in-well method which could measure a large dynamic range of cell survival. Cells were plated at different initial densities of 1,000, 2,000, 3,000, 4,000, 5,000, or 6,000 cells per well. After 7 days of spheroid growth each viability assay was performed and correlated to initial cell seeding number. All four assays showed a strong correlation to the initial plating number ($r^2 > 0.9000$), while CellTiter-Glo had the highest dynamic range (**Figure 23B**). CellTiter-Glo and the luciferase reporting vector were the only two assays that did not require spheroid transfer. To correlate the luminescence from CellTiter-Glo to cell number, total DNA was measured across each spheroid and the DNA concentration (in nanograms) was compared to the luminescence read out. CellTiter-Glo luminescence significantly correlated to total DNA content using Pearson’s correlation (**Figure 23C**, $p < 0.05$). Finally, drug response to carboplatin was measured in 2D and 3D cultures using CellTiter-Glo and showed a dosage response that would not have been observed using spheroid size (**Figure 23D**). Taken together, these data supported using CellTiter-Glo, which provided an in-well, lytic measurement with a large dynamic range.

MCTS Drug Screening Identified Unappreciated Drug Candidates

To identify unappreciated therapeutics with an accelerated path for clinical development, an FDA-approved drug library available from Selleckchem was evaluated for screening. However, even though the 3D model was developed in a 96-well format to support high-throughput screening, I chose to pilot the screening assay and select a subset of drugs from the original 2,000+ compound library. To select a narrower library RNA, sequencing was performed between 2D and 3D cultures to identify regulatory pathways upregulated in MCTS compared to 2D cultures. Twelve ovarian cancer cell lines were grown to 8% confluence in 2D cultures and for 7 days in 3D cultures and RNAs were isolated for transcriptome sequencing. Following statistical analysis, robust changes under the two different growth conditions were observed. In cell lines grown under 3D conditions, 630 genes showed a 1.5-fold over expression compared to 2D cultures with a False Discovery Rate (FDR) of less than 0.05 (**Figure 24A**, blue points). Likewise, 96 genes showed a 1.5-fold over expression with a FDR of less than 0.05 in 2D cultures when compared to 3D cultures (**Figure 24A**, red points). Ingenuity Pathway Analysis (IPA) of the overexpressed genes in 3D cultures showed that the top upstream regulators were related to cellular hypoxia: HIF1A ($p=1.34E-10$), EPAS1 ($p=4.55E-08$), and cobalt chloride ($p=1.22E-07$) (**Figure 24B**). Interestingly, the drugs predicted to inhibit 3D gene expression were a nonsteroidal anti-inflammatory drugs (NSAID), fluticasone ($p=1.44E-09$), and an anti-diabetic, rosiglitazone ($p=4.55E-07$). Increases in both inflammation and metabolic pathways have both been linked to increased drug resistance in ovarian cancer (Lane, Matte et al. 2015, Rohnalter, Roth et al. 2015, Ai, Lu et al. 2016). Representative drugs representing known cancer therapeutics, and those that target inflammation and metabolic disorders were selected based on this IPA analysis **Figure 24** In order to identify unappreciated drugs with the potential of greater *in vivo* efficacy and activity against stem-like cells, it was hypothesized that the use of MCTS for drug screening will yield candidates that have not been previously selected as anti-cancer agents for ovarian cancer through traditional 2D screening methods. Therefore, the

selected subset of drugs from the Selleckchem library of FDA-approved drugs (n=304) was screened against EOC cells lines growing as 2D and 3D cultures. For this screening, cells were grown in 3D culture for four days or in 2D culture overnight before 10 μ M drug was added and incubated for 72 h (**Figure 25A**). Relative viability to DMSO controls was directly compared between 2D and 3D cultures. Drug hits were classified into three different classes: i) 2D only hits with more than 75% reduction in relative viability in 2D cultures and less than 25% reduction in relative viability in 3D cultures; ii) 2D and 3D hits with more than 75% reduction in relative viability for both 2D and 3D cultures; and iii) 3D only hits with more than 75% reduction in relative viability in 3D cultures and less than 25% reduction in relative viability in 2D cultures (**Figure 25B-C**). Overall, there were 78 drugs classified as hits, with 38 2D only hits, 25 2D and 3D hits, and 15 3D only hits (**Figure 25B-C**). Interestingly, the 3D only hits were mostly comprised of drugs from the inflammatory (47%) and metabolic (26%) groups compared to anticancer drugs (20%) (**Table 5**). When searching these drugs on PubMed for previously studies in ovarian cancer (numerator) or all cancers (denominator), there were very few positive results (**Table 5**), indicating these could be unappreciated candidates for drug repurposing in ovarian cancer. Drug screening in the non-tumorigenic cell lines HIO107 and HIO108 showed no 3D only drug hits (**Figure 26**), indicating these compounds may target the more quiescent drug resistance cells found in the MCTS model. However, through the primary screens at 10 μ M of each drug there were not any 3D only hits across multiple cell lines to select for further development. Since some of these repurposed drugs are used at higher concentrations in patients, the 2D and 3D cultures were again screened using a higher concentration of drug (25 μ M) to validate hits with activity across at least three of the four ovarian cancer cell lines. In the secondary screen the fifteen 3D only hits were rescreened, while only select hits from the 2D only and 2D and 3D hits were rescreened. Two drugs, licofelone (drug 179) and glafenine (drug 296) were validated as 3D only hits across at least three cell lines (**Figure 27**). Interestingly the hits from each class, as defined during primary drug screening, tended to retain the same

activity in secondary screening if they showed enough activity to be classified (**Figure 28A**). Validation of select hits from 2D only hits (afatinib, drug 2) and 2D and 3D (bortezomib, drug 3) hits confirmed the activity across multiple cell lines and in a dose-dependent manner (**Figure 28B-C**). While these hits were outside of the scope of the study, they could be further used to test the ability of 3D cultures to predict *in vivo* drug efficacy and better select which drugs to take forward into pre-clinical development. These results identified licofelone and glafenine, identified as candidate anticancer drugs with specific activity against ovarian MCTS that would not have been identified through traditional 2D cell culture drug screening.

The Top 3D Specific Drugs, Licofelone and Glafenine, Reduced Stemness in Ovarian MCTS

Licofelone, a dual COX/LOX inhibitor (**Figure 29A**) developed by Merckle and Ratiopharm (Germany) to treat osteoarthritis (Laufer, Tries et al. 1994, Gay, Neidhart et al. 2001, Boileau, Martel-Pelletier et al. 2002, Tries, Neupert et al. 2002, Skelly and Hawkey 2003), was evaluated using a dosage response from 195 nM to 50 μ M and showed preferential activity in 3D cultures compared to 2D cultures following 72 h treatments (**Figure 29B**). Specifically, the IC₅₀ values for licofelone were lower in 3D cultures than in 2D cultures across A1847 (21.6 μ M vs 50+ μ M), OVCAR3 (13.5 μ M vs 50+ μ M), OVCAR4 (33.5 μ M vs 50+ μ M), and OVCAR8 (17.4 μ M vs 44 μ M) cell lines. Glafenine (**Figure 29C**), an NSAID from the anthranilic acid derivative, was developed by Alecandria Co. (Egypt) for the relief of all types of pain; however, because of a high incidence of anaphylaxis and kidney failure has been withdrawn from the market in most countries (Raken 1972, Dechezlepretre, Lechat et al. 1975, Boeijinga and van der Vijgh 1977). When evaluated using a dosage response from 195 nM to 50 μ M, glafenine also showed preferential activity in 3D cultures compared to 2D cultures following 72 h treatments (**Figure 29D**). The IC₅₀ for glafenine were consistently lower in 3D cultures as compared to 2D cultures of A1847 (24 μ M vs 33 μ M), OVCAR3 (18.0 μ M vs 50+ μ M), OVCAR4 (30.3 μ M vs 50+ μ M), and OVCAR8 (9.1 μ M vs 50+ μ M) cell lines. To test the effect of both drugs on stem cell gene

expression, A1847 and OVCAR8 MCTS were treated with licofelone and glafenine for 72 h at the calculated IC₅₀ concentrations and RNA was isolated for gene expression analysis using qRT-PCR. In A1847 spheroids, licofelone and glafenine significantly reduced the expression of *CD133*, *ALDH1A*, and *KLF4* while the expression of *NANOG*, *OCT4*, and *SOX2* were either not changed or actually increased (**Figure 30A**, * = p<0.05, t-test). In OVCAR8 MCTS licofelone significantly decreased the expression of *CD133*, *ALDH1A*, *KLF4*, *NANOG*, and *SOX2* while glafenine significantly decreased the expression of only *ALDH1A* and *NANOG* (**Figure 30B**, * = p<0.05, t-test). Immunofluorescence analysis of A1847 spheroid sections following 72 h treatment with IC₅₀ concentrations of either licofelone or glafenine showed an increased number of Ki67 positive cells (63.5% and 67.9%, respectively) when compared to vehicle treated MCTS (41%) (**Figure 31**). In 2D cells derived from spheroids previously treated with paclitaxel for 3 days (which maintain and/or induce a stemness phenotype as shown in Chapter 2), licofelone enhanced efficacy as measured by a reduction in IC₅₀ compared to parental 2D cells (**Figure 32**). Interestingly, licofelone treatment showed stronger response than the COX-2 specific inhibitor celecoxib in A1847 spheroids (**Figure 33**), suggesting dual inhibition of COX/LOX better inhibits ovarian cancer spheroid growth than Cox-2 specific inhibition. Taken together these data support two different anti-inflammatory drugs, licofelone and glafenine, inhibit stem-like properties of ovarian cancer as measured by gene expression. Inhibiting stem-like properties of ovarian MCTS and inducing a more proliferative phenotype could enhance the activity of paclitaxel by circumventing drug resistance in combination with licofelone or glafenine. Licofelone was selected for further development of combination therapy with paclitaxel due to a more favorable clinical profile than glafenine.

Pretreatment of Ovarian MCTS with Licofelone Synergistically Enhanced Paclitaxel Activity

MCTS from A1847 and OVCAR8 cell lines were grown for four days before treatment with licofelone. Two days later serial dilutions of paclitaxel were added for another three days (**Figure 34A**). In A1847 spheroids, the combination of single pretreatment of either 10 μM or 20 μM licofelone (dashed lines represent mean viability and grey bars SD) enhance the dose response to paclitaxel (**Figure 34B**). There was a robust decrease in the IC_{50} for paclitaxel in the 10 μM (27 ± 8.8 nM vs >3 μM) or 20 μM (52 ± 7.1 nM vs >3 μM) licofelone pretreated spheroids. To quantify the resistant population response, the minimum viability was decreased at 3 μM , 1 μM and 333 nM in licofelone pretreated spheroids. The average viability across these dosages was significantly decreased ($p < 0.05$, $n=9$, t-test) for 10 μM licofelone pretreatments ($34.4 \pm 4.1\%$) and for 20 μM pretreatments ($25.6 \pm 6.1\%$) compared to $56.2 \pm 5.2\%$ in paclitaxel alone. For OVCAR8 spheroids, a robust change in dosage response was seen for both 10 μM licofelone and 20 μM licofelone (**Figure 34B**). The IC_{50} values for paclitaxel in both 10 μM and 20 μM licofelone pretreatments were significantly reduced ($p < 0.05$, $n=3$, t-test), 14 ± 4.1 nM vs 448 ± 67 nM and 3 ± 0.8 nM vs 448 ± 67 nM respectively. Similarly, the viability across the three highest concentrations was significantly lower ($p < 0.05$, $n=9$, t-test) in 10 μM licofelone ($29.5 \pm 9\%$) and the 20 μM licofelone ($23.6 \pm 1.12\%$) pretreatments compared to paclitaxel alone ($50.5 \pm .90\%$). To quantify the effect of combining treatments, Combination Index (CI) values were calculated (CI values less than 1 were considered synergistic). In A1847 spheroids, higher dosages of paclitaxel were synergistic with both 10 μM licofelone and 20 μM licofelone (**Figure 34C**). While in OVCAR8 spheroids, every dosage of paclitaxel was synergistic in 20 μM licofelone pretreatments, while only 1.3 nM paclitaxel was not synergistic in 10 μM licofelone pretreatments (**Figure 34C**).

Pretreatment of Ovarian MCTS with Paclitaxel Synergistically Enhanced Licofelone Activity

MCTS from A1847 and OVCAR8 cell lines were grown for four days before treatment with paclitaxel. Two days later serial dilutions of licofelone were added for another three days (**Figure 35A**). In A1847 spheroids, combinations of single pretreatment of either 1 μ M or 250 nM paclitaxel (dashed lines represent mean viability and grey bars SD) enhance the dosage response of licofelone (**Figure 35B**). In A1847 spheroids, the IC_{50} for licofelone was significantly ($p < 0.05$, $n=3$, t-test) decreased following paclitaxel treatments of 1 μ M and 250 nM paclitaxel compared to licofelone alone, 573 ± 187 nM vs 19.4 ± 1.9 μ M and 1.7 ± 0.59 μ M vs 19.4 μ M, respectively. To quantify the resistant population response, the average viability in the three highest dosages of licofelone (50 μ M, 25 μ M, and 12.5 μ M) was significantly lower ($p < 0.05$, $n=9$, t-test) in the 1 μ M paclitaxel pretreatment (11.3 ± 0.87 %) and the 250 nM paclitaxel pretreatment (23.3 ± 1.4 %) when compared to the licofelone alone treatment (73.9 ± 2.1 %). In OVCAR8 spheroids, the IC_{50} for licofelone was significantly ($p < 0.05$, $n=3$, t-test) decreased following paclitaxel treatments (1 μ M or 250 nM) compared to licofelone alone, 235 ± 47 nM vs 35 ± 6.4 μ M and 558 ± 232 nM vs 35 ± 6.4 μ M, respectively. The average viability in the three highest dosages of licofelone (50 μ M, 25 μ M, and 12.5 μ M) was significantly lower ($p < 0.05$, $n=9$, t-test) in the 1 μ M paclitaxel pretreatment (18.1 ± 1.25 %) and the 250 nM paclitaxel pretreatment (22.2 ± 1.4 %) when compared to the licofelone alone treatment (63.0 ± 3.0 %). As before, the CI values were calculated and A1847 spheroids pretreated with 250 nM paclitaxel showed synergy at all concentrations of licofelone, except 390 nM while, pretreatment with 1 μ M paclitaxel showed synergy at 1.56, 3.12, 6.25, 12.5, 25, and 50 μ M licofelone (**Figure 35C**). In OVCAR8 spheroids, 1 μ M paclitaxel and 250 nM paclitaxel pretreatments showed synergy across every dosage of licofelone (**Figure 35C**).

Licofelone Combination Enhanced Apoptosis and Reduced Stemness Induced by Paclitaxel

A1847 and OVCAR8 spheroids were treated with either 1 μ M paclitaxel or a combination of 1 μ M paclitaxel and 20 μ M licofelone for 72 hours. Licofelone combination with paclitaxel significantly reduced the expression of a number of stemness related gene in A1847 MCTS (*ALDH1A*, *CD133*, *NANOG*, *OCT4*, and *SOX2*) and OVAR8 MCTS (*ALDH1A*, *CD133*, and *KLF4*) when compared to paclitaxel treatment alone as measured using Taqman qRT-PCR assay (**Figure 36**, n=3, *= p<0.05, t-test). The same combination therapy of 1 μ M paclitaxel and 20 μ M licofelone in A1847 MCTS significantly increased the expression of cleaved PARP compared to total PARP (3-fold) when compared to vehicle treatment, as measured by Western blot analysis (**Figure 37**, n=3, *=p<.05, t-test). The single treatment of 1 μ M paclitaxel showed a trend towards an increase in the ratio of cleaved PARP to total PARP (2-fold) when compared to vehicle, but this difference was not deemed significant. To test the stem-like function of MCTS cells that survived drug treatments, A1847 or OVCAR8 MCTS were treated with drug (vehicle, 1 μ M paclitaxel, 20 μ M licofelone, or 1 μ M paclitaxel plus 20 μ M licofelone) for 72 hours. Following drug treatment, MCTS were washed and disassociated into single cells with trypsin/accutase. Recovered cells were plated onto 2D cell culture dishes and incubated overnight. The following day cells were collected following trypsinization and the number of viable cells was established in order to evaluate their growth using either a soft agar colony forming assay or a MCTS forming assay. For the A1847 cells, the number of colonies appears to be reduced following treatment with either paclitaxel or the combination, but not in the licofelone alone treatment group when compared to vehicle (**Figure 38**). However, the size individual colonies were increased significantly increased in cells previously treated with paclitaxel when compared to vehicle treatments (**Figure 38**, n=30, p<.05, two way ANOVA). Interestingly, the combination of licofelone with paclitaxel significantly reduced colony size when compared to paclitaxel treatment alone (**Figure 38**, n=30, p<.05, two-way ANOVA). For the

OVCAR8 cells, paclitaxel treatment slightly reduced colony number, while the combination of paclitaxel and licofelone drastically reduced colony formation. In comparison and somewhat unexpected, licofelone treatment completely inhibited colony formation (**Figure 38**). Likewise, the size of colonies formed from paclitaxel treatment in OVCAR8 cells was significantly increased when compared to vehicle treatments and the combination of licofelone with paclitaxel significantly reduced colony size compared to paclitaxel alone (**Figure 38**, n=30, p<.05, two-way ANOVA). Secondary spheroid assays were similarly performed following drug treatment in A1847 and OVCAR8 spheroids. Viable cells were counted, serially diluted, and plated at 3,000, 1,000, 300, 100, 30 or 10 cells per well. Following 7 days of incubation, the spheroid size was measured. A1847 cells treated with 1 μ M of paclitaxel showed a significant increase in spheroid size when compared to vehicle for the wells with 300, 100, 30 or 10 cells (**Figure 39**, n=3, *p< 0.05, two-way ANOVA). Consequently, the combination of 20 μ M licofelone with 1 μ M paclitaxel significantly reduced spheroid size when compared to 1 μ M paclitaxel for the wells seeded with 1,000, 300, 100, 30 or 10 cells (**Figure 39**, n=3, *p< 0.05, two-way ANOVA). OVCAR8 spheroids showed a different pattern where both 20 μ M licofelone and the combination of 20 μ M licofelone with 1 μ M paclitaxel both significantly reduced spheroid size when compared to vehicle in the wells with 1,000, 300, 100, 30 or 10 cells (**Figure 39**, n=3, *p< 0.05, two-way ANOVA). These studies suggest that licofelone combination can inhibit the stem-like phenotype observed to be induced following paclitaxel treatment. Blocking the stemness phenotype that is induced during clinical disease progression could enhance the efficacy of treatment in ovarian cancer and diminish disease recurrence and chemotherapy resistance.

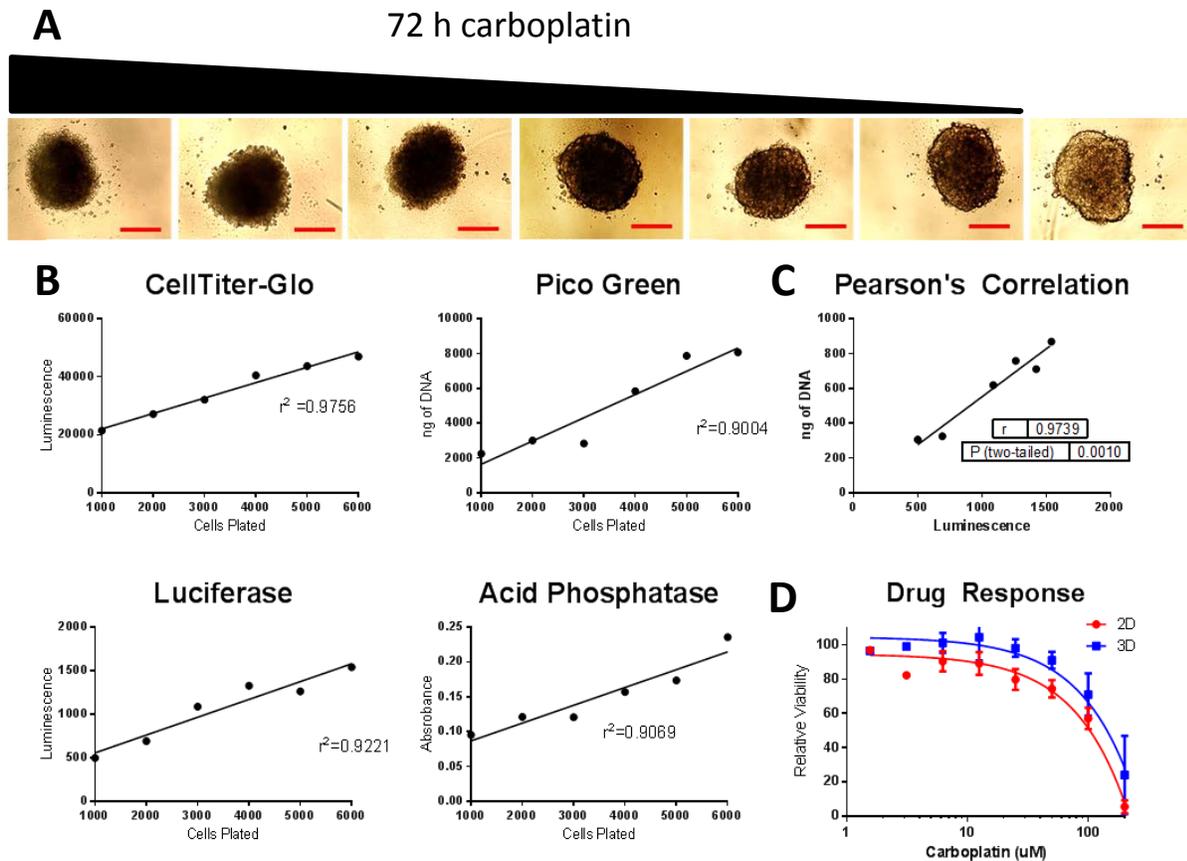
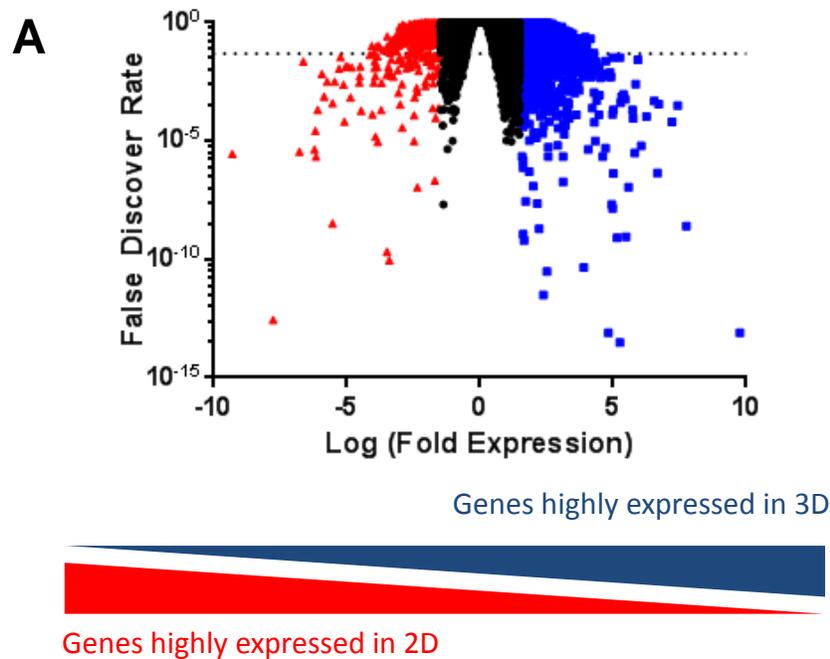


Figure 23: CellTiter-Glo Measured Cell Viability and Drug Response in MCTS.

(A) Spheroid size and volume is a poor predictor of drug response. Light microscopy of MCTS after 72 hr carboplatin treatments in A1847 MCTS (200 μ M, 100 μ M, 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 1.125 μ M and vehicle left to right). (B) Correlation of A1847 spheroid plating density after 72 h and a variety of assays to measure cell viability or absolute DNA amount (r^2 calculated using the linear regression function in GraphPad Prism software). (C) Pearson's analysis shows a strong statistically significant correlation between cell viability measured using CellTiter-Glo and DNA amount measured using the PicoGreen assay. (D) Dosage response to carboplatin in 2D (red) or 3D (blue) cultures measured using CellTiter-Glo.



B **IPA Pathway Regulators**

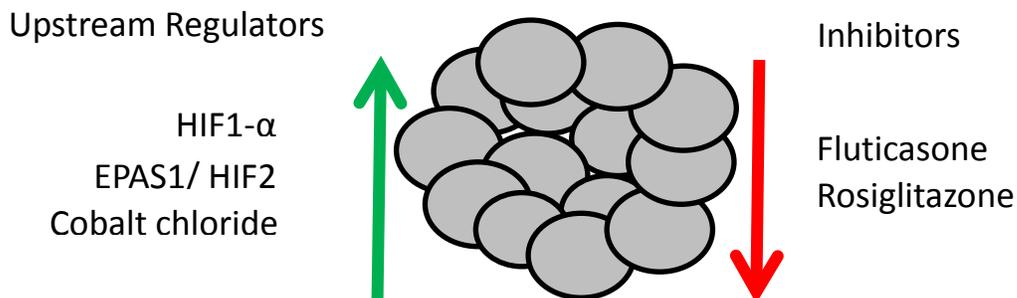


Figure 24: Pathway Analysis of Transcriptome Sequences Identified Inflammation and Metabolism Upregulated in MCTS.

(A) Analysis of RNA-seq data identified genes expressed 1.5-fold greater in 3D cultures (blue) versus 2D and genes 1.5-fold greater in 2D cultures (red) versus 3D with a False Discovery Rates less than 0.05 (dashed line). (B) The top five IPA pathway analysis regulators inferred from the genes identified to be over expressed by at least 1.5-fold in 3D versus 2D culture conditions.

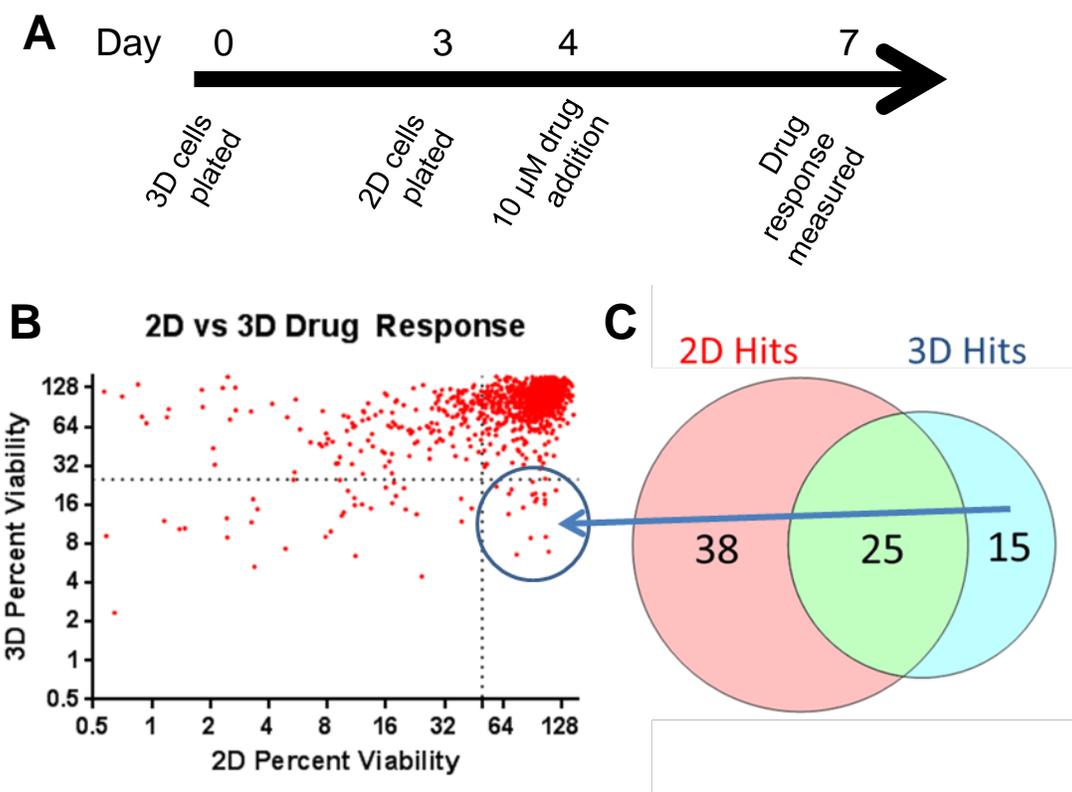


Figure 25: 3D Cell Culture Screening Identified Drug Hits Unappreciated by 2D Cultures.

(A) Representative time-line for drug screening in 2D and 3D cultures. (B) Relative viability compared to vehicle (DMSO) between both 2D and 3D cultures following 72 h treatment of 10 μ M drugs. (C) Venn diagram representing unique and common drug hits: 2D only hits with more than 75% reduction in relative viability in 2D cultures and less than 25% reduction in relative viability in 3D cultures; 2D and 3D hits with more than 75% reduction in relative viability for both 2D and 3D cultures; and 3D only hits with more than 75% reduction in relative viability in 3D cultures and less than 25% reduction in relative viability in 2D cultures.

Table 5: 3D Hits from Primary Screening

Drug	Indication	Target or Use	Pub Med Hits
Pizotifen malate	Inflammation	Serotonin antagonist	0/0
Risedronic acid (Actonel)	Metabolic Disease	Osteoporosis drug	0/109
Hydroxyurea (Cytodrox)	Cancer	Free radical nitroxide	43/4234
Pioglitazone hydrochloride (Actos)	Metabolic Disease	Cytochrome P450 (CYP)2C8 and CYP3A4	3/529
Mecarbinat	Metabolic Disease	Antihypertension	0/0
Licofelone	Inflammation	COX/LOX	0/24
OSI-420 (Erlotinib)	Cancer	EGFR	82/5077
Geniposidie	Inflammation	Glucagon-like peptide-1	0/2
Phenformin HCl	Metabolic Disease	Anti-diabetic. Glycolysis	2/129
Cinepazide maleate	Inflammation	Calcium Channel blocker	0/0
Beclomethasone dipropionate	Inflammation	Glucocorticoid	0/60
Dexamethasone acetate	Inflammation	Glucocorticoid	302/14137
Allylthiourea	Inflammation	Ammonia oxidation	0/3
Bergapten	Cancer	DNA mutagen	0/76
Glafenine	Inflammation	NSAID	0/4

List of 3D specific drug hits (15) identified from the primary drug screen. The disease indication from most drugs was from either inflammation or metabolic disease and not cancer. The specific target or use for each specific drug as listed from the Selleckchem database. PubMed search results for searching each drug with either “ovarian cancer” (numerator) or “cancer” (denominator) indicates most 3D specific drugs have had limited studies in ovarian cancer.

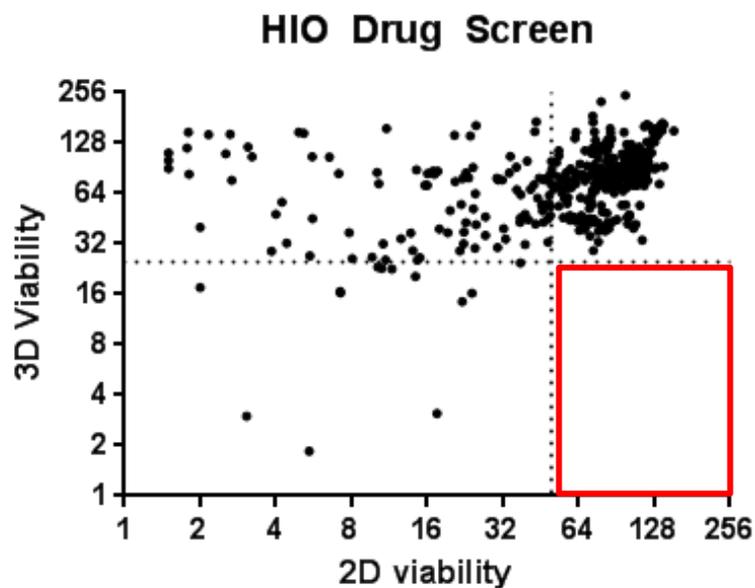


Figure 26: 3D Drug Screening in Non-tumorigenic Cell Lines did not Identify 3D Specific Hits.

Two HIO cell lines (HIO107 and HIO118) were grown as 2D and 3D cultures and screened using the established library of 304 FDA approved drugs. The relative viability was compared to vehicle (DMSO) in both 2D and 3D cultures following 72 h treatment of 10 μ M drug. No 3D specific hits (75% reduction in relative viability in 3D cultures and less than 25% reduction in relative viability in 2D cultures) were observed in HIO cell lines (red box).

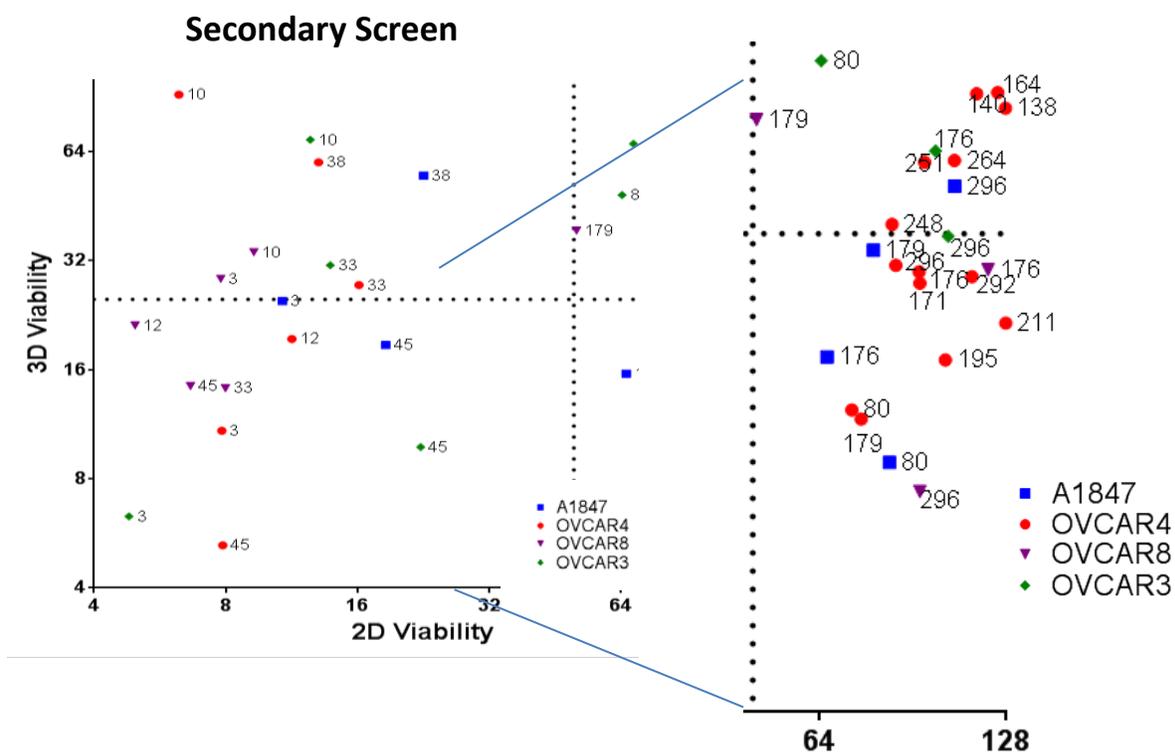
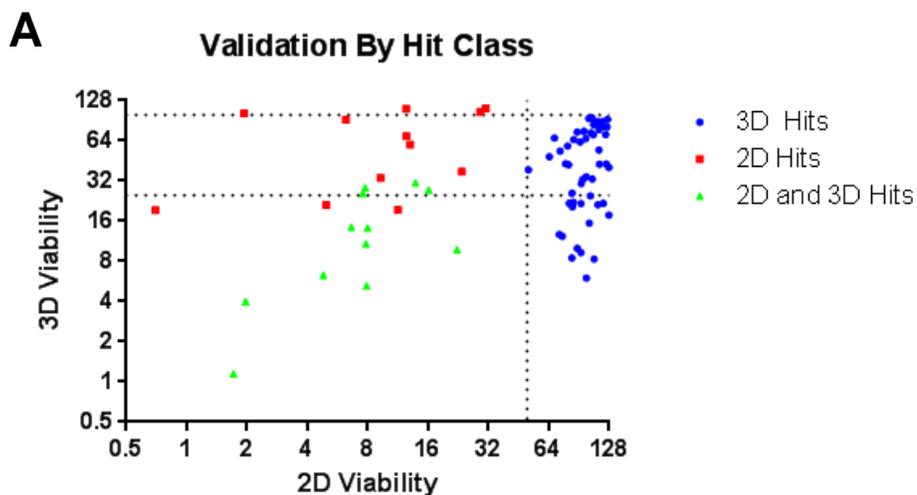
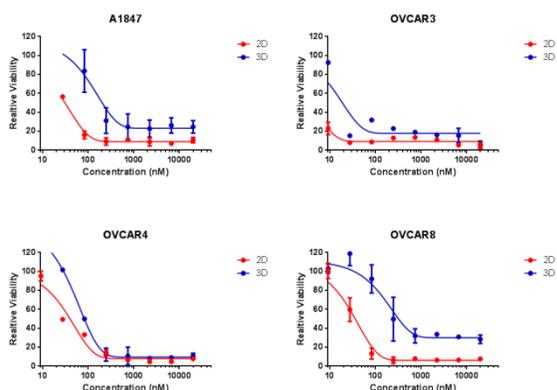


Figure 27: Secondary Screening Validated 3D Specific Hits Across Multiple Cell Lines.

Secondary screening in A1847 (blue squares), OVCAR4 (red circles), OVCAR8 (purple triangles) and OVCAR3 (green diamonds) was performed to validate hits across multiples cell lines. The relative viability was compared to vehicle (DMSO) in both 2D and 3D cultures following 72 h treatment of 25 μ M drug. Secondary screening at 25 μ M validates two hits (drug 176 and 296) with specific activity in 3D cultures across at least three ovarian MCTS as show in the right panel (enlarged section of 3D drug response).



B Bortezomib (2d and 3D Hit)



C Afatinib (2D Only Hit)

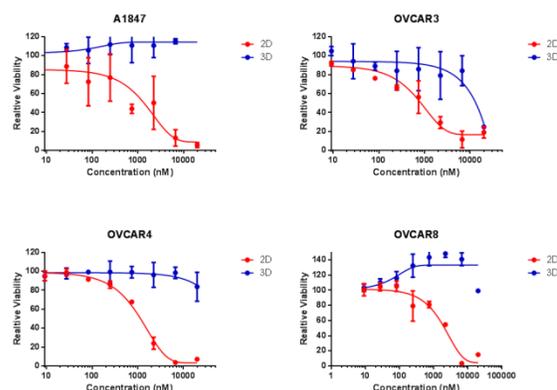
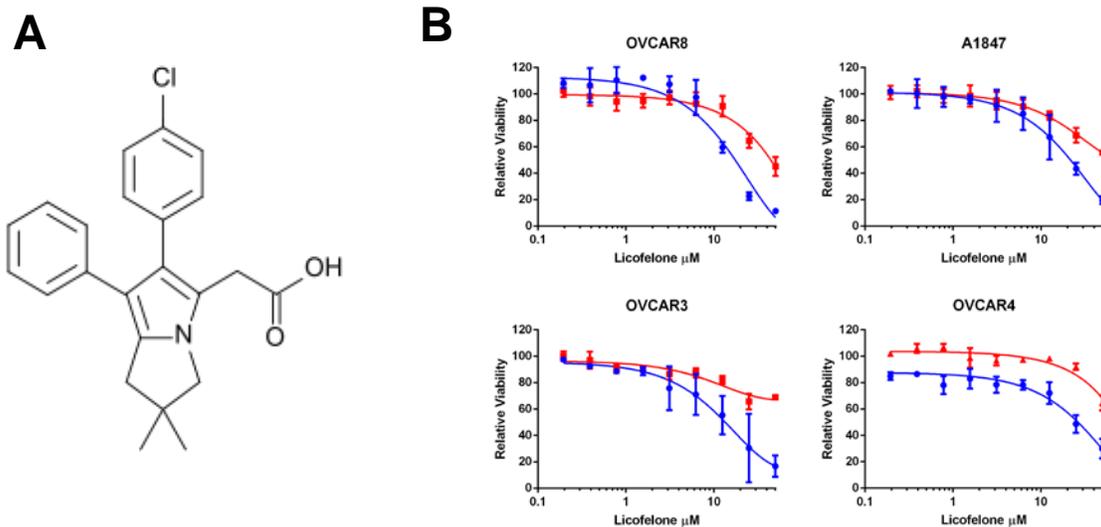


Figure 28: Dosage Response Validation of 2D Only Hit and 2D and 3D Hit.

(A) Plots of relative viability for both 2D and 3D drug response for each hit class identified in the secondary screening using 25 μ M of drug. 2D only hits (red squares) and 2D and 3D hits (green triangles) validated within their own specific class. 3D only hits (blue circles) remained either 3D only hits or showed no change in viability. (B) Bortezomib maintains 2D and 3D activity following dosage response treatment for 72 h. (C) Afatinib was identified as a 2D specific drug as shown in the dosage response curves following 72 h drug treatment.

Licofelone



Glafeanine

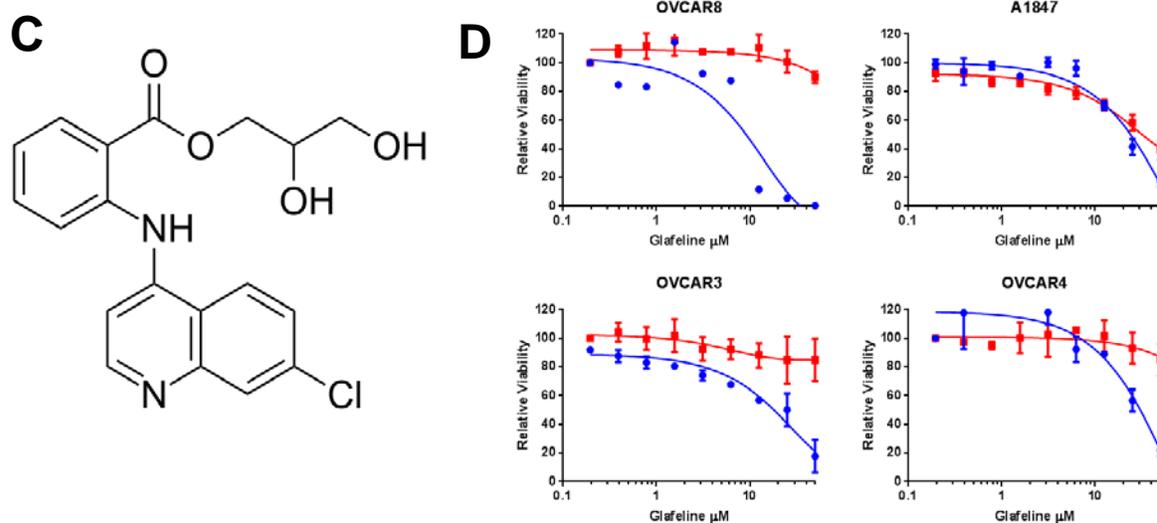


Figure 29: Dosage Response Showed 3D Specific Activity of Both Licofelone and Glafeanine.

(A) The chemical structure of the dual COX/LOX inhibitor licofelone. (B) Dosage response to licofelone as measured using CellTiter-Glo following treatment of the ovarian cancer lines grown as MCTS (blue line) or 2D cultures (red line) at 72 h. (C) The chemical structure glafeanine. (D) Dosage response to glafeanine as measured using CellTiter-Glo following treatment of the ovarian cancer lines grown as MCTS (blue line) or 2D cultures (red line) at 72 h.

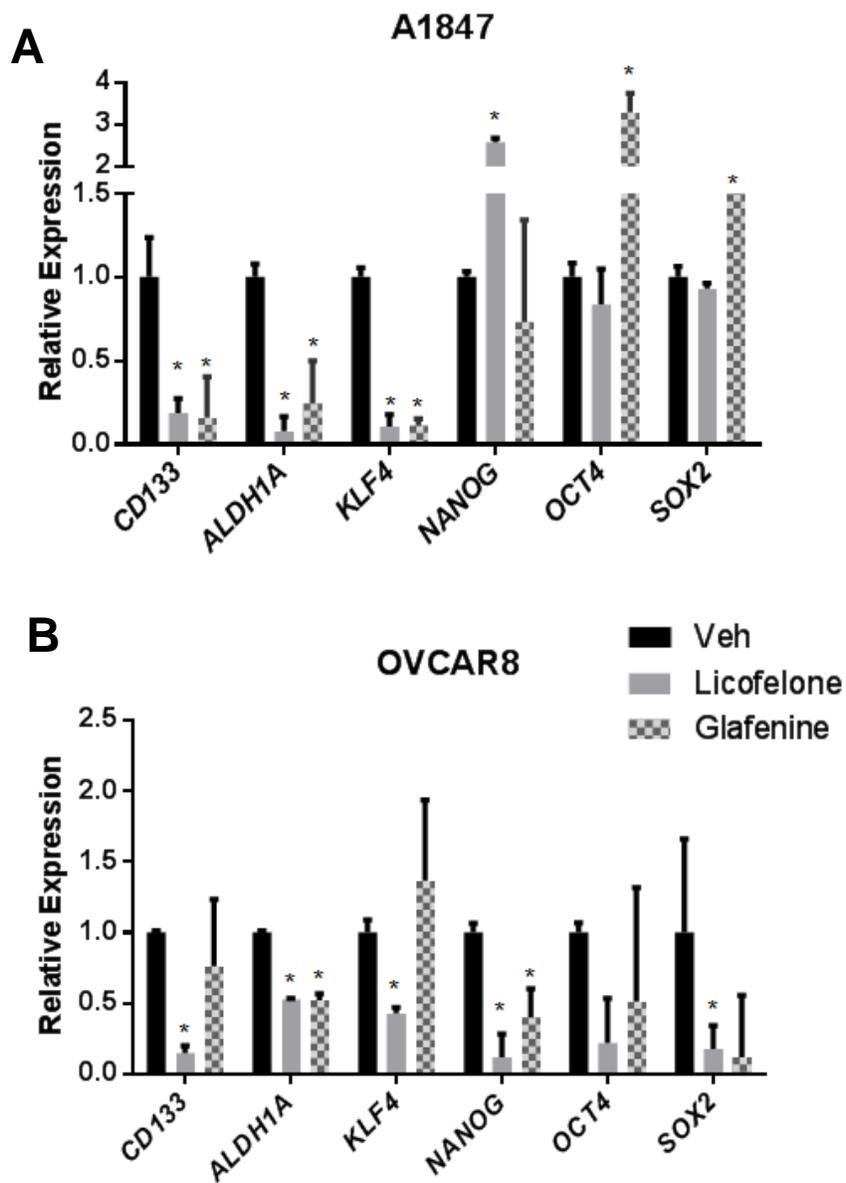


Figure 30: The 3D Specific Drugs Licofelone and Glafenine Reduced Stem-like Gene Expression in Ovarian MCTS.

Expression of stem-like genes (*ALDH1A*, *CD133*, *KLF4*, *OCT4* and *SOX2*) in MCTS following 72 h treatment with IC_{50} concentrations of licofelone (solid bars) or glafenine (checked bars). Expression was measured using qRT-PCR and compared to vehicle only treated spheroids (black bars). (A) *CD133*, *ALDH1A*, and *KLF4* expression is significantly ($*= p < 0.05$) reduced in A1847 MCTS. (B) OVCAR8 MCTS demonstrated significant ($*= p < 0.05$) reduction in the expression of all genes measured except for *OCT4*. All data are represented as mean \pm SD, $n=3$.

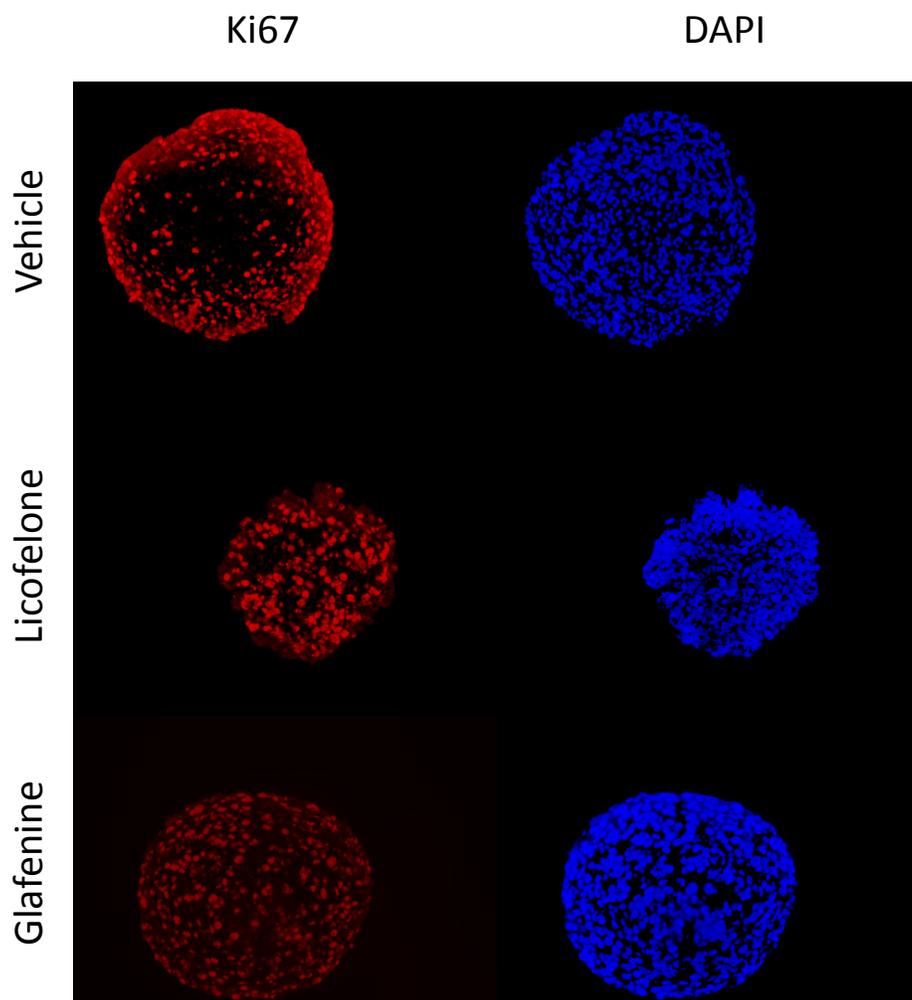


Figure 31: Glafenine and Licofelone Treatment Increased Cell Proliferation in Ovarian MCTS.

Immunofluorescence evaluation of Ki67 (red) and DAPI (blue) reveals an increase in the number of inner proliferative cells following the treatment of spheroids with licofelone or glafenine at previously established IC_{50} concentrations in A1847 cells.

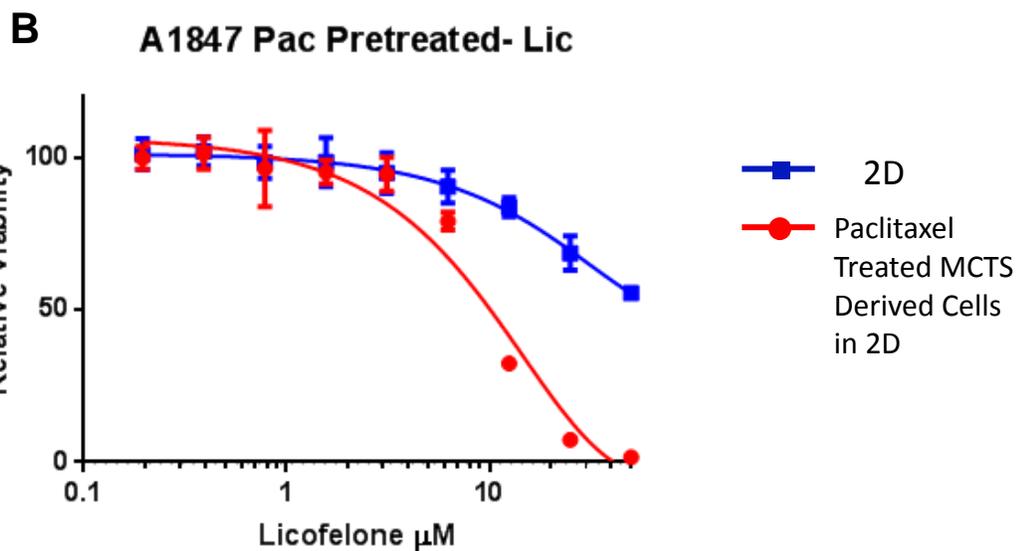
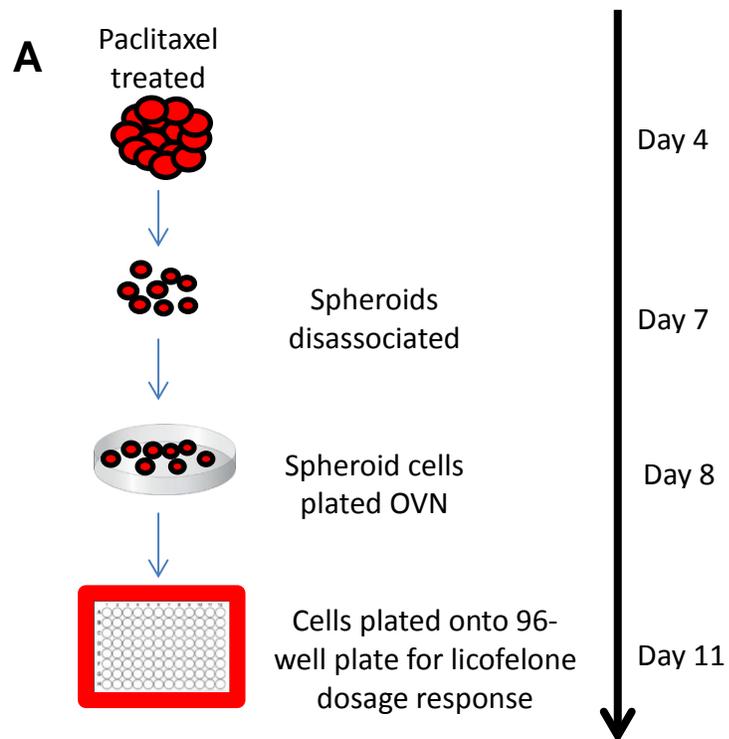


Figure 32: Licofelone Showed Enhanced Activity in Paclitaxel Resistant Cells.

(A) Graphical representation of the method used to measure licofelone response in 2D cells derived from paclitaxel treated MCTS. (B) Dosage response of A1847 cells to licofelone in cells grown in 2D cultures or MCTS derived cells treated with 1 μM paclitaxel. Paclitaxel pretreated cells (red) show enhanced response to licofelone when compared to cells grown in 2D (blue). All data are represented as mean \pm SD, n=3.

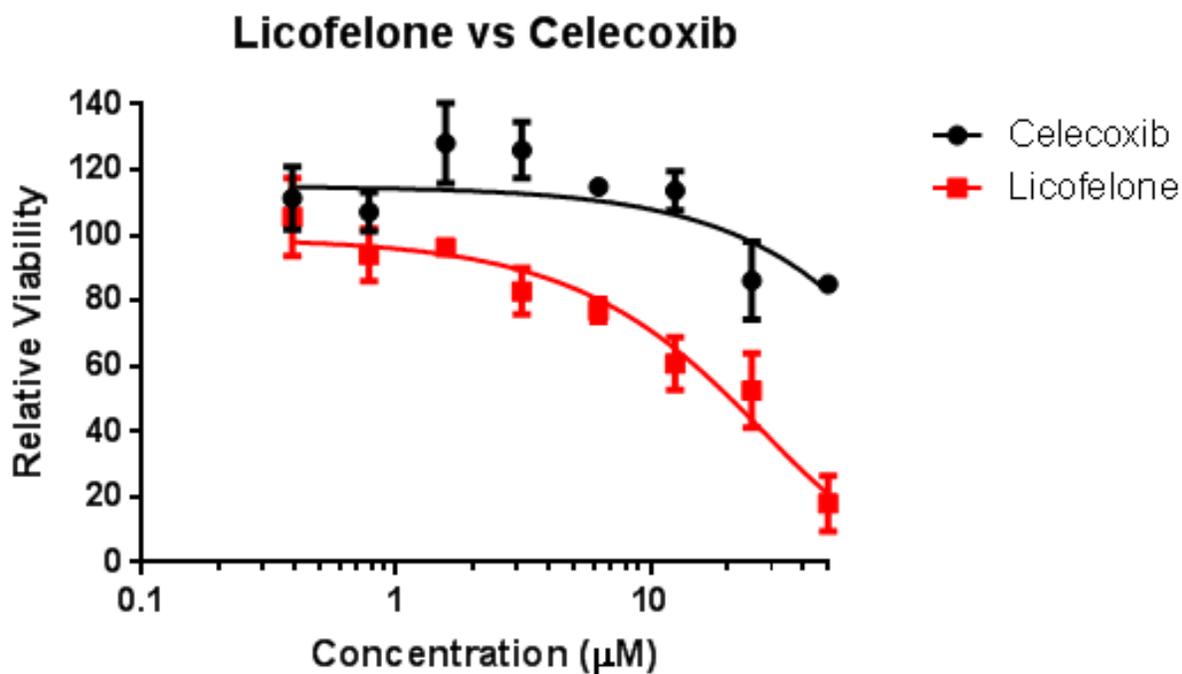


Figure 33: Licofelone had More Activity Against Ovarian Cancer Cell-Derived MCTS than the COX inhibitor Celecoxib.

Comparison of drug response following 72 h treatment in A1847 MCTS to either celecoxib (black) or licofelone (red) as measured by CellTiter-Glo. The dual COX/LOX inhibitor licofelone has stronger activity compared to the COX-2 specific inhibitor celecoxib.

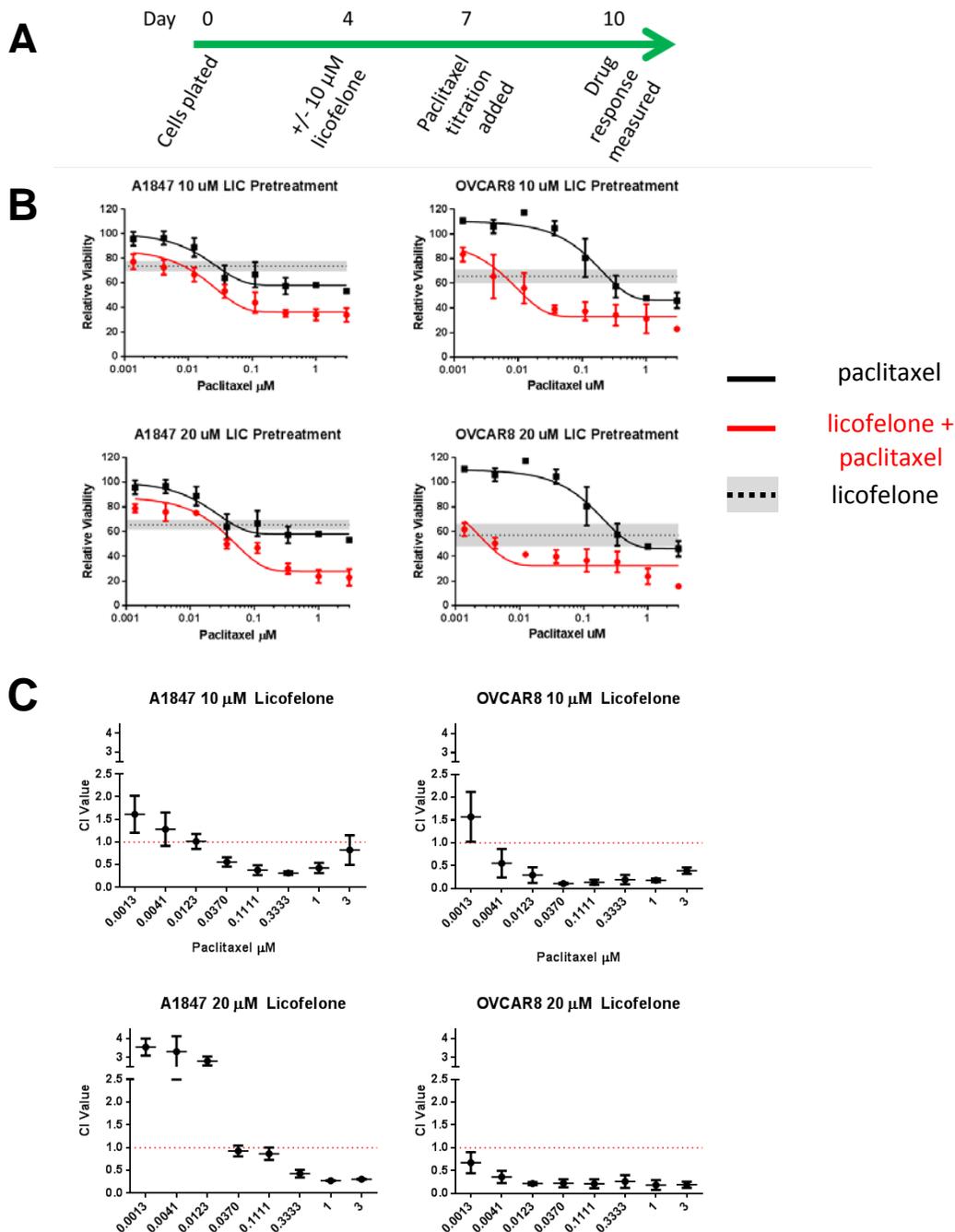


Figure 34: Pretreatment of Ovarian Cancer Cell-Derived MCTS with Licofelone Synergistically Enhanced Paclitaxel Activity.

(A) Representative time-line of drug combinations in A1847 and OVCAR8 spheroids. (B) Dosage response to paclitaxel alone (black line) is lower than paclitaxel following 72h pretreatments with 10 μM licofelone (top) or 20 μM licofelone (bottom) (red lines). Licofelone alone treatments of 10 μM or 20 μM are represented by the horizontal dashed lines (mean) and grey boxes (SD). (C) Combination Index values for A1847 and OVCAR8 spheroids show synergy ($\text{CI} < 1$, red dashed line) across multiple dosages. All data are represented as mean \pm SD, $n=3$.

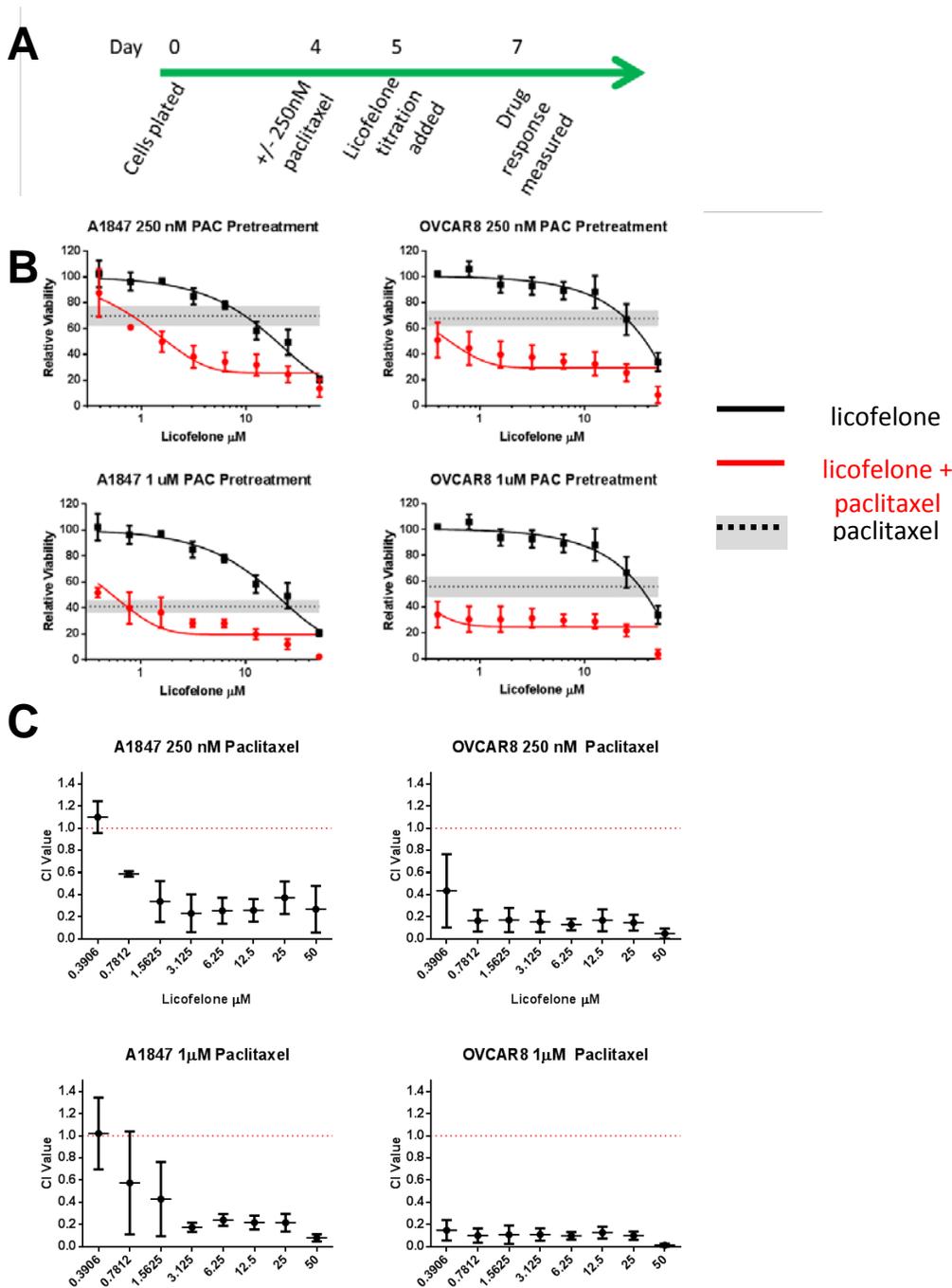


Figure 35: Pretreatment of Ovarian Cancer Cell-Derived MCTS with Paclitaxel Synergistically Enhanced Licofelone Activity

(A) Representative time-line of drug combinations in A1847 and OVCAR8 spheroids. (B) Dosage response to licofelone alone (black line) is lower than licofelone following 72 h pretreatments with 250 nM paclitaxel (top) or 1 μM paclitaxel (bottom) (red lines). Paclitaxel alone treatments of 250 nM or 1 μM are represented by the horizontal dashed lines (mean) and grey boxes (SD). (C) Combination Index values for A1847 and OVCAR8 spheroids show synergy ($\text{CI} < 1$, red dashed line) across multiple dosages. All data are represented as mean \pm SD, $n=3$.

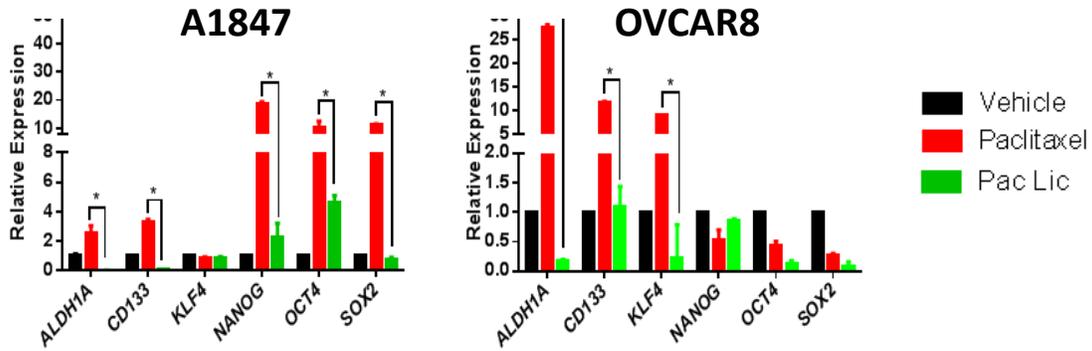


Figure 36: Licofelone Blocked the Induction of Stem-like Gene Expression by Paclitaxel.

Expression of stem-like genes (*ALDH1A*, *CD133*, *KLF4*, *OCT4* and *SOX2*) following a 72 hr treatment with vehicle (DMSO) (black), 1 μ M paclitaxel (red), or the combination of 1 μ M paclitaxel and 20 μ M licofelone (green) in A1847- or OVCAR8-derived spheroids. Licofelone treatment significantly reduces stem cell gene expression induced by 1 μ M paclitaxel in the ovarian cancer cell-derived MCTS (*= $p < 0.05$, t-test). All data are represented as mean \pm SD, $n=3$.

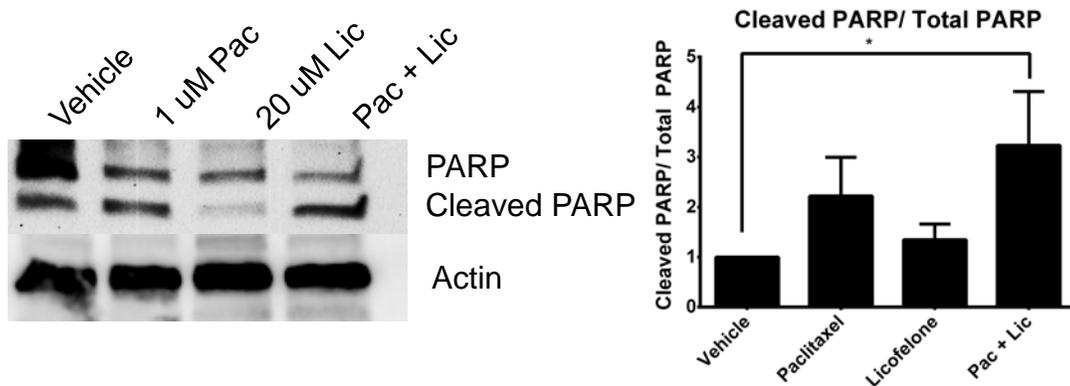


Figure 37: Combination of Licofelone and Paclitaxel Increased Apoptosis in Ovarian Cancer Cell-Derived MCTS.

Western blot analysis of PARP and cleaved PARP in A1847 spheroids (left panel) and densitometry analysis (right panel) show a significant increase of cleaved PARP to total PARP in the combination treatment of 1 μM paclitaxel and 20 μM licofelone. Single treatment of 1 μM paclitaxel shows a slight, yet not significant increase in cleaved PARP.

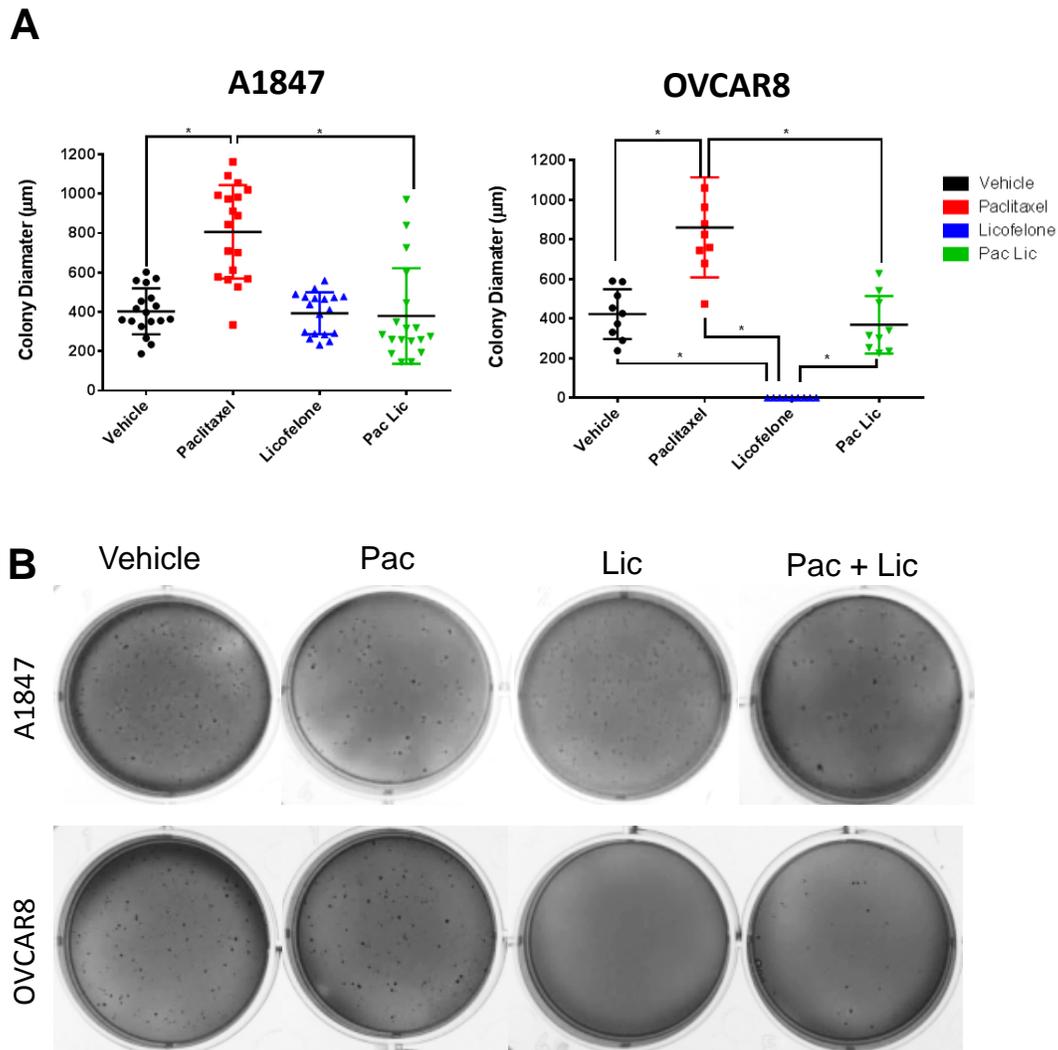


Figure 38: Licofelone Decreased Stem-like Function of Paclitaxel Treated MCTS.

(A) Relative size (μm) of colonies grown using a soft agar forming assay following drug treatment of A1847 or OVCAR8 MCTS with vehicle (DMSO) (black), 1 μM paclitaxel (red), 20 μM licofelone (blue), or the combination of 1 μM paclitaxel and 20 μM licofelone (green) and disassociation into single cells. Colony size was significantly increased following 1 μM paclitaxel and was decreased following 1 μM paclitaxel and 20 μM licofelone treatments ($n > 10$, $* = p < 0.05$, two-way ANOVA). (B) Representative images of colonies formed using a soft agar forming assay following drug treatment of A1847 or OVCAR8 MCTS with vehicle (DMSO), 1 μM paclitaxel, 20 μM licofelone, or the combination of 1 μM paclitaxel and 20 μM licofelone and disassociation into single cells.

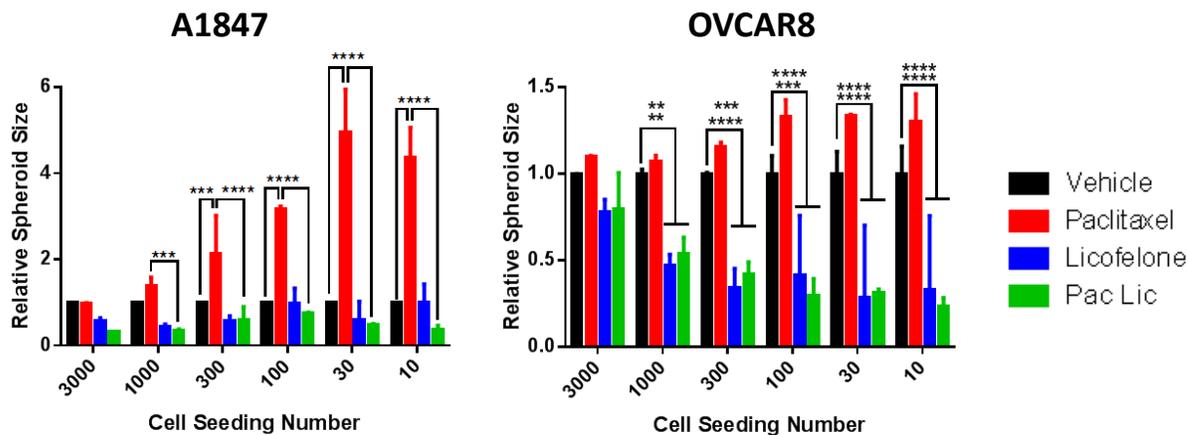


Figure 39: Licofelone Reduced the Secondary Spheroids Forming Ability of Paclitaxel Treated MCTS.

Spheroid size (μm) of secondary A1847 or OVCAR8 spheroids formed from viable cells plated at 3,000, 1,000, 300, 100, 30 or 10 per well following drug treatment of MCTS with vehicle (DMSO) (black), 1 μM paclitaxel (red), 20 μM licofelone (blue), or the combination of 1 μM paclitaxel and 20 μM licofelone (green) and enzymatic disassociation. Spheroid size from both A1847 or OVCAR8 cells was significantly increased following 1 μM paclitaxel and was decreased following 1 μM paclitaxel and 20 μM licofelone treatments ($n=3$, $*=p<0.05$, two-way ANOVA).

Discussion

Studies have used the *in vivo* representation of solid tumors in three-dimensional cell culture to validate primary drug screens (Fayad, Rickardson et al. 2011, LaBarbera, Reid et al. 2012, Kenny, Lal-Nag et al. 2015). However, these approaches still rely on traditional drug screening methods in two-dimensional cultures. The use of three-dimensional cell culture in primary screening has shown there could be drug targets that favor hypoxic and drug resistant properties demonstrated by MCTS (Wenzel, Riefke et al. 2014, Rotem, Janzer et al. 2015, Zhang, Hua et al. 2016). Therefore, the activity of clinically repurposed drugs across 2D and 3D cultures was directly compared to identify new drug candidates, unappreciated by traditional screening in 2D cultures. Developing a drug from the bench side to the clinical approval and use can take over 10 years and cost over 1 billion dollars (Paul, Mytelka et al. 2010). Drug repurposing provides a cheaper and accelerated pathway to the clinic for drugs with new indications since they have known delivery and toxicity profiles from previously studies that were used to gain clinical approval (Weir, DeGennaro et al. 2012, Strittmatter 2014). A previous study I participated in from my laboratory identified repurposed drugs that synergized with frontline chemotherapy (imatinib) in gastrointestinal stromal tumors (Pessetto, Ma et al. 2014), indicating repurposed drugs could be a promising pipeline for development in treating chemotherapy resistant ovarian cancer as well. To compare drug response between these two methods, a subset of compounds were “cherry picked” from the Selleckchem FDA-Approved drug library based on the IPA Pathway analysis of genes selectively expressed in ovarian cancer cells grown in 3D cultures. This selected drug library includes current cancer drugs as well as metabolic and inflammatory drugs, based on the top pathway regulators identified via IPA analysis. The use of clinically repurposed drugs provides a more direct and cost effective

route to clinical development, since they have already been through toxicity testing and other pre-clinical development.

Through my drug screens, compounds classified as 2D and 3D hits were predominantly from the current cancer therapeutic class in the drug screening. Many of these drugs would have been developed in traditional 2D cultures and then brought to the clinical after they showed efficacy in animal models. In fact, the top two hits from this class were the proteasome inhibitor bortezomib and the DNA intercalating agent, doxorubicin. Both drugs have been evaluated in clinical trials with varying degrees of success (Aghajanian, Blessing et al. 2009, Gibson, Alzghari et al. 2013, Staropoli, Ciliberto et al. 2014). Bortezomib and doxorubicin were used in combination in a phase II clinical trial in patients with relapsed ovarian cancer, but showed minimal antitumor activity (Parma, Mancari et al. 2012). Our study showed three-dimensional cell culture drug screening is an alternative to traditional screens by comparing the drug efficacy of a selected FDA-approved library in 2D cultures and 3D cultures. Most of the 3D only hits came from metabolic or anti-inflammatory drugs that had not been extensively studied in cancer before based on literature searches and clinical trials. To my knowledge, the top two 3D only hits, licoferone and glafenine, have not been evaluated in preclinical or clinical studies for ovarian cancer. This approach identified alternative drug hits, unappreciated by traditional drug screens.

Inhibiting stemness in ovarian cancer could enhance chemotherapy response and improve progression free survival (Landen, Goodman et al. 2010, Silva, Bai et al. 2011, Wang, Yo et al. 2012). It is clear stem-like properties are a promising area for drug targeting in ovarian cancer. While specific inhibitors of CD133 are not available, genetic targeting CD133 reduced ovarian cancer progression in mice (Skubitz, Taras et al. 2013). My thesis research identified two anti-inflammatory drugs, licoferone and glafenine, through drug screening using ovarian cancer cells grown as 3D spheroids that inhibited stem cell properties, including altered expression of

ALDH1A, *CD133*, *KLF4*, *NANOG*, *OCT4*, and *SOX2* and decreased colony forming ability. OVCAR8 spheroids showed stronger reduction in the level of stem cell gene transcripts following licofelone treatment, which also corresponded to a complete loss of colony forming ability. A1847 spheroids maintained some colony forming ability following licofelone treatment, which could be driven by the expression of *NANOG*, *OCT4*, and *SOX2* which form a positive feedback loop (Boyer, Lee et al. 2005, Ivanova, Dobrin et al. 2006, Loh, Wu et al. 2006). Interestingly, licofelone and glafenine also induced proliferation, indicating they might not be promising single agents, but could enhance paclitaxel activity by driving a less stem, more proliferative phenotype. Indeed, there was a synergistic combination between licofelone and paclitaxel following consequential addition of either drug. Order of addition favored licofelone as a secondary therapy, reducing viability of ovarian MCTS to approximately 1% at higher dosages of licofelone. However, both combinations regardless of the treatment scheme showed drug synergy. Importantly, the combinations of licofelone with paclitaxel blocked the stem like properties induced by paclitaxel treatment as measured by gene expression, colony forming ability and the size of secondary spheroids following drug addition.

Licofelone is a novel non-steroidal anti-inflammatory (NSAID) that inhibits both COX-2/5-LOX that was developed for the treatment of arthritis. COX-2, or cyclooxygenase-2, is prostaglandin synthase that converts arachidonic acid to PGH₂. COX-2 is overexpressed in ovarian cancer patients and may play a role in tumor progression (Singhal, Spiegel et al. 2005, Lee, Choi et al. 2006). Interestingly, COX-2 has shown to induce stem cells in breast cancer cell lines (Majumder, Xin et al. 2016). 5-LOX, or 5-lipoxygenase, is overexpressed in ovarian cancer and contributes to the HIF-1 α mediated hypoxia response (Ji, Wang et al. 2013, Wang, Ma et al. 2014). Likewise, LOX expression was significantly increased in ovarian MCTS when compared to 2D cultures. The only cell line without significant overexpression of LOX, was OVCAR3 and this cell line showed the lowest response to licofelone. While the use of licofelone

in cancer therapy has been limited, it has been shown to inhibit cigarette smoke induced lung cancer (Balansky, Ganchev et al. 2015) and blocked pancreatic cancer induction with a marked decrease in stem cell markers (Mohammed, Janakiram et al. 2015). While licofelone has not been evaluated clinically for ovarian cancer, celecoxib, a COX-2 specific inhibitor, enhanced chemotherapy in Phase II trial (Legge, Paglia et al. 2011). However, licofelone showed enhanced efficacy in ovarian MCTS when compared to celecoxib and is known to have fewer side effects (Bias, Buchner et al. 2004). In this study, licofelone was shown to inhibit stem-cell like characteristics of ovarian MCTS. Glafenine is also a NSAID and was recently identified as an ABCG2 inhibitor in a drug repurposing screen (Zhang, Byun et al. 2009). ABCG2, a member of the ATP Binding Cassette Sub-Family, is highly expressed in subpopulations of ovarian cancer stem cells (Dou, Jiang et al. 2011, Kryczek, Liu et al. 2012). ABCG2 is highly expressed, along with other stem cell markers, in recurrent, chemotherapy resistant prostate cancer (Guzel, Karatas et al. 2014). However, I chose to further evaluate licofelone as compared to glafenine which has been discontinued based on its associated toxicities. While licofelone has not achieved FDA approval, it has been extensively studied in Europe for its role in arthritis and reduced side effects compared to COX-2 specific inhibitors (Bias, Buchner et al. 2004).

Alternatively, both drugs are NSAIDs, which might indicate a role of mitochondrial regulation by blocking stemness in ovarian cancer MCTS via decreased mitochondrial function and metabolism (Vaish, Tanwar et al. 2011). The use of NSAIDs, specifically aspirin, has been retrospectively shown to lower the risk of ovarian cancer in a dose and frequency dependent manner (Murphy, Trabert et al. 2012, Trabert, Ness et al. 2014). Ovarian cancer stem cells have enhanced mitochondrial metabolism as noted by increased oxidative phosphorylation metabolism compared to glucose metabolism, shunted pyruvate to the Krebs cycle, and an increase in ROS production (Pasto, Bellio et al. 2014). Ovarian cancer spheroid stem cells

favor the Krebs cycle to induce hypoxia resistant metabolism (Liao, Qian et al. 2014). Targeting mitochondrial function by inhibiting complex III with atovaquone, a repurposed malaria drug, showed a reduction in OXPHOS and inhibited breast cancer stem cells (Fiorillo, Lamb et al. 2016). Licofelone is able to induce mitochondrial apoptosis in colon cancer cells independent of arachidonic acid cascade activity (Tavolari, Bonafe et al. 2008). Taken together with these data, NSAIDS could be blocking mitochondrial function and limiting stem-like cell metabolism to inhibit stem-like cells. This could help provide insight into the regulation of stem-like cell expansion or induction in ovarian cancer patient tumors.

Conclusion

The studies reported in this thesis shows that three-dimensional culturing of ovarian cancer cell lines provides an alternative to traditional drug screening models. By screening a select drug library chosen based on identified pathway regulators in MCTS, drugs that specifically inhibit 3D cell cultures were identified. Drugs with specific activity in ovarian MCTS showed the ability to inhibit stemness and to synergize with paclitaxel, as well as inhibit the stem-like phenotype induced in cells that survive paclitaxel (**Figure 40**). These data support the further clinical development of licofelone for the treatment of patients with ovarian cancer and the use of 3D models in the identification and validation of newly formulated anti-cancer drugs. By reversing the stem-like phenotype with licofelone, there were fewer viable cells that survive paclitaxel. If this phenomenon was replicated in patients, it could decrease the cells that survive chemotherapy long term and replenish the tumor when therapy is complete and prevent disease recurrence and drug resistance, the two primary factors in patient mortality in ovarian cancer.

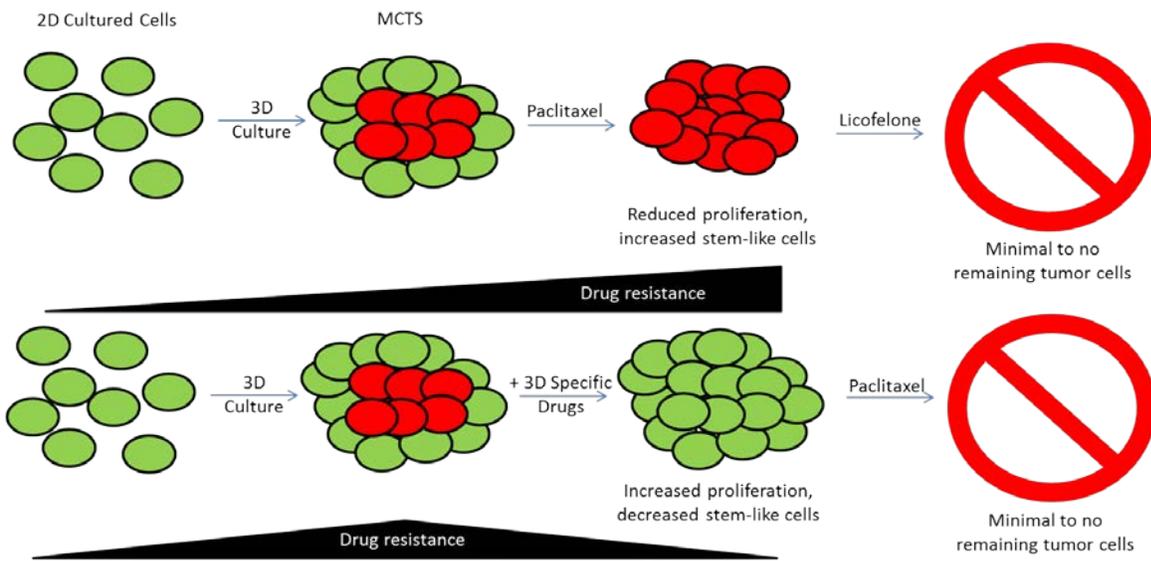


Figure 40: Summary of 3D Specific Drug Activity in Ovarian MCTS.

Chapter 4: Summary, Discussion, and Future Directions

Summary

My thesis provides a scientific rationale and supporting data to indicate that the 2D *in vitro* method used to screen for drug activity in ovarian cancer cells can provide many false positives and missteps in the drug development process. Drug development for the treatment of primary ovarian cancer has been stagnant since the approval of paclitaxel in 1992. This lack of success could be due in part to discrepancies between preclinical *in vitro* ovarian cancer models and the highly heterogeneous disease diagnosed in patients and treated by oncologist. In this thesis I combined knowledge of clinical ovarian cancer and chemotherapy resistance, with a three-dimensional cell culture approach to provide an alternative method to screen for drugs that might have better therapeutic activity in the clinic. This approach led to the identification of a drug, licofelone, a dual COX/LOX inhibitor developed as a potential treatment for osteoarthritis. I found that licofelone can specifically target cells grown in 3D cell culture and reverse mechanisms of drug resistance such as stem-like characteristics, which could improve the efficacy of paclitaxel in patients with recurrent disease.

In Chapter 1, I provided a broad background of ovarian cancer and discussed the myriad of clinical trials using targeted therapies and the combination studies of cytotoxic drugs with these molecular therapies, which have for the most part failed to greatly improve the PFS or OS of patients with epithelial ovarian tumors (Bell, Brady et al. 2006, Bookman, Brady et al. 2009, Pecorelli, Favalli et al. 2009, Mannel, Brady et al. 2011). Recent studies have called into question the capacity and usefulness of traditional 2D cell cultures in mimicking clinical disease, which is thought to be the limiting factor in the success of current drug screening and development approaches (Domcke, Sinha et al. 2013), and worse yet, providing false positive leads that use critical time and valuable resources. Therefore, when I was contemplating

various research projects for my thesis, I decided to focus on applying knowledge of chemotherapy resistance in a clinical setting to improve the *in vitro* representation of drug-resistant disease using ovarian cancer cell lines. Designing cell culture models that better reflect cells that survive chemotherapy and repopulate tumors could identify unappreciated drugs that would not have otherwise been selected using traditional screening methods which have thus far not produced any efficacious drugs to date.

Towards this goal, in Chapter 2 I described the development and optimization of a 3D cell culture model that reflected various aspects of drug resistant ovarian cancer that cannot be appreciated using traditional 2D cultures and that can be easily and reproducibly adapted for further drug screening. By using a 3D culture method, ovarian cancer cell lines formed MCTS that induced drug resistant mechanisms such as reduced cell proliferation, cellular hypoxia, and the expression of stem cell-related transcripts (Huang, Ao et al. 2010, Steg, Bevis et al. 2012, Dobbin, Katre et al. 2014, Liao, Qian et al. 2014). Consequently, MCTS from 3D cultures of ovarian cancer cell lines were significantly more resistant to paclitaxel than their 2D cultured counterparts. Mimicking progression of disease in a clinical setting, following paclitaxel treatment, cells within MCTS showed significantly higher expression levels of stem cell genes and reduced cell proliferation (Steg, Bevis et al. 2012). The cells from MCTS maintained their stemness and drug resistance characteristics following disassociation and reseeded into 2D cultures, indicating that the drug resistant phenotype was stable and not just a phenomenon of 3D culture alone.

Finally, in Chapter 3 I used the MCTS 3D drug screening model to screen a subset of a drug repurposing library in order to identify unappreciated drug candidates that target the stem-like cells within MCTS and thereby improve the efficacy of chemotherapy in ovarian cancer. Several rounds of drug screening of these clinically relevant drugs revealed that two anti-

inflammatory compounds, licofelone and glafenine, reduced cell viability in 3D cultures more so than in 2D cultures. Additional validation and follow-up experiments measuring gene expression and cell proliferation revealed that these two anti-inflammatory drugs reversed the stem-like properties of MCTS. Furthermore, I showed that licofelone synergized with paclitaxel and reversed paclitaxel resistance and associated phenotypes in ovarian MCTS.

In summary, 2D cell cultures predominantly used for the vast majority of drug screening studies do not accurately represent the drug resistance present in recurrent clinical samples and therefore, produce misleading drug hits that ultimately do poorly when given to patients with ovarian cancer. Using a 3D MCTS model with ovarian cancer cell lines for drug screening results in preferential selection of drugs, which target stem cell-like properties and synergize with paclitaxel. While the top drug hits have potential for clinical development, application of this 3D model in large scale drug screening of chemical libraries could yield additional drug and chemical probe hits with far more potential at improving patient survival than what has been achieved thus far using the classical 2D screen models. Adapting the 3D culture approach to drug screening for ovarian cancer has the potential to aid in the identification and development of new drugs that complement existing front line therapy and improve patient survival for the first time in almost thirty years.

Discussion and Future Directions

With the completion of the proof-of-principle (PoP) mid-throughput drug screen in my thesis, I have provided sufficient evidence for the feasibility of successful and rapid 3D drug screen without the requirement of any special equipment. My studies indeed identified two previously unappreciated drugs with anti-cancer activity against ovarian cancer cell. However, the PoP studies were performed using a cherry-picked library of 304 drugs, a smaller number compared to those of other high-throughput published studies which exceed 16,000 drugs (Gupta, Onder

et al. 2009, Visnyei, Onodera et al. 2011). The use of a smaller library was necessary in my screening method due to two factors both related to drug delivery. Firstly, the 3D MCTS were formed using 96-well plates, which are less efficient than 384- and 1536-well plates for drug screening. However, 96-well plates were needed for these screens as they provided a larger surface area to coat with agarose, while 384-well plates required a very small amount of agarose to cover the wells and thus lead to rapid solidification of the agarose in the wells and subsequent improper coating. Secondly, in my screening model the drug was added on top of previously plated cancer cells, which limits the volume of additional liquid that could accurately be applied by robotics when using 384-well plates. For volumes less than 5 μ L, accurate delivery in mid- or high-throughput methods is typically performed by coating plates with microsprays and adding cell media on top of the drug. In the absence of ready access to such sophisticated instruments, the 96-well platform was used for my PoP studies. And while the use of 96-well plates was sufficient for the small scale drug library that was used in my screens, it is clear that any expanded scale drug screen will require technological modifications to the MCTS drug screening platform accommodate 384-well or 1536-well plates. Combining my PoP data, the clinical properties of 3D cell culture, and its use in drug screening will aide in developing these more efficient screening techniques for large chemical compound libraries such as those available on the Lawrence campus of KU in the High Throughput Screening facility (<https://hts.ku.edu/>). Furthermore, rapid adaptation of this model for other solid tumors will help development of additional unappreciated cancer therapeutics for other devastating diseases.

Based on my studies, there are several strategies that could be adapted for the current 3D screening platform to help improve its throughput. One of these strategies is to form MCTS, treat with vehicle (or paclitaxel) (such as in Chapter 2, **Figure 18**), dissociate the cells and plate under 2D growth conditions for typical high throughput drug screening methods. While I have shown that these cells retain paclitaxel resistance and express stem cell markers, other drug

resistance mechanisms such as cellular hypoxia signatures might not be retained in this method. This method would retain the advantage of the low cost of agarose coated plates, while adding about 10 days to the cell preparation method compared to traditional 2D culture screening. Secondly, using another 3D culture method that is adaptable to 384 or 1536 well plates could provide a more efficient model to apply drug directly to spheroids. However, this could require high cost, low attachment plates and would not solve the problem of adding drug on top of the already plated media. Lastly, since the spheroids do not invade the solid agarose, spheroids could be transferred following formation on top of plates with drugs that have been coated with small volumes of drug. While each of these methods add either extra time or cost to my established model, the data I have shown support that the investment could be worthwhile in terms of identifying more unappreciated drugs to the treatment of ovarian cancer. My proof-of-principle screen provides further rationale to use this type of 3D cell culture model for future drug discovery and development in ovarian and potentially other cancers.

While the spheroid disassociation and re-plating experiments described above and in Chapter 2 could be used for scaling drug screening, they also provide a platform for studying long term drug resistance and how cells may survive chemotherapy and repopulate tumors several months later. Maintaining drug resistance and stem cell gene expression from 3D cells into 2D culture could provide insight into longer term studies. My study did not investigate how long this phenotype is stable and whether it is being driven by positive feedback loops between stem cell transcription factors (Boyer, Lee et al. 2005, Ivanova, Dobrin et al. 2006, Loh, Wu et al. 2006) or other long term factors such as epigenetic regulation. Long term studies of drug resistance indicate drug resistance is likely driven by combinations of multiple mechanisms (Rohnalter, Roth et al. 2015). Determining how long the drug resistant phenotype is maintained following 3D culture and paclitaxel treatment would require time course studies to identify specific cellular alterations that might maintain or regulate drug resistance. Using my 3D culture

to study cells that survive paclitaxel in my model is also advantageous since there can be around 3- to 5-fold more cells that survive compared to 2D culture, increasing the amount of biological and cellular assays that could be applied.

Another area that is supported for further development by my thesis is the pre-clinical and clinical development of licofelone for the treatment of ovarian cancer. I showed promising data that both licofelone and glafenine can both rescue the stem-like phenotype of ovarian MCTS while also increasing cellular proliferation. Since licofelone is better tolerated in the clinic (glafenine has been pulled from the shelf due to the degree of severe side effects) I chose to evaluate it in combination with paclitaxel. Licofelone showed synergy with paclitaxel regardless of the order of addition and could be included in front-line therapy or developed as a therapeutic approach to resensitize tumors once they have become refractory to chemotherapy. Both of these approaches could seek to eliminate the cells that survive chemotherapy and eventually lead to disease recurrence and death of ovarian cancer patients. The primary advantage of drug repurposing is that it creates an accelerated pathway to the clinic due to completion of previous clinical studies and a co-operative funding mechanism between academic laboratories and pharmaceutical companies (Weir, DeGennaro et al. 2012). Since pharmaceutical companies own the intellectual property to the drug, they often collaborate with academic labs that have found off label uses to develop the product for other maladies by providing the compound or other financial support. However, while licofelone was included in the Selleckchem FDA-approved drug library, it has only gone through clinical studies and achieved clinical approval in Europe, not the USA. Working with pharmaceutical companies to obtain licofelone has also been problematic since it was developed in part by two German pharmaceutical companies, Merckle and Ratiopharm, which were later purchased by Teva Pharmaceuticals (Petah Tikva, Israel). Identifying who owns the intellectual property and

creating contacts from there will be the key going forward. Efforts to gain support for potential clinical development of licoferone for the treatment of ovarian cancer patients are still ongoing.

While my primary focus was on unappreciated drugs identified by 3D culture screening, another promising development based on my thesis would be the predictive value of 3D culture compared to 2D culture for *in vivo* drug delivery. *In vivo* animal models are much more expensive and time consuming than *in vitro* cell models. However, they are necessary for pre-clinical drug delivery and development studies. Using 3D culture to better predict the success of drugs in *in vivo* models could save valuable time and money invested in drugs that could be predicted not to have success. When validating hits from primary drug screening in 2D cultures, one study found that the more hydrophobic the mitotic inhibitors identified from 2D cultures were the better they performed in 3D cultures (Fayad, Rickardson et al. 2011). Interestingly, I found differences between the 3D culture responses between drugs with strong activity in 2D cultures (Chapter 3, **Figure 28**). Specifically, afatinib had strong activity against ovarian cancer cells in 2D culture but minimal response in 3D cultures while bortezomib had strong response in both 2D and 3D cultures. Validating more hits from both “2D only” and “2D and 3D” hits from my study could provide multiple drugs to compare the *in vivo* predictive value of 3D cultures.

Finally, the mechanism by which both anti-inflammatory drugs, licoferone and glafenine, inhibit ovarian cancer stem cells could provide insight into the progression of clinical disease. Specific targets of licoferone, COX/LOX, or glafenine, ABCG2, have roles in ovarian cancer progression, stemness, and drug resistance (Singhal, Spiegel et al. 2005, Lee, Choi et al. 2006, Dou, Jiang et al. 2011, Kryczek, Liu et al. 2012, Ji, Wang et al. 2013, Wang, Ma et al. 2014). However, general effects of anti-inflammatory drugs could be to block mitochondrial function which has also been related to cancer stem cell function and metabolism (Vaish, Tanwar et al. 2011, Murphy, Trabert et al. 2012, Liao, Qian et al. 2014, Trabert, Ness et al. 2014). Expanding the specific knowledge of how these drugs are regulating ovarian cancer stem cells could

identify targets for either drug repurposing or unappreciated drug development. Likewise, if these drugs are targeting stem cells through independent, specific pathways, selecting patient cohorts in clinical trials could be performed based on target expression.

Perspective

The last front-line chemotherapy drug approved for ovarian cancer was almost 30 years ago (Einzig, Wiernik et al. 1992), before technological phenomena such as the iPhone (2007), Facebook (2004), Google (1998), Windows NT (1993), and home dial-up internet service, America Online (1993). In the time from the internet being introduced to people's homes (AOL and Windows NT) to it being at the tip of the fingers for almost 70% of the United States population with the advent of smartphones (Anderson 2015), no major drug discoveries were made for the treatment of ovarian cancer; the fifth leading cause of cancer-related death among women in the United States (Siegel, Naishadham et al. 2013, Howlader, Noone et al. 2015). In my thesis I detail how this could be attributed to failures in clinical trials for ovarian cancer driven in part by culture models that do not accurately represent the most common causes of death in clinical disease, resistance to front line chemotherapy. While many attempts have been made to continue to use cytotoxic agents that target many aspects of cell division and have failed (Bell, Brady et al. 2006, Bookman, Brady et al. 2009, Pecorelli, Favalli et al. 2009, Mannel, Brady et al. 2011), my study provides an alternative method to drug screening that identifies drugs that target mechanisms of long term cell survival and stemness. By identifying a frontline drug screening method that targets populations of ovarian cancer cells not represented by traditional methods, I have provided a potential pathway for developing new drugs for the treatment of ovarian cancer for the first time in almost 30 years.

In summary, through my thesis studies I developed a 3D cell culture model of ovarian cancer which results in cells demonstrating reduced sensitivity to paclitaxel and alterations in

associated drug resistance pathways. This 3D cell culture method provided a drug screening platform for identification of unappreciated, anti-inflammatory drugs, with unappreciated anti-cancer activities which could synergize with paclitaxel. This thesis research studies provide the rationale for further exploration of 3D cell culture for the development of unappreciated drug therapies to treat ovarian cancer.

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