Patient-Derived Xenografts as Pre-Clinical Models of Response to Chemotherapy

by

Paulina Cybulska

A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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2014

Abstract

Ovarian high-grade serous cancer (HGSC) is the most lethal gynecologic malignancy and well-characterized models may improve patient outcomes. Patient-derived xenografts (PDXs) recapitulate disease heterogeneity; however, to be useful in predicting response to novel chemotherapeutics, they must reflect the response of the donor tissue to standard chemotherapy. The objectives of this study were: first, to evaluate the response of PDXs’ to platinum therapy and compare this response to that of the donor; and second, to determine whether treatment with chemotherapy enriches for tumourigenic cells. Eighteen samples formed tumours in the mammary fat pads of NOD-Scid-IL2Rγnull mice and were treated with Carboplatin. There was a 100% concordance between sample status and PDXs response to chemotherapy. HGS histology was confirmed for all cases. A conclusion regarding post-chemotherapy tumourigenicity could not be made due to inadequate statistical power. PDXs represent useful tools for evaluation of novel therapies and identification of patients who are platinum-resistant/sensitive.
Acknowledgments & Contributions

First, I would like to thank both of my supervisors, Drs. Benjamin Neel and Marcus Bernardini. Ben’s incredible scientific knowledge inspires the pursuit of answers. Ben taught me to always be an inquisitive, critical thinker, not afraid of asking questions or challenging concepts. He also taught me the value of collaboration and scientific ‘consultation’. It has been a memorable, privileged journey.

I cannot understated the impact that Dr. Bernardini has had on my training, both during residency and graduate school. I look up to him professionally, as a brilliant surgeon and clinician, and personally, as a dedicated spouse and parent. I am so grateful for his encouragement, praise, and motivation (‘stay cool!’).

In addition, I am so grateful to all members of my committee Dr. Blaise Clarke, Dr. Micheline Piquette, and honorary member, Dr. Amit Oza. I truly value the time you committed these projects. Your ideas and suggestions were always mindful, sensible and instrumental to completing the project.

Thank you to my defense examiners: Dr. Catherine O’Brien, Dr. Ted Brown and Dr. Barbara Vanderhyden. Thank you to Dr. Howard Mount for your time and input.

Thank you Dr. Marcus Bernardini for obtaining all of the clinical patient information. Thank you to Dr. Blaise Clarke for all of the histological annotations and scoring. Thank you to Dr. Kwan Ho Tang for all of the virus work required for the bioluminescence project and to Dr. James Jonkman for your BLI teaching.

My research could not have been initiated if it were not for the wonderful support I received from the department of Obstetrics and Gynecology. Thank you to Dr. Heather Shapiro for encouraging me to pursue this goal. My extreme gratitude to Dr. Donna Steele for her constant wise words, mentorship, support, laughs and tears. You are an amazing woman and I am blessed to have you on my side. Thank you to Drs. Alan Bocking and John Kingdom for making the clinician investigator program an option. Thank you to Dr. Satkunaratnam who allowed me to continue working in the OR, despite nervous hands.

To all of my labmates – none of my achievements (or failures!) could have been possible without your help. I am so lucky to have had the pleasure of working with you all. Thanks to Sherifa Mohamed for dealing with everything administrative. Cathy Iorio, Richard Marcotte, Gordon Chan, Kwan Ho Tang, Angel Sing, Shengqing Gu, Xionan Wang, Raquel De Souza – thank you for all of your help, particularly in the days when I didn’t know how to do simple tasks! Cathy, it has been a tremendous gift to get to know you and your family.

To Dr. Laurie Ailles and members of the Ailles lab, particularly Dr. Craig Gedye, Ella Hyatt and Keira Pereira. To Dr. Gordon Keller and members of the Keller lab- thank you for always including me in your social events. Your friendships are blessings!

Dianne Chadwick (and the Biobank team), Golnessa Mojtahedi and Sarah Rachel Katz, thank
you for putting up with my constant emails and questions. Carl Virtanen- thank you for always making time for me despite your busy schedule.

Thank you to all of my new grad school friends: Alicia Tone, Jennifer Woulter, Tracy Liu, Alec Witty, Keira Pereira, Shawn Stapleton, TD MacDonald. I am grateful for all of the science discussions and social events. To my residency family: thank you for always including me in activities and remembering that I am still part of the program (both my ‘former’ colleagues and ‘new’ colleagues). Special thanks to Laura Sovran, Kelsey Mills, Louise-Helene Gagnon, Helena Frecker, Karli Mayo, and Brian Liu.

To my Ottawa family: Jennifer Brodeur, Nadine Doris, Erin Lamont, Peter Unger, Courtney Maskerine, Julie Hakim, and Robyn Phanouvong; my life would be empty without you. To my Toronto family: Justin Weissglas, Jenny Ferguson, Christa Favo, Caitlin McKeever, Adrian Sacher, Lauren Lapointe-Shaw, Shannon Corbett, Jennica Platt: you brighten my days.

Dr. Susan Goldstein – your words of wisdom have been indispensible.

Finally, to the sister I never knew I had – Jocelyn Stewart, my pip. Getting to know and love you has been one of the greatest gifts. You made this project possible through your scientific input and amazing personality (you are the reason I dance with my arms up). I cannot wait to share more experiences with you. Thank you to Mamma Lynne and Papa Tom for incorporating me into the family.

Lastly, to my mom: nothing could be possible without you. You have always made me believe that I can achieve anything. Thanks to your courage, I am able to succeed. Kocham mocno.
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<th>Definition</th>
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<tbody>
<tr>
<td>AMH</td>
<td>Anti-Müllerian Hormone</td>
</tr>
<tr>
<td>AA</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AMG</td>
<td>Medical Oncology trial drug</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
</tr>
<tr>
<td>BRAF</td>
<td>Proto-oncogene B-Raf</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer 1, early onset</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer 2, early onset</td>
</tr>
<tr>
<td>BSA</td>
<td>Body Surface Area</td>
</tr>
<tr>
<td>BSO</td>
<td>Bilateral salpingo-oophorectomy</td>
</tr>
<tr>
<td>C.I.</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>CI</td>
<td>Combination Index</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>DSF</td>
<td>Disulfiram</td>
</tr>
<tr>
<td>ED</td>
<td>Estimated dose</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial Ovarian carcinoma</td>
</tr>
<tr>
<td>FA</td>
<td>Fanconi anemia</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug administration</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<tr>
<td>FT</td>
<td>Fallopian tube</td>
</tr>
<tr>
<td>FTE</td>
<td>Fallopian tube epithelium</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>Gamma-histone 2AX</td>
</tr>
<tr>
<td>GEMM</td>
<td>Genetically engineered mouse model</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GOG</td>
<td>Gynecologic Oncology Group</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HBOC</td>
<td>Hereditary breast and ovarian cancer</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
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<tr>
<td>HGSC</td>
<td>High-grade serous cancer</td>
</tr>
<tr>
<td>HMG</td>
<td>High mobility group</td>
</tr>
<tr>
<td>HOXA</td>
<td>Homeobox A</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IB</td>
<td>Intrabursal</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitory Concentration</td>
</tr>
<tr>
<td>ICL</td>
<td>Interstrand crosslink</td>
</tr>
<tr>
<td>IDS</td>
<td>Interval debulking</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IUD</td>
<td>Intrauterine device</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LAR</td>
<td>Low anterior resection</td>
</tr>
<tr>
<td>LDA</td>
<td>Limiting dilution assay</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDA</td>
<td>Microtubule disrupting agents</td>
</tr>
<tr>
<td>MFP</td>
<td>Mammary fat pad</td>
</tr>
<tr>
<td>MIS</td>
<td>Müllerian Inhibiting Substance</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerable dose</td>
</tr>
<tr>
<td>MUC 16</td>
<td>Mucin 16</td>
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<tr>
<td>NACT</td>
<td>Neoadjuvant chemotherapy</td>
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<td>NER</td>
<td>Nucleotide excision repair</td>
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<tr>
<td>NFkB</td>
<td>Nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD-scid-IL2Rγnull</td>
</tr>
<tr>
<td>OCP</td>
<td>Oral contraceptive pill</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian Surface epithelium</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP)-ribose polymerase</td>
</tr>
<tr>
<td>PAX2</td>
<td>Paired-box 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDS</td>
<td>Primary debulking surgery</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFI</td>
<td>Platinum free interval</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td>PIK3</td>
<td>Phosphatidylinositol 3'-kinase</td>
</tr>
<tr>
<td>PLD</td>
<td>Pegylated liposomal doxorubicin</td>
</tr>
<tr>
<td>PM</td>
<td>Princess Margaret Cancer Centre</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria in Solid Tumours</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SCOUT</td>
<td>Secretory cell outgrowth</td>
</tr>
<tr>
<td>SO</td>
<td>Salpingo-oophorectomy</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex-determining region</td>
</tr>
<tr>
<td>STIC</td>
<td>Serous tubal intraepithelial carcinoma</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein 53</td>
</tr>
<tr>
<td>TAH</td>
<td>Total abdominal hysterectomy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
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<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TIC</td>
<td>Tumour initiating cell</td>
</tr>
<tr>
<td>TILT</td>
<td>Intraepithelial lesions in transition</td>
</tr>
<tr>
<td>TVUS</td>
<td>Transvaginal ultrasound</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
1.1 Embryology of Gonadal Development

Knowledge of the embryology of the reproductive tract is important for understanding the genes and cell signaling pathways involved in normal development and the alterations that lead to disease. The third week of human embryonic life is marked by gastrulation, a process wherein the bilaminar embryonic disk transforms into a trilaminar disk (composed of ectoderm, mesoderm and endoderm). By the end of the third week, the mesoderm subdivides further into 3 distinct components, from medial to lateral: paraxial mesoderm, intermediate mesoderm and lateral plate mesoderm. The urogenital ridge originates from the intermediate mesoderm and will ultimately form the reproductive and excretory systems (DeCherney and Nathan, 2007). At approximately 40 days of gestation, primordial germ cells migrate from the yolk sac to the urogenital ridge, which divides further into the nephrogenic and genital ridges, respectively. The mesonephric (Wolffian) ducts and mesonephric kidneys develop from the nephrogenic ridge, while paired paramesonephric (Müllerian) ducts develop from invaginations of the coelomic epithelium.

Sex determination is based upon the presence or absence of the sex-determining region (SRY) gene and anti-Müllerian hormone (AMH). Female embryos lack the SRY gene and AMH, which cause the development of the gonad into the ovary and the persistence of the Müllerian ducts, respectively. The Müllerian ducts ultimately grow and fuse caudally to form the proximal vagina, while their unfused cephalad portions become the fallopian tubes (Hoffman et al., 2012). All proposed sites of serous cancers: the OSE, fallopian tubes and the lining of the peritoneal cavity are derived from the coelomic epithelium (Auersperg, 2013b).

Although the embryology of the female reproductive system is fairly well described, the cellular and molecular mechanisms that regulate its development are poorly understood. Our current knowledge stems largely from mouse knockout studies. In mice, Müllerian duct formation
requires a small set of homeodomain-containing transcription factors and signaling molecules. One of these is paired-box gene 2 (Pax2). Pax2-null mice die soon after birth and lack a reproductive tract due to the degeneration of both the mesonephric and the paramesonephric ducts (Kobayashi and Behringer, 2003). Similarly, Lim-1 and Emx2 are required for Müllerian duct formation; Emx2 and Lim-1-null mutant mice lack fallopian tubes, a uterus and the upper portion of the vagina (Kobayashi and Behringer, 2003).

Retinoic-acid signaling also plays a critical role in Müllerian duct development. Females with compound mutations in retinoic-acid receptor genes (RARα1, RARα2, RARβ2 and RARY) lack reproductive organs (Lohnes et al., 1994; Mendelsohn et al., 1994a; Mendelsohn et al., 1994b). Likewise, Wnt gene family members, specifically Wnt4, Wnt5a and Wnt7a, are required for normal Müllerian development in females and Müllerian duct regression in males (Miller et al., 1998).

As stated above, female embryos lack AMH (a.k.a. Müllerian inhibiting substance, MIS), a hormone that is both necessary and sufficient for the regression of the Müllerian duct system. Mis-mutant male mice have testes and are virilized but have a uterus and fallopian tubes. When Mis is overexpressed in female mice, Müllerian duct-derived organs are absent (Behringer, 1994; Behringer et al., 1994).

The Homeobox A (Hoxa) genes are a family of highly conserved transcription factors. They are expressed along the anterior-posterior axis of the Müllerian ducts according to their 3’-5’ order in the HOX cluster, and specify the identity of each segment: HOXA9 is expressed in the oviduct, HOXA10 in the uterus, HOXA11 in the uterus and cervix and HOXA13 in the cervix and upper vagina (Taylor et al., 1997). The reproductive tract is unique in that HOX genes continue to be expressed in these tissues in adulthood and are important not only for proper reproductive tract development but also for appropriate adult function (Taylor, 2000). High-grade serous ovarian cancers express HOXA9 and forced expression of Hoxa9 in undifferentiated murine ovarian surface epithelium cells can induce the formation of ovarian HGSC (Bast et al., 2009).
The current classification scheme for ovarian tumours is based on histogenesis of the normal ovary. Tumours of the ovary were initially thought to originate from one of the three cell types found within gonadal tissue: sex cord-stromal cells, germ cells and surface-epithelial cells. However, recent evidence suggests that most EOC, and likely all HGSC, do not originate from the surface-epithelium, but rather from the fallopian tube epithelium (FTE). These details will be discussed further in Sections 1.4 and 1.6. Furthermore, epithelial tumours are not a single disease, but rather comprise a diverse group of tumours that can be classified based on distinctive morphologic and molecular genetic features (Kurman and Shih Ie, 2010). These can be further subdivided into serous, mucinous, endometrioid, clear cell and transitional cell (Figure 1.0).
1.3 Epidemiology

According to the WHO, ovarian cancer is the 6th most common cancer in women and the 7th most common cause of cancer death. In 2008, about 225,000 women were diagnosed and 140,000 died from this disease worldwide (Jemal et al., 2011; Organization, 2008). In Canada, it is the 7th most common cancer type and the 5th most common cause of cancer death, representing 2600 new cases and 1750 deaths annually, thus making it the most lethal gynecologic malignancy (Society, 2012). Epithelial ovarian cancer is the most common gynecologic cancer, with an overall incidence of 9.4/100,000 and an incidence of 33/100,000 in women over age 50 (Hennessy et al., 2011; Schorge et al., 2010). Based on the US cancer data, the annual incidence is highest in white women (13.4 per 100,000), compared with Hispanic (11.3 per 100,000), black (9.8 per 100,000) or Asian (9.8 per 100,000) women (Edwards et al., 2002). The median age of patients with ovarian cancer is 60 years, and the average lifetime risk for women is about 1 in 70 (Cannistra, 2004). The age of diagnosis is younger in women with a hereditary ovarian cancer syndrome. For example, women with Lynch syndrome are typically diagnosed between the ages of 43-50 (Watson et al., 2001). Similarly, women with a BRCA1 mutation are younger at diagnosis, as their risk of ovarian cancer is 2-3% by age 40 (King et al., 2003). Lifetime risks are also significantly higher, with an estimate in the range of 28-66% for BRCA1 mutation and 16-27% for BRCA2 mutation (Satagopan et al., 2002).

1.4 Pathogenesis

As mentioned, ovarian tumours are classified according to the cell types from which they are derived (Figure 1.0). Ninety percent of ovarian tumours are classified as epithelial (Hennessy et al., 2011); however, most recent evidence suggests that epithelial neoplasms can be further divided into two groups: those that arise from the ovary, and those that arise from the fallopian tube (Tone et al., 2012a). The first group, which likely originates in the ovary, consists of: low-grade endometrioid, mucinous, clear cell, borderline and low grade serous. This group is often classified as Type I, as they are low grade, present at an early stage and behave in a relatively indolent manner. They develop in a slow, step-wise fashion from well-defined precursor lesions.
Genomically, they are characterized by mutations in *KRAS, BRAF, ERBB2, PTEN*, or *PIK3CA* and display a low level of chromosomal disruption (Vang et al., 2013).

Conversely, the second type of EOC (Type II), typically appears in the absence of a well-defined precursor, presents at an advanced stage and are high-grade (Kurman and Shih Ie, 2010). They metastasize early in the disease course, with tumours found on the ovarian surface, fallopian tube and peritoneal cavity (Crum et al., 2007b; Shih Ie and Kurman, 2004). Ovarian HGSC is largely synonymous with Type II tumours (Bowtell, 2010). Originally thought to be mainly ovary-derived, Type II tumours are now thought to originate from both the FTE and OSE (Auersperg, 2013a, c; Tone et al., 2012a; Tone et al., 2012b) (discussed further in Section 1.6). In contrast to Type I tumours, molecular abnormalities in Type II tumours include mutations in the tumour suppressors *TP53 (>95% of cases) and BRCA1 or BRCA2 (20-50% of cases)* (Hennessy et al., 2010; Network, 2011; Press et al., 2008a; Schrader et al., 2012). Amplification and overexpression of the genes in the *PI3K* family occur in more than 40% of type II cancers, conferring activation of the *PI3K* pathway and consequently, cell proliferation and survival. Additionally, the *RB* pathway is altered in >65% of cases, leading to aberrant cell cycle regulation. Less than 1% of Type II tumours have mutations of *BRAF, KRAS* and *PIK3CA*, which are typically found in type I tumours (Romero and Bast, 2012).

Recently, the Cancer Genome Atlas (TCGA) analyzed the genomes of 489 high-grade serous ovarian adenocarcinomas. They concluded that the mutation spectrum of ovarian HGSC is completely distinct from other EOC subtypes. Furthermore, on the basis of gene expression profiles, the TCGA found at least four subtypes within ovarian HGSC, consisting of: immunoreactive, differentiated, proliferative and mesenchymal; however, survival was not dictated by the transcriptional subtype (Network, 2011). Verhaak et al. expanded the analysis of the TCGA and showed that individual ovarian HGSC samples often express multiple subtype signatures. Using this analysis, they were also able to develop the Classification of Ovarian Cancer (CLOVAR) survival signatures with significant ability to predict outcome to therapy (Verhaak et al., 2013).
1.4.1 Role of TP53 in Ovarian Cancer Pathogenesis

The Cancer Genome Atlas study showed that >95% of ovarian HGSC have TP53 mutations (Network, 2011). TP53 mutation is an early and obligatory event in ovarian HGSC development (Bowtell, 2010). Wild-type P53 is a nuclear phosphoprotein, encoded by a highly conserved 20-Kb gene. The central core of the protein is made up primarily of the DNA-binding domain required for sequence-specific DNA binding. It is largely within this binding domain that most missense mutations occur and result in a dysfunctional protein (Bai, 2006). As a tumour suppressor, TP53 is essential for preventing inappropriate cell proliferation. It does so by activating diverse downstream targets that, in turn, elicit various responses such as cell cycle check points, cell survival and apoptosis (Christie and Oehler, 2006; Hofseth et al., 2004). Mutations that lead to loss of TP53 function, as seen in ovarian HGSC, result in failure to activate these responses, leading to un repaired DNA damage, increased chromosomal instability, and ultimately, tumour formation (Buttitta et al., 1997; Tomasini et al., 2008). TP53 loss associated with chromosomal instability is believed to contribute to the widespread copy number change seen in ovarian HGSC (Bowtell, 2010).

1.4.2 Role of BRCA1 and BRCA2 in Ovarian Cancer Pathogenesis

Germline or somatic mutations in BRCA1 and BRCA2 are found in approximately 20-50% of ovarian HGSCs (Castellarin et al., 2013; Hennessy et al., 2010; Network, 2011; Pal et al., 2005; Press et al., 2008a; Zhang et al., 2011). The BRCA1 and BRCA2 genes are located on chromosomes 17q21 and 13q12.3, respectively, and their proteins prevent malignant transformation by helping to maintain genomic integrity through their respective roles in DNA repair, particularly homologous recombination (HR) (Powell et al., 2013).

BRCA1 comprises 24 exons encoding a protein of 1863 amino acids (Miki et al., 1994). It mediates HR repair of double strand breaks (DSBs) by binding directly to DSBs through association with the abraxas-RAP80 macro-complex. This complex is ultimately responsible for DSB resection, and functions with cell cycle checkpoints to prevent propagation of DNA damage (Powell et al., 2013). BRCA1 has also been shown to regulate cell cycle progression via roles in the G1/S, G2/M and spindle checkpoints (Deng, 2006).
BRCA2 consists of 27 exons encoding a protein of 3418 amino acids and is a member of the Fanconi Anemia (FA) DNA repair pathway (Ramus and Gayther, 2009). The FA-BRCA DNA repair pathway comprises 13 proteins, which act together to repair DNA interstrand crosslinks (ICLs) and respond to stalled replication forks (Abraham et al., 2011). BRCA2 (a.k.a. FANCD1) is integral to this process through recruitment of the recombinase RAD51 to DSBs. RAD51 is essential for HR and is responsible for the tumour suppressive function of the repair process (Moynahan et al., 2001).

1.5 Risk Factors

1.5.1 Patient Characteristics

Epidemiologic studies examining typical cancer risk factors, such as socioeconomic status, alcohol consumption, smoking, and physical activity have been inconsistent with respect to ovarian cancer risk (Zografos et al., 2004). On the other hand, age and family history have been consistently identified as major risk factors. HGSC rates increase with older age, with mean age at diagnosis of 55-65 years (Vang et al., 2009). Furthermore, women with a family history of ovarian cancer are three to four times more likely to develop ovarian cancer than those without such history (Banks, 2001). A geographic distribution for ovarian cancer has also been found, with incidence increasing with latitudinal distance away from the equator. Some evidence point to vitamin D deficiency as a potential mechanism for this distribution, however, the underlying mechanisms have not been described (Bakhru et al., 2010; Garland et al., 2006; Lefkowitz and Garland, 1994). Additionally, the risk of ovarian cancer increases for women who migrate from a country with a low-risk incidence to a country with a higher risk incidence (Organization, 2008).

1.5.2 Risk from Familial Cancer Syndromes

Although the vast majority (~90%) of ovarian cancer cases are sporadic, the greatest and most reliable risk factors for ovarian HGSC are a positive family history and/or a known BRCA1/2 mutation. If either gene is mutated in the germ line, the result is hereditary breast and ovarian cancer (HBOC) syndrome, which causes a high lifetime risk to not only breast and ovarian
cancers, but also pancreatic, stomach, laryngeal and prostate cancers (Roy et al., 2012). The most commonly reported BRCA1 mutations are 185delAG and 5382insC, while the most common BRCA2 mutation is 6174delT (Boyd, 2003). These mutations are inherited in an autosomal dominant pattern. More than 1200 mutations have been reported for BRCA1/2 with ~80% being either frameshift or nonsense, causing the formation of a truncated protein (ACOG, 2009; Boyd, 2003).

A meta-analysis of six studies showed that 5.7% and 3.8% of all ovarian cancers are associated with germline BRCA1 and BRCA2 mutations, respectively, although more recent studies have found a higher mutation prevalence of 13-20% (Boyd, 2003; Schrader et al., 2012; Zhang et al., 2011). Mutations in BRCA1 confer a risk of ovarian cancer of ~50% by age 70, with a lifetime risk of 16-63% (Easton et al., 1995; Ford and Easton, 1995). BRCA2 mutations confer a ~10% risk of ovarian cancer by age 70 and a lifetime risk of 20-30% (Boyd, 2003; Lakhani et al., 2004). Risks of ovarian cancer are significantly higher in BRCA1 mutation carriers, compared with BRCA2 (cumulative risk of 0.21 vs. 0.02 by age 50), and tumours tend to occur at a younger age (mean age 40s/50s vs. 60 years for BRCA2 carriers) (Boyd and Rubin, 1997; Gayther et al., 1995; King et al., 2003). These figures stand in stark contrast to the lifetime risk of ovarian cancer of 1.4% in the general population.

Approximately 1 in 280 women carry a germline BRCA1/2 mutation; however, approximately 40% of all Ashkenazi Jewish ovarian cancer patients are BRCA1/2 mutation carriers, putting this population at particularly high risk (Boyd, 2003). Other populations with more frequent BRCA1/2 mutations include French Canadians and Icelanders (ACOG, 2009).

Whereas 90% of hereditary ovarian cancers are associated with a mutation in BRCA1/2, the remainder can be attributed to mutations in the DNA mismatch repair (MMR) genes, primarily seen in association with Lynch Syndrome (LS) (formerly: hereditary nonpolyposis colon cancer, HNPCC). Lynch syndrome occurs due a germline mutation in one of several mismatch repair genes (MMR): MLH1, MSH2, MSH6, PMS1 or PMS2, and like BRCA1/2, it is inherited in an autosomal dominant pattern (Crispens, 2012). The MMR pathway is involved in the removal of DNA base mismatches that arise either during DNA replication or as a result of DNA damage. Mutation rates are 100- to 1000-fold greater in MMR-deficient tumour cells compared with
normal cells (Martin et al., 2010). As a result, the lifetime risk of ovarian cancer in women with LS has been estimated to be 7-12%, with a younger age at diagnosis (mean age 43-49 years) (Ketabi et al., 2011; Watson et al., 2008). Although most of these tumours are endometrioid, a small fraction (~10%) has high-grade serous histology (Grindedal et al., 2010).

1.5.3 Reproductive History Risk

Studies examining the relationship between age of menarche and ovarian cancer risk have been conflicting. Rodriguez et al. found that menarche after age 12 correlates significantly with lower ovarian cancer rates, compared with menarche at a younger age, but other studies have been inconclusive (Franceschi et al., 1991; Rodriguez et al., 1995).

The data surrounding age of menopause are similarly conflicting. A large European study found that, compared with women whose menopause occurred at age 44 or earlier, the relative risk (RR) for ovarian cancer was 1.4 for women having menopause between the ages of 45 and 49, 1.6 for women with menopause between 50 and 52, and 1.9 if menopause occurred after the age of 52 (Franceschi et al., 1991). Similarly, women who had surgical menopause saw protective effects, with a RR= 0.7 (Hartge et al., 1988). However, parity-adjusted data from the USA and Australia found no trend in ovarian cancer risk with increasing age of menopause (Purdie et al., 1995; Whittemore et al., 1992).

Unlike menarche and menopause, the association between parity/gravidity and ovarian cancer is well established (Adami et al., 1994). In the 1970s, Beral et al. found a clear inverse relation between average completed family size and mortality from ovarian cancer in various populations of women and successive generations (Beral et al., 1978). In the Nurses Health Study, a RR of 0.84 per pregnancy was observed (Hankinson et al., 1995). Furthermore, Albrektsen et al. showed that the RR of epithelial ovarian cancer was 0.56 for women with three pregnancies but did not decrease with additional pregnancies (Albrektsen et al., 1996). Conversely, nulliparous women have a marked increase in risk, reaching three times that of parous women (Bandera, 2005; Stewart et al., 2013).
Like gravidity, lactation suppresses ovulation and has a favorable role in ovarian cancer risk reduction. The protection against ovarian cancer is related to total lactation time, with longer durations conferring the greatest risk reduction (Greggi et al., 2000; Yen et al., 2003).

Likewise, use of the oral contraceptive pill (OCP) has a substantial protective effect on ovarian cancer. Several cohort and case-control studies have demonstrated a 40% reduction of ovarian cancer in OCP users, and this number increases to 50% if OCPs have been used for 5 years or longer (Bosetti et al., 2002; Chiaffarino et al., 2001; La Vecchia and Franceschi, 1999). Similar protective effects are seen with the use of an intrauterine device (IUD) (Ness et al., 2011). Gravidity, lactation and OCPs all suppress ovulation and are thought to be protective through a common mechanism of either ovulation suppression or altered levels of steroid hormones (Ness and Cottreau, 1999).

Unfortunately, the link between hormone replacement therapy (HRT) and ovarian cancer has not been as clear. Several case-control studies have shown that any history of HRT-use is related to a modest increase (RR 1.4) in ovarian cancer risk, (Chiaffarino et al., 1999; Riman et al., 2002a; Riman et al., 2002b); however, other studies found no such association (La Vecchia, 2006; Lukanova and Kaaks, 2005; Sit et al., 2002). Two large prospective studies did find an association between HRT use and ovarian cancer. The first, a Danish study looking at >900,000 women, found that compared with women who never took hormone therapy, current users of hormones had EOC incidence rate ratios of 1.44 (95% C.I., 1.30-1.58). The researchers estimated that 1 extra ovarian cancer occurred in approximately 8300 women taking hormone therapy each year (Morch et al., 2009). The second study, the UK Million Women Study, found a similarly increased risk of EOC for current HRT users, with a relative risk of 1.53 (95% C.I., 1.31-1.79) (Beral et al., 2007). Neither of these studies examined the risk specifically for ovarian HGSC.

Lastly, tubal ligation (TL) reduces ovarian cancer risk. This procedure does not entail removal of any part of the fallopian tube, but simply creates an interruption in the fallopian tube lumen. The Nurses’ Health Study reported a dramatic protective effect of TL, with an odds ratio of 0.33 (95% C.I., 0.16-0.64) (Hankinson et al., 1995). Several mechanisms have been suggested to explain the protective effects of TL. TL may reduce blood flow to the ovary resulting in anovulation and altered levels of hormones and growth factors. Alternatively, TL could block the
retrograde flow of carcinogenic or inflammatory agents into the peritoneal cavity (Sieh et al., 2013). TL has been suggested as a feasible risk-reducing option for certain at-risk women (Narod et al., 2001).

1.5.4 Inflammation

A growing body of literature suggests that the OSE and FTE are chronically exposed to an inflammatory environment, due to normal physiological events (ovulation, menstruation), as well as pathological states (endometriosis, pelvic inflammatory disease). Pro-inflammatory cytokines, such as C-reactive protein (CRP), are present in ovulatory fluid and menstrual effluent. CRP also is increased markedly in endometriosis (implantation of ectopic endometrial tissue in the abdomen and pelvis) and pelvic inflammatory disease (PID; ascension of sexually transmitted infections into the pelvis) (Hunn and Rodriguez, 2012). Inflammation produces toxic oxidants intended to kill pathogens; however, these oxidants can also damage host DNA, proteins and lipids, and could play a direct role in carcinogenesis (Dreher and Junod, 1996). Elevated serum levels of CRP, prostaglandins and cytokines (specifically interleukins, IL-2, IL-4, IL-6, IL-12, and IL-13) are elevated in women who subsequently develop EOC (Clendenen et al., 2011; McSorley et al., 2007; Toriola et al., 2011).

Environmental toxins can enter the genital tract and also cause inflammation. This association was first seen between talc use and EOC. A meta-analysis of 16 studies demonstrated a 33% increase risk of EOC in talc users, particularly in HGSC (Gertig et al., 2000; Huncharek et al., 2003). Talc particles have also been seen in ovarian neoplasms, providing some further evidence that these can act as direct carcinogens (Henderson et al., 1979).

1.6 Ovarian Cancer Etiology

1.6.1 The Menstrual Cycle: A Brief Overview

Understanding the proposed etiologies and pathogenesis of ovarian cancers requires knowledge of the normal physiology of the reproductive tract. The production and release of an ovum is a
carefully regulated process coordinated between the hypothalamus, pituitary and ovaries. In humans, the typical 28-day cycle consists of follicle development, ovulation and luteinization (Figure 1.1). The follicular phase comprises the first half of the cycle, during which a dominant follicle is selected from a pool of candidate follicles. During the follicular phase, estradiol levels rise and the endometrium proliferates. Ovulation is defined by the release of the oocyte from the dominant follicle, and formation of the corpus luteum. In the luteal phase, the corpus luteum produces high levels of estradiol and progesterone in order to support an impending pregnancy. Accordingly, the endometrium transforms into a secretory state to facilitate implantation. In the absence of pregnancy, menstruation occurs as the corpus luteum regresses. Similarly, the fallopian tube epithelium undergoes cyclical changes comparable with the proliferative and secretory patterns of the endometrium, with the follicular phase being ‘secretory’, and the luteal phase being a ‘recovery’ stage (Crow et al., 1994).

**Figure 1.1 The Menstrual Cycle**

Coordination of the ovarian-uterine cycle involves complex interplay between the hypothalamic release of gonadotropin-releasing hormone (GnRH), pituitary gonadotropin release and ovarian sex steroid production (Bieber, 2006). Neurons in the arcuate nucleus of the mediobasal
hypothalamus secrete GnRH in a pulsatile manner into the hypophyseal portal circulation. These act to stimulate the anterior pituitary to release gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on their respective target cells in the gonad. The rise of FSH in the follicular phase, which begins on the first day of menstruation, stimulates the granulosa cells of the ovary to proliferate, and express aromatase. LH stimulates the theca cells of the ovary to produce androgens, which are then, aromatized in the granulosa cells to produce estradiol. Ovulation occurs ~38 hours after the LH surge and the corpus luteum (CL) develops from the ruptured ovarian follicle. This marks the beginning of the luteal phase. Progesterone is released from the CL and slows GnRH drive, which leads to decreased LH and FSH release. If pregnancy does not occur, the CL regresses causing a drop in progesterone and estradiol, and subsequent menstruation (Bieber, 2006).

1.6.2 Ovarian Cancer Etiology: The Cell-of-Origin Controversy

The cell-of-origin and pathogenesis of ovarian cancer continue to be current topics of debate. The traditional view of ovarian carcinogenesis has been that all EOC tumours are derived from the OSE. Specifically, following ovulation, inclusion cysts form during repair of ovulatory ‘wounds’ in the OSE. These form invaginations into the ovarian cortex, pinch off from the surface of the ovary and sit within the ovarian stroma (Fathalla, 1971). Subsequently, these inclusion cysts are subjected to the stimulative influence of stromal growth factors and were proposed to undergo metaplasia, dysplasia and eventually, malignant transformation (Lukanova and Kaaks, 2005; Risch, 1998). Supporting this, several studies reported focal p53 immuno-positive inclusion cysts in the ovaries of women with serous carcinomas, suggesting an origin from these sites (Folkins et al., 2008; Jarboe et al., 2008). Moreover, inactivation of Brca1 in mouse OSE results in pre-neoplastic changes that resemble pre-malignant lesions (Clark-Knowles et al., 2007).

More recently, an alternative hypothesis was proposed, which suggests that ‘ovarian’ HGSCs are actually derived from the epithelium of the distal, fimbrial end of the fallopian tube (Crum et al., 2007a; Kindelberger et al., 2007; Lee et al., 2006; Medeiros et al., 2006). This concept emerged
from studies identifying dysplastic lesions and *in situ* carcinomas in prophylactic salpingooophorectomy (SO) specimens removed from high risk women (Auersperg, 2013b).

As described in Section 1.1, the fallopian tubes are paired organs derived from the Müllerian ducts. Their main role is to transport the oocyte from the ovary for subsequent fertilization. The most distal ends of the FT, termed fimbriae, are finger-like projections that sweep the released ovum into the tube. The FT comprises two main epithelial cells: secretory FTE cells, which line the tube and support oocyte survival; and ciliated cells, which facilitate transport of the oocyte through their sweeping action (Gordts *et al.*, 1998).

The FT model of ‘ovarian’ HGSC pathogenesis describes a step-wise process. First, FT secretory cells are exposed to genotoxic injury, leading to *TP53* mutations. Next, clonal expansion of *TP53*-mutated cells results in a ‘p53 signature’ (the ‘p53 signature’ describes 12 consecutive nuclei with strong TP53 immunopositivity in an otherwise benign-appearing FTE). A subset of cells with the p53 signature might undergo cellular proliferation and result in fully developed serous tubal intraepithelial carcinomas (STICs). These are characterized by frequent *TP53* mutations and increased FT secretory cell proliferation (Bowtell, 2010). STICs have the capability of either expanding locally or metastasizing to the ovarian surface or peritoneum as a HGSC (Crum *et al.*, 2007b). Extensive examination of fimbria in patients with ‘ovarian’ HGSC has revealed the presence of STICs in 40-60% of FT (Kindelberger *et al.*, 2007). Prophylactic SOs for high-risk women have identified STICs in 5-10% of cases, with no analogous lesions found in the ovaries of the same women (Hunn and Rodriguez, 2012).

Two additional pre-malignant lesions have also been identified. Secretory cell outgrowth (SCOUT) is defined as a discrete expansion of 30 secretory cells without the involvement of *TP53*. Tubal intraepithelial lesions in transition (TILTs) have nuclear atypia and p53 positivity but lack the proliferation rate seen in STICs, and may represent the precursor between the development of p53 signature and STICs. Knowledge of lesions on the SCOUT-STIC continuum, and how each participates in carcinogenesis is limited (Delair and Soslow, 2012).

Debate surrounding the ovarian HGSC cell-of-origin continues. Those supporting the FTE-cell-of-origin model argue that prophylactic SOs have failed to reveal a pre-malignant ovarian pathology (Tone *et al.*, 2012a). Others maintain that there is histologic evidence of early lesions
in OSE-lined inclusion cysts, with ‘p53 signatures’ analogous to those found in STICs (Auersperg, 2013a). While arguments in favour of ovarian HGSC originating in the fallopian tube are compelling, the possibility that the OSE and peritoneum are additional and significant sources of these carcinomas remains (Auersperg, 2013b; Delair and Soslow, 2012).

Recently, a stem cell niche has been identified in the hilum region of the mouse ovary, in the junction between the OSE and FTE. This niche contains cells responsible for OSE regeneration that are prone to malignant transformation (Flesken-Nikitin et al., 2013). It remains unclear if this niche contributes to ovarian carcinogenesis.

1.6.3 Ovarian Cancer Etiology: Incessant Ovulation

Historically, incessant ovulation has been proposed as a major contributor to the development of EOC. Rupture of the ovulating follicle damages the OSE, requiring immediate repair, and frequent ovulation causes continuous damage to the OSE, increasing the likelihood of mutations during the repair process (Fathalla, 1971). This model is supported by evidence of a decrease in EOC risk in women with suppressed ovulation as result of oral contraceptive use, pregnancy or lactation (Section 1.5.3). A positive association between lifetime ovulatory years and risk of ovarian cancer in pre-menopausal women has been consistently demonstrated (Tung et al., 2005). Furthermore, EOC is rare in non-primate mammals, and this is thought to be because humans are one of the few species that cycle continuously. The only other non-primates to develop EOC are hens, especially those that have been hyper-ovulated to produce eggs (Fredrickson, 1987; Land, 1993).

The ovulatory process causes a pro-inflammatory state, characterized by infiltration of leukocytes, ROS generation and production of inflammatory cytokines (Bonello et al., 1996; Rizzo et al., 2012). The OSE proliferates rapidly in the presence of cytokines and oxidative stress resulting from ovulation. King et al. showed that oxidative stress causes normal mouse OSE to undergo transformative changes (King et al., 2013). DNA damage caused by ovulation-induced inflammatory factors could be an important element in the transformation of damaged OSE/FTE (Fleming et al., 2006). This possibility is supported by the decreased risk of EOC associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs) (Wernli et al., 2008).
1.6.4 Ovarian Cancer Etiology: The Role of Hormones

The OSE and FTE are responsive to the local hormonal environment (Risch, 1998). Multiple studies have shown that progesterone protects against ovarian cancer development, whereas androgens, estrogens and gonadotropins increase ovarian cancer risk (Ho, 2003; Lukanova and Kaaks, 2005; Risch, 1998). Cramer and Welch suggested that EOC was the result of excessive stimulation of the OSE by LH and FSH. They suggested that gonadotropins either directly activated gonadotropin-responsive genes, contributing to malignant transformation, or indirectly stimulated ovarian steroidogenesis (Cramer and Welch, 1983). Several observations argue against this proposed mechanism. First, estrogen levels are highest during pregnancy, a protective event for EOC (Risch, 1998). Second, one study found no estrogen receptors on the OSE or in inclusion cysts (Zeimet et al., 1994). Third, women who develop EOC do not have elevated serum gonadotropin levels (Helzlsouer et al., 1995). Conversely, progesterone is believed to be protective against EOC. This association has been seen in multiple births (twins, triplets, etc), which have significantly higher circulating progesterone levels and exert a higher protective effect against EOC compared to singleton pregnancies (Whiteman et al., 2000).

1.7 Screening

The small proportion of patients diagnosed with stage 1 EOC have a 5-year survival in excess of 90% (Jacobs and Menon, 2004). However, because the large majority of patients are diagnosed with advanced (> stage 3) disease, numerous efforts have been made to develop screening tools utilizing imaging techniques and/or serum markers. A screening test for a disease with low incidence, like ovarian cancer, must have good sensitivity in addition to very high specificity in order to achieve an acceptable positive predictive value (PPV) (Skates et al., 1995). The consequence of a false positive screening test in this case is surgical intervention with its inherent risks. Numerous studies have looked at screening with CA125, with and without transvaginal ultrasound (TVUS) (Jacobs et al., 1993; Menon et al., 1999). Unfortunately, elevated CA125 (> 35 U/mL) is not specific for EOC and can be found in a variety of other gynaecological conditions, including endometriosis, pregnancy, adenomyosis and polycystic
ovarian syndrome. In addition, CA-125 levels are typically low in early stage ovarian cancer (Cannistra, 2004). Multiple other biomarkers, including prostasin, osteopontin, inhibin and kallikrein have been proposed to have a role in EOC, but their utility as screening tools has not been established (Jacobs and Menon, 2004).

The Prostate, Lung, Colon and Ovary trial looked at combining CA125 with TVUS, but found a PPV for invasive cancer of only 23% if both tests were used in combination (Buys et al., 2005). Most recently, Lu et al. evaluated a 2-stage ovarian cancer screening strategy, which incorporates age and changes in CA125 over time. Women with high risk scores based on CA125 changes were then referred for TVUS and to a gynecologic oncologist for further evaluation. With this model, they were able to achieve a PPV of 40% and all cancers were discovered at an early stage (Lu, 2013). Whether this will have an impact on survival remains unanswered.

1.8 Presentation

Symptoms of ovarian cancer are related to patterns of disease spread. EOC disseminates throughout the abdominal cavity, forming nodules on the surface of the visceral and parietal peritoneum and omentum. EOC can also travel through lymphatics and blood vessels to nodes and parenchyma of distant organs, most commonly, the liver and spleen. Blockage of diaphragmatic lymphatics prevents outflow of proteinaceous fluid from the peritoneal cavity, causing the accumulation of ascites fluid in advanced disease (Romero and Bast, 2012).

The clinical presentation of EOC may be either acute or subacute, but typically is related to advanced stage disease, as over 80% of women have stage 3 or greater disease at diagnosis (Clegg et al., 2002; Institute, 2013). Diagnoses based on subacute presentations are typically made due to the incidental finding of an adnexal mass either on physical examination or by imaging. Symptoms associated with subacute presentations are generally vague, consisting of bloating, urinary urgency/frequency, pelvic/abdominal pain or early satiety, and symptoms do not typically correlate with stage of disease. Conversely, acute presentations are related to
symptoms of pleural effusion (shortness of breath), bowel obstruction (obstipation, constipation) or venous thromboembolism (leg swelling, leg pain, shortness of breath).

1.9 Diagnosis

Given the frequent absence of symptoms or the presence of vague complaints, clinicians must have a high clinical suspicion to investigate and diagnose ovarian cancer. A thorough history and physical are required, including pelvic and rectovaginal examinations. A palpable pelvic mass, especially in the context of a post-menopausal female, must be carefully evaluated. The definitive diagnosis requires a tissue diagnosis, obtained either at the time of surgery, from paracenthesis/thoracenthesis or via an imaging-guided biopsy; however, radiologic and serum studies assist in confirming the diagnosis.

1.9.1 Cancer Antigen 125

Cancer Antigen 125 (a.k.a. Mucin 16) is a serum biomarker that is elevated in greater than 80% of EOC patients and plays an important role in detection and disease management (Duffy et al., 2005; Hogdall et al., 2007). With sensitivity and specificity of ~70% and ~85%, respectively, serum CA125 is used to assess the risk of malignancy in women with an adnexal mass (Nolen et al., 2010; O'Connell et al., 1987; Partheen et al., 2011). Once diagnosed with EOC, CA125 measurements are performed routinely and are used to monitor response to treatment, progression and recurrence (Aggarwal and Kehoe, 2010; Diaz-Padilla et al., 2012).

CA125 is a trans-membrane glycoprotein that is shed from ovarian cancers and circulates in serum (Romero and Bast, 2012). Physiologically, it is expressed in a number of tissues derived from coelomic epithelium, including trachea and pericardium, where it functions to provide a lubricating barrier at mucosal surfaces (Hori et al., 2008). In ovarian cancer, CA125 is thought to have a role in tumour cell growth, tumourigenesis, cell adhesion and metastases (Rump et al., 2004; Theriault et al., 2011). Furthermore, in vitro, it has been implicated in sensitivity of ovarian cancer to chemotherapy, with higher levels suggestive of platinum resistance (Boivin et
Persistent elevation of CA125 after chemotherapy indicates residual disease with greater than 90% accuracy (Romero and Bast, 2012).

### 1.9.2 Radiologic Studies

Imaging studies can help to assess the presence of ascites and the extent of disease. Abdominal and pelvic computerized tomography (CT) and transvaginal US are the main imaging modalities. Several characteristics found on TVUS are highly suggestive of malignancy, these include: size, morphology (septation, papillation, solid components), and Doppler flow within the mass. These characteristics provide 90% and 88% sensitivity and specificity, respectively, when combined with other clinical variables, such as age (Twickler and Moschos, 2010).

Computed tomography (CT) is the preferred technique for pre-surgical evaluation of patients, as it defines the extent of disease and likelihood of successful surgical cytoreduction (Axtell et al., 2007). On CT, advanced ovarian cancer typically appears as thick-walled, septated cysts, with papillary projections. These are optimally visualized after administration of CT contrast dye. Additional CT findings often include pelvic organ involvement, sidewall or peritoneal implants, adenopathy and ascites (Iyer and Lee, 2010). Most recently, positron emission tomography (PET) imaging has been combined with CT imaging. This combination provides the most accurate radiologic evaluation of suspected recurrence, with a recent meta-analysis showing that it has a sensitivity and specificity of 91% and 88%, respectively (Gu et al., 2009).

Magnetic Resonance Imaging (MRI) is used occasionally, particularly when the patient has a CT contrast dye allergy. MRI is not as sensitive as Doppler TVUS for identifying malignant lesions (TVUS 100%, MRI 96.%) but it is more specific (TVUS 40%, MRI 84%) (Iyer and Lee, 2010). Consequently, MRI is typically reserved for patients with a low risk of malignancy, but an indeterminate lesion on TVUS.
1.10 Management of Disease

1.10.1 Management of Primary Disease: Surgery

Surgical management is the mainstay of treatment for ovarian cancer. A surgical procedure is necessary to obtain tissue for diagnosis, assess extent of disease and attempt optimal cytoreduction (Mann, 2013). The standard surgical procedure includes exploratory laparotomy, total abdominal hysterectomy (TAH), bilateral salpingo-ooporectomy (BSO), pelvic and peri-aortic lymphadenectomy, omentectomy and multiple peritoneal biopsies. Confirming the diagnosis is critical, as several tumours can mimic ovarian cancer, including gastrointestinal tract tumours (with Krukenberg ovarian metastases) and breast cancer with ovarian metastases.

Extent of disease (surgical staging) determines initial treatment. Less than 25% of patients with EOC present at early stage disease (stages 1 or 2) (Table 1.0), and their management differs from patients with advanced (stage ≥3) disease (Young et al., 1983; Young et al., 1990). The ACTION trial showed that after a median follow-up of 10.1 years, there were no differences between the observation and the adjuvant chemotherapy arms in cancer-specific survival if early stage patients were optimally debulked. However, when non-optimally debulked patients were analyzed, cancer-specific survival was better in the adjuvant chemotherapy arm than in the observation arm (Trimbos et al., 2010). This study examined all EOC patients and did not look specifically at HGSC.

Unfortunately, the majority of EOC patients present with advanced stage disease. The standard of care for these patients consists of a combination of aggressive debulking surgery followed by platinum-based chemotherapy. Multiple studies have shown that minimum residual tumour post-operatively is one of the most powerful determinants of survival (Allen et al., 1995; Bristow et al., 2002; Hoskins et al., 1994). Residual tumour size greater than 2 cm is associated with a survival of 12–16 months, compared with 40–45 months if the tumour is less than 2 cm (Mutch, 2002). Bristow et al. showed that every 10% increase in cytoreduction was associated with a 5.5% increase in median survival (Bristow et al., 2002). The goal of every surgery is for ‘optimal cytoreduction’; however, that definition remains debatable. The most widely used definition for ‘optimal’ is residual disease less than 1 cm (Del Campo et al., 1994; Eisenkop and Spirtos,
In addition to increasing survival, there are other benefits of surgery, including decreasing disease-related symptoms, improving host immune competence and maximizing the effect of chemotherapy (Covens, 2000; Merogi et al., 1997). Patients who are not good surgical candidates due to medical comorbidities, can be considered for neoadjuvant chemotherapy (NACT) with interval debulking surgery (IDS); however, whether this is a reasonable alternative for all patients is currently being investigated (Morrison et al., 2012).

### Table 1.0 Ovarian Cancer Staging (Adapted from Mann, 2013)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Tumour limited to ovary; negative washings</td>
</tr>
<tr>
<td>1b</td>
<td>Tumour limited to both ovaries; negative washings</td>
</tr>
<tr>
<td>1c</td>
<td>Tumour limited to both ovaries with either tumour on the surface of the ovary or positive washings</td>
</tr>
<tr>
<td>2a</td>
<td>Implants on uterus and/or tube(s); negative washings.</td>
</tr>
<tr>
<td>2b</td>
<td>Implant on other pelvic tissues; negative washings.</td>
</tr>
<tr>
<td>2c</td>
<td>Pelvic extension and/or implants; positive washings.</td>
</tr>
<tr>
<td>3a</td>
<td>Microscopic peritoneal metastasis beyond pelvis (no macroscopic tumour)</td>
</tr>
<tr>
<td>3b</td>
<td>Microscopic peritoneal metastasis beyond pelvis ≤2 cm</td>
</tr>
<tr>
<td>3c</td>
<td>Peritoneal metastasis beyond pelvis &gt; 2 cm and/or regional lymph node metastasis</td>
</tr>
<tr>
<td>4</td>
<td>Distant metastasis (excludes peritoneal metastasis)</td>
</tr>
</tbody>
</table>

### 1.10.2 Management of Primary Disease: Chemotherapy

Cisplatin has been used since the 1970s for the treatment of various cancers, including ovarian, testicular, head and neck and cervical. Its second-generation analog, Carboplatin, forms identical lesions on DNA but has a notably lower spectrum of toxicities (Rabik and Dolan, 2007). Unlike cisplatin, Carboplatin rarely results in nephrotoxicity, ototoxicity or peripheral neuropathy, but can cause myelosuppression, particularly when administered in high doses (Rabik and Dolan,
A large meta-analysis showed that patients with advanced ovarian cancer had virtually identical survival durations when treated with Carboplatin or cisplatin-containing regimens, and as such, Carboplatin use is preferred (Alberts, 1995).

Upon entering the cell, all platinating agents are aquated and become positively charged. This highly reactive species is then capable of interacting with nucleophilic molecules within the cell, such as DNA and RNA (Siddik, 2003). When binding to DNA, platinating agents favor the imidazole rings of guanosine and adenosine and can form three different lesions: mono-adducts, intra-strand crosslinks, and inter-strand crosslinks (Rabik and Dolan, 2007).

The most abundant adducts are intrastrand crosslinks between adjacent purines (Pt-GG and Pt-AG), representing 65% and 25%, respectively, of total adducts formed (Crul et al., 2003). The majority of monoaadducts also react to form crosslinks, and these all cause steric changes in the DNA. The physical distortions in the DNA are then recognized by over 20 individual proteins, which transduce DNA damage signals to downstream effectors. The downstream effects activated by cisplatin can be pro-survival or pro-apoptotic, and the relative intensity and/or duration of each signal determines the final fate of the cell (Siddik, 2003). Intra-strand crosslinks are believed to be the most cytotoxic because of their ability to recruit high mobility group (HMG) proteins, which in turn recognize and bind DNA and prevent replicative bypass (Vaisman et al., 1999). Increased HMGB1 expression is associated with platinum sensitivity (He et al., 2000; Reeves and Adair, 2005).

Activation of cell cycle checkpoints occurs with platinum-induced DNA damage. One of these is a transient S-phase arrest, followed by inhibition of Cdc2-cyclin A or B kinase to cause a long-lasting G2/M arrest (He et al., 2005). Cell-cycle arrest is necessary to enable the nucleotide excision repair (NER) complex to remove adducts and promote cell survival. However, DNA damage exceeding a certain threshold results in activation of the caspase 9 - caspase 3 pathway and causes cell death (Siddik, 2003).

Inter-strand crosslinks represent 5-10% of the total number of Pt-DNA adducts. DSBs resulting from platinum-induced inter-strand adducts are thought to be repaired predominantly by homologous recombination, which explains the increased drug sensitivity seen in cells lacking a functional HR pathway (Crul et al., 2003). As such, women with a germline mutation in BRCA1
or BRCA2 show higher response rates to platinum-based chemotherapy and therefore, a longer progression-free survival and improved overall survival (Berns and Bowtell, 2012).

Paclitaxel is the standard taxane used to treat ovarian HGSC. Taxanes are microtubule-disrupting agents (MDA), whose cytotoxicity is not the result of any one single parameter, but rather a combination of effects on the cell cycle and apoptosis. Paclitaxel works mainly by binding to intracellular β-tubulin, which leads to microtubule stabilization, G2/M arrest and apoptosis (Wang et al., 1999).

The preferred route of administration and timing of chemotherapy has recently come into question. Standard intravenous (IV) treatment of Carboplatin (AUC 4-6) with paclitaxel (175 mg/m²) is given every 21 days for six cycles (Hennessy et al., 2009). Treatment for longer durations has been evaluated and no clear benefit has been shown by extending beyond six cycles (Muggia, 2011). For optimally debulked patients, intraperitoneal (IP) chemotherapy has been shown to have an advantage over standard IV chemotherapy. GOG 172 compared an IV regimen (IV paclitaxel and IV cisplatin) to IV and IP (IV paclitaxel, IP cisplatin, IP paclitaxel) regimen: the IV and IP regimen showed significant benefit in both overall and progression free survival, but had significantly higher toxicity (Armstrong et al., 2006). With standard-of-care chemotherapy, more than 80% of patients have a significant reduction in tumour size, with 40-60% of patients showing complete response.

### 1.10.3 Management of Recurrent Disease

Overall survival (OS) is measured as the time from treatment to death from any cause, and progression free survival (PFS) is measured as the time from treatment to documented disease progression. In ovarian cancer, PFS correlates well with OS and is often used as a surrogate to measure clinical benefit (Bast et al., 2007). Eighty-five percent of women with stage 3 or greater disease at presentation will recur, and 20-30% will relapse within 6 months (Berns and Bowtell, 2012). Due to these recurrence rates, the median PFS is only 18 months (Greenlee et al., 2001). PFS is most strongly linked to the length of the platinum-free interval (PFI) as well as debulking status (Huang et al., 2012; Rump et al., 2004; Theriault et al., 2011). The PFI is defined as the time from first-line platinum chemotherapy to relapse. The longer this interval, the better the response to subsequent chemotherapy and length of survival (Huang et al., 2012).
Patients who recur within 6 months of completing platinum therapy are classified as platinum-resistant, and if disease progression occurs during the course of platinum therapy, these cases are classified as platinum-refractory (Yap et al., 2009). Primary platinum-resistance occurs in 20-40% of patients. Re-treatment with platinum in these instances yields a response rate of only 10-20% and a response rate to other chemotherapeutic agents of only 10-25% (Agarwal and Kaye, 2003). Platinum-resistant tumours present a therapeutic dilemma, and thus, the focus with these patients tends to be palliative rather than curative (Muggia, 2011). Second-line therapy is not well established but typically consists of doxorubicin (caelyx), etoposide or topotecan. Given the low success rates of these agents, patients are typically enrolled into clinical trials for multi-drug treatment.

Women with a PFI of greater than 6 months can expect to respond to further chemotherapy. For example, a PFI of 12 months yields a platinum retreatment response rate of 27%; conversely, a PFI of 24 months gives a 72% response rate (Parmar et al., 2003). The term ‘platinum-sensitive’ is often given to women who recur ≥6 months after completing platinum therapy. Several trials have looked at platinum-sensitive recurrent disease in an effort to maximize platinum therapy. Most recently, the CALYPSO trial studied the combination of Carboplatin with either pegylated liposomal doxorubicin (PLD) or paclitaxel. The Carboplatin/PLD arm had a longer PFS and a better toxicity profile (Pujade-Lauraine et al., 2010). Re-treatment with platinum or platinum analogs remains the preferred treatment choice, but carries a risk of hypersensitivity, which can be fatal. In recurrent platinum-sensitive disease, re-treatment with non-platinum agents also provides benefit (Muggia, 2011).

Secondary surgery in recurrent disease has a role in women with platinum-sensitive recurrence, who have a single recurrent lesion and a prolonged PFI (Hennessy et al., 2009). In the setting of platinum-resistant disease, secondary surgery is often palliative.

To monitor for recurrence, the National Comprehensive Cancer Network has recommended serum CA125 assessment every 3-4 months (Mann, 2013). A rise in serum CA125 predicts clinical relapse within 2-6 months; however, treatment on the sole basis of elevated CA125 has not shown a survival benefit (Rustin, 2010; Rustin et al., 2010).
1.11 Mechanisms of Therapy Resistance

As most patients relapse, re-treatment options are important. Resistance to platinum-based chemotherapy limits the efficacy and use of these compounds, and is believed to cause treatment failure and death in more than 90% of patients (Agarwal and Kaye, 2003). Chemotherapy failure can be classified into three categories: pharmacokinetic, tumour micro-environment dependent and cancer cell-specific. Pharmacokinetic resistance is a function of drug concentration and time of exposure. Drug resistance can result from inadequate intra-tumour drug concentration due to patient variables, such as first-pass metabolism, renal clearance, hepatic drug metabolism and tumour vascularity. The tumour micro-environment triggers signaling pathways in response to various stressors. For example, cellular attachment has been shown to affect chemosensitivity, with cell monolayers displaying different sensitivities to drugs compared to cells grown as spheroids (Francia et al., 2005). Furthermore, hypoxia can induce chemo-resistance. Using an ovarian cancer cell line, Tomida and Tsuruo found that drug resistance was induced in all cell lines under stress conditions (hypoxia, glucose deprivation), and that this resistance was reversible once the stress conditions were removed (Tomida and Tsuruo, 1999). Cancer cell-specific resistance pertains to acquired somatic mutations and epigenetic changes within tumour cells as they evolve. Bernardini et al., found that resistant tumours were found to have twice as much allelic imbalance as sensitive tumours (Bernardini et al., 2005). Finally, cell specific drug resistance can develop as a result of decreased influx or increased efflux of drug, and/or increased drug detoxification (Rabik and Dolan, 2007). Furthermore, certain cancer cells (e.g., tumor-initiating cells) might be intrinsically more resistant to chemotherapy, as described in Section 1.12.

1.12 Tumour Heterogeneity

Solid tumours display intra-tumour heterogeneity. Two models have been proposed to explain this heterogeneity: the stochastic model and the hierarchical model. The stochastic model holds that all malignant cells within a tumour are equally capable of generating the tumour. Conversely, the hierarchical, ‘cancer stem cell’ model suggests that only a subset of tumour cells
have this capacity (Figure 1.2). These ‘cancer stem cells’ (CSCs) are defined by their distinct gene expression, which confers the ability to self-renew, recapitulate the original tumour, proliferate and promote recurrence (Alvero et al., 2009a; O'Brien et al., 2009a). Given these properties, it should be possible to prospectively isolate and purify this population of tumour cells. Tumour-initiating cells (TICs), often used as a surrogate for CSCs, are defined operationally by their capacity to generate tumours in immunocompromised mice.

Figure 1.2 A) Stochastic and B) hierarchical models of tumour heterogeneity.

Multiple studies have shown that within a single tumour, tremendous genetic heterogeneity exists (Anderson et al., 2011; Gerlinger et al., 2012; Notta et al., 2011; Shah et al., 2012). This heterogeneity is further seen in primary versus metastatic tumours, at presentation versus recurrence and even in tumours taken at the same time from different anatomic sites (Campbell et al., 2010; Ding et al., 2012; Gerlinger et al., 2012; Yachida et al., 2010). Because the majority of patients with ovarian HGSC have resistant and/or recurrent disease, interest into whether CSCs are responsible for drug-resistance in ovarian cancer has grown. In this scenario, although bulk tumour shrinkage occurs with chemotherapeutic treatment, recurrence and metastasis arise from failure of chemotherapy to eradicate CSCs. According to the CSC model, treatment with chemotherapy allows reservoir of CSCs to persist and subsequently reproduce the tumour phenotype (O'Brien et al., 2009a; Zhang et al., 2008). Consequently, eradicating the tumour would require specifically targeting the CSC population.
Current chemotherapeutics are thought to be unable to target CSCs; therefore, treatment with chemotherapy is thought to actually enrich for tumourigenic cells. This has been reported in several cancers, including pancreatic, breast and brain cancer models (Bao et al., 2006; Hermann et al., 2007; Li et al., 2008). Dylla et al. showed that treatment of a colon cancer xenograft model with standard chemotherapy not only enriched for a CSC-phenotype in the residual tumour, but also increased tumourigenic cell frequency (Dylla et al., 2008).

In ovarian cancer, several studies have attempted to isolate ‘CSCs’, but most have utilized cultured primary cells or immortalized cell lines. The first report of ‘CSCs’ was by Bapat et al. who isolated a single tumourigenic clone from a mixed population of cells derived from the ascites (Bapat et al., 2005). Next, Alvero et al. reported the molecular characterization of ovarian cancer stem cells as CD44+. Again, this was primarily done in vitro with insufficient in vivo analysis (Alvero et al., 2009a). Subsequently, using more robust assays, it was found that both the CD44+ and CD44- fractions of primary ovarian HGSC are tumourigenic in immunocompromised mice (Stewart, 2013).

CD133 has been studied as a marker of the CSC population in a variety of solid cancers, including brain, colon, pancreas and lung (Dalerba et al., 2007; Eramo et al., 2008; Hermann et al., 2007; O’Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004). Stewart et al., found that ovarian HGSC conforms to the CSC model, but that there are at least two (CD133+ and CD133-), and possibly three (CD133+, CD133-, CD133+/-) CSC phenotypes in this disease (Stewart et al., 2011). This was performed using limiting dilution assays (LDA), the gold standard method for assessing CSC.

Although it is not within the scope of this thesis to debate the validity of the CSC model, it should be mentioned that several strong criticisms against the model have been made. Most notably, Kern and Shibata argue that the “mathematical support for the concept of therapeutically useful stem cells is weak” (Kern and Shibata, 2007).
1.13 Current Models of Ovarian Cancer

1.13.1 In Vitro Models of Ovarian Cancer

To unravel the key determinants in ovarian cancer initiation, progression and response to treatment, a model that faithfully recapitulates ovarian HGSC is necessary. Most preferable would be in vitro and in vivo models that reflect the cell biology and heterogeneity of the disease. Most studies of ovarian cancer have utilized immortalized cancer cell lines as in vitro tumour models. There are several problems with the current in vitro models. First, cancer lines are derived from cancer cells adapted to grow outside a normal tumour microenvironment, resulting in changes that are distinct from the genetic stress imposed on tumours in patients (Tentler et al., 2012). Moreover, many of these cell lines are unlikely of high-grade serous origin, and as such, are badly suited for investigating ovarian HGSC. This is supported by the work of Domcke et al., who found that the most frequently cited ‘high-grade’ ovarian cancer cell lines (SKOV3, A2780, OVCAR-3, CAOV3 and IGROV1), have genomic profiles that do not reflect those of the primary tumour (Domcke et al., 2013). The authors argue that using cell lines with genomic backgrounds similar to those in patient samples could at least increase the likelihood that conclusions reached in the in vitro setting be transferable to the clinical setting. Furthermore, many of these ‘ovarian HGSC’ cell lines either do not reproduce serous histology when implanted into immune-compromised mice or do not give rise to xenografts at all (Press et al., 2008b; Vaughan et al., 2011). Of the cell lines that do generate the appropriate histology in vivo, their capacity for predicting drug response is severely lacking (Jin et al., 2010). There are a number of problems inherent to ‘predictive’ in vitro assays, making the relationship between tumour inhibition in vitro and a patient’s response complex (Fiebig et al., 2004).

Another in vitro method, developed with the aim of predicting drug response, is the ATP-based tumour chemosensitivity assay (ATP-TCA). This assay measures the viability of dissociated tumour cells, cultured in serum-free medium, against a panel of single agents or drug combinations. Although this method indicates platinum resistance with reasonable accuracy (~90%), this only applies to primary and not recurrent cases (Andreotti et al., 1995; Kurbacher et al., 1995). Because no reliable data have shown this assay strategy to be reliably, clinically useful, it has not been incorporated into clinical practice (Markman, 2011).
1.13.2 In Vivo Models of Ovarian Cancer

Few animal models develop spontaneous ovarian tumours, with the exception of hens. The laying hen provides insight into the role of incessant ovulation in the development of spontaneous ovarian adenocarcinomas (Ricci et al., 2013; Vanderhyden et al., 2003). Forty percent of hens will develop ovarian tumours by age 4, with certain mutations analogous to human ovarian HGSC (Mullany and Richards, 2012). Although the hen provides a good model of determining disease initiation, progression and drug testing, the cost makes these models fairly prohibitive. Also, a challenge of working with hens is the limited knowledge of their molecular biology and difficulty with genetic manipulation (Lengyel et al., 2013).

1.13.2.1 Genetically Engineered Mouse Models

Mice are the primary mammalian model in cancer research due to their short generation time, ability to bear large litters, relative ease of breeding, and advances in mouse genomics (Edinger et al., 2002). Genetically engineered mouse models (GEMMs) were developed 20 years ago in order to provide an efficient system in which to analyze the specific roles of oncogenes and tumour suppressor genes in tumourigenesis in vivo (Mullany and Richards, 2012; Talmadge et al., 2007). In GEMMs, the genetic profile of the mice is altered such that one or several genes thought to be involved in transformation or malignancy are mutated, deleted or overexpressed (Richmond and Su, 2008). Moreover, using Cre-lox conditional targeting, these genes can be altered in a precise, tissue-specific manner (Garson et al., 2012).

Using this technology, two studies showed that concomitant inactivation of Brca1 and Trp53 or Rb1 and Trp53 in mouse OSE can give rise to serous carcinomas; however, conflicting results were found by other studies, which showed that specific conditional inactivation of Brca1 and Trp53, in the presence or absence of Rb, resulted in ovarian leiomyosarcomas (Clark-Knowles et al., 2009; Flesken-Nikitin et al., 2003; Quinn et al., 2009; Xing and Orsulic, 2006). Most recently, Szabova et al., found that inactivation of Rb-mediated tumour suppression induced surface epithelial proliferation and additional bi-allelic inactivation and/or missense p53 mutation in the presence or absence of Brca1/2 caused progression to stage IV disease. As in human ovarian HGSC, these mice developed peritoneal carcinomatosis, ascites, and distant metastases (Szabova et al., 2012).
Despite these impressive advances, GEMMs have not been shown to be superior to xenograft models in a variety of tumour types and have not shown to predict clinical trial results (Francia and Kerbel, 2010). Overall, GEMMs have seldom been used to test novel anti-cancer drugs and the few studies that have used GEMMs against clinically effective agents have not been encouraging. As such, these models have not yet demonstrated a role in drug discovery (Talmadge et al., 2007).

1.13.2.2 Xenografts

The ability to successfully engraft surgically derived tumours from cancer patients has been established for decades. A commonly used strain for maximizing engraftment is the non-obese diabetic severe combined immunodeficiency (NOD-scid) mouse. Non-Obese Diabetic (NOD) is an inbred mouse strain, which has low Natural Killer (NK) cell activity, and lacks circulating complement and proper function of antigen-presenting cells (Bastide et al., 2002). The Scid mutation is a loss-of-function allele of the Prkdc gene. Prkdc encodes the catalytic subunit of DNA-dependent protein kinase, which has a role in resolving DNA DSBs that occur during variable, diverse and joining [V(D)J] recombination. In the absence of V(D)J recombination, mice lack T- and B-lymphocytes but retain innate immune functions (Laboratory, 2013). Together, the NOD-Scid model combines multiple functional defects in adaptive and innate immunity to maximize engraftment efficiency. Unfortunately, NOD-Scid mice are limited by their relatively short life span (due to lymphoma), residual NK cell activity, and other components of innate immunity, which can impede engraftment (Shultz et al., 2007).

To mitigate these problems, an immunodeficient mouse homozygous for targeted mutations at the interleukin-2 receptor (IL-2R) γ-chain locus was generated. These mice have increased engraftment of human tissue compared with all previously developed immunodeficient mouse models (Ito et al., 2002). The IL-2R γ-chain is a crucial component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, required for signaling by these receptors. The absence of the IL-2R γ-chain blocks NK cell development and results in additional defects in innate immunity (Shultz et al., 2007; Shultz et al., 2005). NOD-Scid-IL2Rγnull (NSG) mice lack mature lymphocytes and NK cells, have severe defects in innate immunity, do not develop thymic lymphomas (unlike NOD-Scid), and engraft human tissues at high levels (Shultz et al., 2005).
Although their lack of immune response translates into high engraftment rates, it also means that aspects of the host-tumour interaction cannot be studied using xenograft models.

 Typically, the process of generating a patient-derived xenograft (PDX) consists of obtaining fresh surgical tissue, sectioning it into small pieces, and implanting these pieces into an immunodeficient mouse (Tentler et al., 2012). One of the main advantages of PDX models is maintenance of the original tumour architecture and histological characteristics (Gray et al., 2004). Unlike xenografts using tumour cell lines, the PDX-process of engraftment and expansion appears to maintain the majority of the key genes and global pathway activity in tumours. This has been shown in lung, colorectal, and pancreatic cancer PDX models, where a high degree of genetic similarity between the primary cancer and the corresponding PDX tumour was observed (Daniel et al., 2009; Fichtner et al., 2008; Jones et al., 2008; Tentler et al., 2012). In addition to recapitulating the original tumour biology, there are several other key advantages of using PDX: results can be obtained in a few months (compared to a GEMM, which often require as long as a year to develop), and multiple therapies can be tested in a single tumour sample (Richmond and Su, 2008).

 For ovarian cancer, injection into either the ovarian bursa (intrabursal; IB) or the FT fimbriae would constitute an orthotopic PDX. An intraperitoneal (IP) PDX is also considered orthotopic and is easily generated, but represents advanced metastatic disease. A disadvantage of both IB and IP models is difficulty of monitoring disease progression. Even with recent advances in mouse imaging technology, the ability to accurately measure disease has not been optimized (Ricci et al., 2013). Furthermore, the requirement of an experienced operator combined with low throughput of the technology (typically allowing only one animal to be imaged at a time) diminishes its practicality and affordability (Garson et al., 2005; Kung, 2007).

 Due to the challenges seen with orthotopic PDXs, the standard PDX is ectopic, with cells injected typically implanted subcutaneously (SC) or into the mammary fat pad (MFP). This model allows close monitoring of superficial tumours but the predictive capability of SC PDXs has been questioned (Kung, 2007). Although SC/MFP PDXs recapitulate many aspects of the tumour, it is likely that the tumour-host microenvironment is most similar when cells are implanted into the organs/anatomic sites from which the tumour originally arose.
The advantages of MFP over orthotopic PDX were previously demonstrated by the Neel laboratory, which evaluated the peritoneum, ovarian bursa, kidney capsule and mammary fat pad of mice as sites for xenotransplantation. They found high tumour takes from each site but tumours were most readily and reliably detected while still relatively small in the mammary fat pad. Mammary fat pad xenografts also recapitulated the inter- and intra-tumour heterogeneity of primary HGSC, as assessed by histology and surface immunophenotype (Stewart, 2013; Stewart et al., 2011). These results, combined with the impracticality of using orthotopic models, provided the rationale for using the MFP model in my experiments.

To take advantage of the orthotopic site but to avoid some of the issues seen with ectopic PDXs, fluorescent or bioluminescent technology has been employed. Bioluminescence imaging (BLI) is a powerful tool for imaging of small laboratory animals, enabling the study of ongoing biological processes in vivo. This has been increasingly adopted for tumour burden quantification in mice (Sadikot and Blackwell, 2005). This technology will be further described in Appendix 1.

Despite these challenges, many researchers continue to utilize ectopic PDX for drug testing. Several large-scale PDX programs have been established, and the activity of drugs in these models was scored and compared with subsequent activity in phase II clinical trials. The general conclusion from some of these trials was that the predictive power of PDX is variable. For example, PDX derived from breast carcinomas predict poorly whereas PDX derived from lung tumours have a good predictive value (Johnson et al., 2001). The criticism of those experiments is that the pharmacology of the therapeutic was poorly understood and not optimized, thus making the drug dosing schedule in humans unrealistic (Sausville and Burger, 2006). Two trials showed high predictive results with PDX used agents where the pharmacology was well understood and the human tumours were directly transplanted from the patient, not first cultured in vitro (Fiebig et al., 2004; Peterson and Houghton, 2004). Recently, Hidalgo et al. reported a pilot study using ectopic PDX from patients with advanced cancers, whose treatments were selected on the basis of activity seen in the PDX. Their findings showed a remarkable correlation between drug activity in the PDX and clinical outcome (Hidalgo et al., 2011). Ovarian cancers were not tested in any of these studies, providing a rationale for this project.
Several investigators have generated PDX from ovarian cancer. The first were Ward et al. and Massazza et al., who used fresh primary tumour ‘slurries’ and injected these IP into nude mice (Massazza et al., 1989; Ward et al., 1987). Schumacher et al. reported that IP injection of ovarian cancer cells from patients’ ascites into Scid mice resulted in tumour formation (Schumacher et al., 1997; Schumacher et al., 1996). Xu et al. implanted intact ovarian cancer samples (all EOC) into the peritoneum of Scid mice (Xu et al., 1999). In recent years, several groups have used bulk tumour specimens to generate PDX. First, Lee et al. grafted tumour specimens under the renal capsule of NOD-Scid mice and found that these tumours retained major histopathological characteristics of the original tissue (Lee et al., 2005). Next, Press et al. showed that PDX grown under the renal capsule of NOD/SCID mice showed minimal genetic change from the original tumour based on array comparative genomic hybridization (Press et al., 2008b). Most recently, Bankert et al. injected tumour cell aggregates IP into NSG mice and observed tumour progression and metastasis mirroring patient disease (Bankert et al., 2011). Although all of these authors state that these PDX are valuable for testing novel chemotherapeutics, no one to date has validated this model for this purpose. Moreover, all previous methods have utilized tissue fragments, which cannot be used to measure TIC frequency. Stewart et al. established robust conditions to generate PDXs from ascites and solid tumours in the MFP of mice, with the capacity to assess tumourigenicity (Stewart et al., 2011). As such, this protocol was utilized for the project.

1.14 Current State of the Problem

The major problem with treatment of ovarian HGSC is that all patients receive platinum and Taxol therapy. This ‘one size fits all’ approach is largely to blame for overall survival being unchanged over the last 30 years. We know that the length of the treatment-free interval (TFI) positively correlates with overall survival, and this is largely due to platinum-resistant/refractory tumours having low response rates to re-treatment (Yap et al., 2009). It is my belief that three major changes can alter the outcomes of patients with ovarian HGSC: improved therapeutics, early identification of platinum-resistant/refractory patients, and the individualization of cancer treatment.
Our recent expanded understanding of the pathogenesis and evolution of ovarian HGSC has led to the development of many new molecular targeted therapies, including those targeting angiogenesis, cell proliferation and metastases. However, individualized patient selection for the application of these targeted therapies is essential (Yap et al., 2009).

One of the most frequently cited reasons for high failure rates of new agents in oncology clinical trials is the paucity of pre-clinical models that recapitulate the heterogeneity of patient disease (Johnson et al., 2001; Tentler et al., 2012). For successful drug development and establishment of treatment strategies, pre-clinical in vivo models must have a good probability of being predictive of similar activity in humans. Ovarian HGSC PDXs could potentially resolve some of these issues, as these tumours have been shown to recapitulate the biological characteristics of the primary tumour.

1.15 Research Questions & Thesis Overview

New models of ovarian HGSC are required that can ultimately improve management of this disease. In order to improve survival, these models should identify platinum-resistant/refractory patients and have the capacity to be used to study new therapeutic cancer treatments. To this end, this thesis is a culmination of addressing several elements of the problem:

1) Can ovarian HGSC PDXs predict response to platinum therapy? (Chapter 2)

2) Does chemotherapy enrich for TICs in ovarian HGSC PDXs? (Chapter 2)

3) Can I generate luminescent ovarian PDXs to utilize bioluminescent imaging for disease monitoring? (Appendix 1)

4) Can we use ovarian HGSC PDXs to evaluate response to novel anti-tumour agents? (Appendix 2)
1.16 Research Objectives & Hypotheses

Because our model of ovarian HGSC PDXs recapitulates disease heterogeneity (Stewart, 2013), I expect that these PDXs also will recapitulate response to chemotherapy. Over the past 6 years, Dr. Neel’s laboratory has procured a large collection of primary ovarian HGSC samples, with clinical follow-up. From this collection, I identified sensitive, resistant and prospective tumours and engrafted these into NSG mice. The PDXs received treatment with Carboplatin and response was measured. I hypothesized that PDXs derived from platinum-sensitive tumours would respond to platinum therapy by displaying a reduction in tumour volume. Conversely, I expected that PDXs derived from platinum-resistant/refractory tumours would show little or no response when treated with platinum.

Next, I utilized these PDXs to ask whether treatment with chemotherapy enriches for CSCs. The CSC model predicts that CSC and non-CSC have differences in biological properties and CSCs might be intrinsically more resistant to conventional chemotherapy than non-CSCs. Each of my PDXs was treated with Carboplatin or vehicle control, and effects on TICs was assessed by limiting dilution assays. As reported for other tumour types, I expected TICs would be enriched following treatment with chemotherapy.

As previously discussed, the orthotopic PDX-model (IB and/or IP) does not allow easy or practical evaluation of disease burden and response to treatment. For these reasons, an ectopic (mammary fat pad, MFP) model was used to evaluate chemotherapeutic response in my cohort of PDXs. However, I also wanted to generate a luminescent ovarian PDX model to monitor disease by imaging (Appendix 1).

Lastly, I used PDXs to evaluate the effects of drug shown to have anti-tumour effects in other cancer types. Disulfiram (DSF), a drug commonly used to treat alcohol abuse, works by causing an accumulation of acetaldehyde, and causing ICLs. By combining this drug with Carboplatin, I hypothesized that the drug combination would increase the number of DNA adducts formed, thereby enhancing the toxic effects of platinum (Appendix 2).
Chapter 2
Patient-Derived Xenografts as Pre-Clinical Models of Response to Chemotherapy
2.1 Introduction

Ovarian cancer is the 6th most common cancer in women and the 7th most common cause of cancer death worldwide (Organization, 2008). The clinical presentation of ovarian cancer is typically related to symptoms of advanced stage disease, as over 80% of women have stage 3 or greater disease at diagnosis (Clegg et al., 2002; Institute, 2013). Epithelial ovarian cancer (EOC) is the most common form of ovarian cancer, and HGSC is the commonest and most aggressive subtype. Aggressive surgical cytoreduction and platinum-based chemotherapy are the mainstays of treatment; however, overall survival for these patients has remained largely unchanged over the last thirty years (Winter et al., 2007).

Clinical outcomes in ovarian HGSC are most strongly related to the length of the platinum-free interval (PFI) as well as debulking status (Huang et al., 2012; Rump et al., 2004; Theriault et al., 2011). Several studies have shown that minimum residual tumour post-operatively is one of the most powerful determinants of survival (Allen et al., 1995; Bristow et al., 2002; Hoskins et al., 1994). For example, residual tumour size of greater than 2 cm is associated with a survival of only 12–16 months, compared with 40–45 months if the tumour is less than 2 cm (Mutch, 2002). The platinum-free interval is defined as the time from first-line platinum chemotherapy to relapse; the longer this interval, the better the response rate to subsequent chemotherapy and the chance for improved survival (Huang et al., 2012).

Unfortunately, over 85% of women with advanced disease at presentation will recur. Thirty-percent will relapse within 6 months of completing platinum therapy, and such patients are termed ‘platinum-resistant’ (Yap et al., 2009). These patients will have response rates of only 10-20% to additional platinum therapy, and 10-25% to other chemotherapeutic agents (Agarwal and Kaye, 2003). Early identification of platinum-resistant patients is critical in order to triage these patients into clinical trials. Moreover, appropriate and individualized patient selection for the application of these novel and targeted therapies is critical (Yap et al., 2009).

High failure rates plague oncology clinical trials, and one of the most frequently cited reasons is the paucity of pre-clinical models that recapitulate the heterogeneity of patient disease (Johnson et al., 2001; Tentler et al., 2012). According to Cespedes et al., the ideal cancer model should
show histologic similarity to the human tumour, progress through the same stages of disease, involve the same genes and biochemical pathways, closely reflect the response of the human tumour to a particular therapy and finally, predict therapeutic efficacy in assays (Cespedes et al., 2006). The creation of such cancer models is essential for advancing translational ovarian cancer research (Press et al., 2008b).

Traditionally, in vivo experimental approaches have relied on the use of GEMMs or xenografts established from ovarian cancer cell lines. Neither of these types of models has demonstrated a valuable role in drug discovery, likely because they do not reflect the genetic and cellular diversity of human HGSC (Talmadge et al., 2007). PDXs potentially resolve some of the issues seen with other in vivo models. Establishment of PDXs consists of obtaining surgical tissue and implanting it into an immunodeficient mouse (Tentler et al., 2012). PDXs’ tumours have been shown to maintain the architecture, biological properties and histological characteristics of the original tumour (Gray et al., 2004; Stewart et al., 2011).

A robust PDX model could alter the course of patients with ovarian HGSC in two ways. First, if PDXs reproduce the patient’s response to platinum treatment, this could allow for identification of platinum-resistant/refractory patients. Identifying such ‘high-risk’ patients could change the way they are triaged, followed and managed, and ultimately could increase the patient’s quality and quantity of life. Second, these models could be used to develop and test new therapeutics and predict the best course of treatment for a given patient. Several therapies could be tested at the same time on a ‘library’ of PDXs derived from the same tumour, thereby tailoring the patient’s treatment in order to establish effective drug response.

### 2.1.1 Drug Dose Translation

First-line chemotherapy for advanced ovarian cancer consists of Carboplatin and paclitaxel administered IV at the maximum tolerable dose (MTD), followed by a treatment-free interval to allow recovery of healthy tissues. The efficacy of Carboplatin is directly related to its plasma concentration. Since Carboplatin is eliminated primarily via glomerular filtration, the pharmacokinetics and, ultimately, the pharmacodynamics of Carboplatin are highly dependent on renal function. Conventional fixed dosing based on body surface area (BSA) has led to Carboplatin overdosing or, more commonly, under-dosing, which has resulted in less than
optimal treatment results (Alberts and Dorr, 1998). Consequently, dosing is typically individualized by using the Calvert formula: target AUC x (creatinine clearance +25). The area under the curve (AUC) is based on pharmacokinetic studies of the dose effect. The AUC level of drug and/or its active metabolites correlates with toxicity and therapeutic effect and is a product of desired serum concentration (mg/mL) and time (min) (Frei and Antman, 2000). Typically, an AUC of 4-6 is used for treating ovarian HGSC patients, with doses corresponding to 600-900 mg/treatment (Calvert et al., 1989).

**Table 2.1 Previously published Carboplatin doses administered to mice.**

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Mouse strain</th>
<th>Cancer model</th>
<th>Carboplatin Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Jandial et al., 2009; Press et al., 2008b)</td>
<td>NOD/SCID</td>
<td>Ovarian &amp; Uterine</td>
<td>Carboplatin IP 80 mg/kg Qweek x2</td>
</tr>
<tr>
<td>(Jandial et al., 2009)</td>
<td>nu/nu</td>
<td>Ovarian</td>
<td>Carboplatin single dose: 85 mg/kg IP</td>
</tr>
<tr>
<td>(Merk et al., 2009)</td>
<td>NMRI:nu/nu</td>
<td>NSCLC</td>
<td>Carboplatin IP 75mg/kg d1 and d8</td>
</tr>
<tr>
<td>(Fichtner et al., 2008)</td>
<td>NMRI:nu/nu</td>
<td>NSCLC</td>
<td>Carboplatin IP 75 mg/kg d1 and d8</td>
</tr>
<tr>
<td>(Harrap et al., 1990)</td>
<td>unspecified</td>
<td>Ovarian cancer cell line</td>
<td>Carboplatin 100 mg/kg d0 &amp; d7</td>
</tr>
<tr>
<td>(Das et al., 2008)</td>
<td>BALB/c-nu/nu</td>
<td>Neurblastoma, Medulloblastoma</td>
<td>Carboplatin 100mg/kg IP Qweek x 3 weeks</td>
</tr>
<tr>
<td>(Merk et al., 2011)</td>
<td>NOD/SCID</td>
<td>NSCLC</td>
<td>Carboplatin 75 mg/kg/d Qd 1 and 8 IP</td>
</tr>
</tbody>
</table>

NSCL: non-small cell lung cancer

Typically, mouse-to-human drug dose translation is obtained through normalization of the dose to the BSA and multiplication by the \( Km \) factor (Human equivalent dose = Animal dose x \( Km_{animal}/Km_{human} \)). The \( Km \) factor, body weight (kg) divided by BSA (m\(^2\)), is used to convert the mg/kg dose to a mg/m\(^2\) dose. The BSA correlates well with parameters of mammalian biology (blood volume, renal function, etc.), which makes BSA normalization logical for scaling drug doses between species (Reagan-Shaw et al., 2008). However, because dosing of Carboplatin uses the Calvert formula rather than BSA dosing, using the \( Km \) factor for drug translation into mice is
adequate but imperfect. As such, I performed a thorough literature search to determine the Carboplatin doses that had previously been used in other studies (Table 2.1).

### 2.1.2 CA125

CA125 (MUC 16) is a trans-membrane glycoprotein with a short intracellular domain, and a large 22,097 amino acid extracellular domain, and an average molecular weight of ~2.5 million Dalton (Theriault et al., 2011). It is aberrantly expressed and cleaved from the surface of ovarian cancer cells and shed into the blood, providing a useful biomarker for disease monitoring (Wang et al., 2008). CA125 is elevated in greater than 85% of serous HGSC patients and plays an important role in the detection and management of patients (Duffy et al., 2005; Hogdall et al., 2007).

CA125 is also thought to have a role in tumour cell growth, tumourigenesis, cell adhesion and metastases (Rump et al., 2004; Theriault et al., 2011). MUC16 extends from the surface of ovarian cancer cells and has been shown to bind to mesothelin, a protein found on the surface of mesothelial cells lining the peritoneal cavity (Scholler et al., 2007). Theriault et al. showed that knockdown of MUC16 in ovarian cancer cell lines inhibited tumour growth in vitro and in vivo, and conversely, that overexpression increased tumourigenicity and metastases (Theriault et al., 2011). Furthermore, in vitro, it has been implicated in sensitivity of ovarian cancer to chemotherapy, with higher levels being suggestive of platinum resistance (Boivin et al., 2009).

Physiologically, mucins are involved in coating, lubricating and protecting epithelial surfaces of internal tracts (Bafna et al., 2010). During embryonic development, CA125 is expressed in fetal coelomic epithelia and its derivatives. In adulthood, MUC16 is normally expressed by the lining of pleural, peritoneal and pelvic cavities, as well as in the pericardium of the heart, ocular surface and upper respiratory tract.

Given its expression in a variety of tissues and organs, CA125 serum levels have been detected in other types of cancers (endometrial, colon, pancreatic, breast) and in some benign conditions (endometriosis, pelvic inflammatory disease), making it a non-specific marker for ovarian cancer (Bast et al., 1998; Wang et al., 2008). With a positive and negative predictive value of 60% and 90%, respectively, serum CA125 levels are used to assess the risk of malignancy in women with
a pelvic mass (O’Connell et al., 1987). Once diagnosed with ovarian cancer, CA125 measurements are performed routinely and are used to monitor response to treatment, progression and recurrence (Aggarwal and Kehoe, 2010; Diaz-Padilla et al., 2012). The positive predictive value of elevated serum CA125 at suspected recurrence is >95% (Prat et al., 2009). Decisions to continue or cease chemotherapy are made on the basis of rising CA125, even in the absence of radiological progression. Given the importance of CA125 serum values in managing ovarian cancer patients, I wanted to assess whether PDXs similarly express CA125. Moreover, I wanted to see if the change in CA125 following chemotherapy could be used in conjunction with tumour volume measurements as a corollary measure of drug response.

2.1.3 Tumour Initiating Cells

Over 85% of patients will recur following initial therapy, and the challenge in treating HGSC is that recurrences tend to be platinum-resistant, limiting further treatment options (Kulkarni-Datar et al., 2013). Understanding how chemo-resistant tumour cells develop and potentially cause disease resurgence could provide a strategy to modify the current therapeutic management of both primary and recurrent ovarian cancer.

Solid tumours, and in particular HGSC, display tremendous inter- and intra-patient cellular heterogeneity (Bashashati et al., 2013; Burgos-Ojeda et al., 2012). Two models exist to explain these distinct tumour cell populations. The stochastic model holds that all cells within a tumour are equally capable of generating and sustaining the tumour. This model argues that cancer heterogeneity is the result of selection pressures exerted on tumour cells, causing stochastic genetic and epigenetic changes. These changes confer a survival advantage to certain cells and the development of an increasingly more aggressive cancer over time (Aparicio and Caldas, 2013).

By contrast, the CSC model suggests that only a subpopulation of tumour cells, termed Cancer Stem Cells (CSC), possess self-renewal ability and the capacity to initiate tumour growth and drive tumour progression. In the CSC model, tumour heterogeneity results from differentiation of CSC into phenotypically diverse non-tumourigenic cells (Kulkarni-Datar et al., 2013). An essential feature of the CSC model is that tumours are organized hierarchically, so that
tumourigenic and non-tumourigenic cells can be prospectively differentiated from one another according to their unique phenotypes (Burgos-Ojeda et al., 2012).

Importantly, a hierarchical organization of cells, intrinsic to the CSC model, has not been described in ovarian cancers (Dyall et al., 2010). Furthermore, no single marker clearly identifies the ovarian CSC population and the use of combinations of markers has yielded inconclusive results (Foster et al., 2013). Burgos-Ojeda et al., proposed a hierarchy of ovarian CSC based on the current literature; however, they acknowledge that conclusive markers and definitive data have yet to be identified (Burgos-Ojeda et al., 2012).

Recent attention has focused on ovarian CSCs as a mechanism behind disease relapse and drug resistance. It is believed that CSCs in other tumour sites evade the toxicity of standard chemotherapy due to their slow rate of cell division and enhanced drug efflux properties (Rizzo et al., 2011; Steg et al., 2012). As such, following treatment with chemotherapy, a reservoir of CSCs could persist and subsequently reproduce the tumour phenotype (O'Brien et al., 2009; Zhang et al., 2008). As a result, treatment with chemotherapy is thought to actually enrich for tumourigenic cells. This has been shown in several cancers, including colon, pancreatic, breast and brain cancer models (Bao et al., 2006; Hermann et al., 2007; Li et al., 2008; Dylla et al., 2008).

Although often used interchangeably, CSCs differ from tumour initiating cells (TICs) operationally. TICs are cells capable of initiating a tumour in immunodeficient mice. However, according to the CSC model, TICs are organized hierarchically and can be distinguished prospectively from non-TICs. Many cancers might contain TICs, but some could also contain tumorigenic cells, without hierarchical organization (Zhou et al., 2009).

The ability to identify and isolate CSCs in other tumours was made possible by knowledge of surface markers, expression patterns, and immunophenotyping of normal stem cells in some organs or tissues (Bapat, 2010). Lack of information regarding normal stem cells in ovarian biology has been a limiting factor in the identification of CSCs in ovarian cancer (Alvero et al., 2009a). Several groups have identified CD133, ALDH, CD44, CD117 and CD24 as ‘stem-like’ markers; however, a hierarchy using these markers has yet to be defined (Curley et al., 2009; Gao et al., 2010; Luo et al., 2011; Zhang et al., 2008). Stewart et al., found that ovarian HGSC
conforms to the CSC model, but that there are at least two (CD133+ and CD133-), and possibly three (CD133+, CD133-, CD133+/-) CSC phenotypes in this disease (Stewart et al., 2011).

To date, enrichment or isolation of these CSCs has been carried out via multiple strategies, most frequently utilizing immortalized cell lines (which typically are not HGSC) or cultured cells, which calls into question the relevance of these results to primary human tumours. By definition, CSC must have the capacity to form tumours in vivo, which again, argues for caution in interpretation of many studies that have used in vitro experiments (Zhou et al., 2009).

Some of these studies have suggested that there is an enrichment of ovarian cancer stem-like cell populations post-chemotherapy, supporting the concept that these cells resist conventional chemotherapy and contribute to resurgence of disease (Figure 1.2). Alvero et al., identified a CD44-positive cell population with CSC-like properties, that when treated with Carboplatin and paclitaxel, differentiated to become even more resistant in response to chemotherapy (Alvero et al., 2009b). Again, these studies were done in vitro and did not properly examine the CD44-negative cell compartment.

The gold standard assay to measure the frequency of cells possessing a unique functional property within a heterogeneous cell population is in vivo limiting dilution analysis (LDA). In normal and cancer stem cell biology, this is an experimental method that is used to detect and quantify cells with the ability to engraft a recipient mouse. For CSCs, this assay measures the frequency of cells that are able to give rise to tumours (Eirew et al., 2010). I performed LDAs with tumour cells obtained from Carboplatin-treated PDXs or control (saline-treated) PDXs in order to ascertain whether treatment with Carboplatin enriches for the TIC population.

2.2 Methods

2.2.1 Identification of Patient Samples

Patient samples were identified retrospectively, to be either platinum-resistant (3654, 3875, 6447, 2555, 2028, 2462, 2803, 2489, 3444, 2903, 6259, 2685, 2753, 2261, 4070, 62143, 6259) or platinum-sensitive (3748, 3670, 61382, 63867, 3028, 3336) at the time of acquisition. In
addition, samples were required to have sufficient clinical information and must have been previously viably frozen in ample quantities in the Neel laboratory’s HGSC repository. Prospective samples (67199, 67326, 6565, 6775) were chemo-naïve, primary samples from patients likely to be followed at The Princess Margaret Cancer Centre.

2.2.2 Tumour Processing & Establishment of Xenografts

Human tumour specimens were obtained with informed consent from patients undergoing surgery at the PM following a protocol approved by the University Health Network Research Ethics Board (REB #06-0903T).

HGSC tissue samples were obtained from the University Health Network Tissue Bank. Once confirmed by a pathologist, tumours were obtained within 5 hours of excision. To establish first generation xenografts (X1), solid tumours were minced and digested with collagenase-hyaluronidase (Stem Cell Technologies) in DMEM at 37°C for two hours. Red blood cells were lysed in 0.16M ammonium chloride for approximately 5 minutes. The remaining cells were filtered through a 70µm mesh and viable cells were counted using trypan blue exclusion. Ascites cells were collected by centrifugation at 300xg and processed as above. Anti-CD45 microbeads were used to deplete leukocytes, as per the manufacturer’s instructions (Miltenyi Biotec). CD45-depleted cells (10^6) in 1:1 HBSS:growth factor-reduced Matrigel (BD Biosciences) were injected into the mammary fat pads (1 pad/mouse) of healthy, 6-8 week old, female NSG mice. Tumours obtained from xenografts were processed as described above. Single cells were frozen viably in 10% DMSO, 15% FBS, 50% DMEM, and stored in liquid nitrogen.

To generate X2 (or later passage) xenografts, previously frozen cells were thawed rapidly in 37°C and washed in HBSS + 2% serum. Anti-H2K-biotin microbeads were used to deplete mouse stroma, as per the manufacturer’s instructions (Miltenyi Biotec). H2K-depleted cells (10^6) in 1:1 HBSS:growth factor-reduced Matrigel (BD Biosciences) were injected into the right mammary fat pad of 10-15 NSG mice. When tumours became palpable (~200 mm³), mice were treated with Carboplatin. Mice were sacrificed when tumours exceeded the size of 2.5cm or if mice looked unwell or fell under criteria indicated in the humane endpoints.

Upon reaching the endpoint (either termination of treatment or humane endpoint), all mice were
sacrificed in accordance with the protocol procedures. Tumours were excised, a portion was snap-frozen at −80 °C, and some tissue was fixed in 10% neutral buffered formalin and paraffin-embedded. If there was sufficient material, cells were viably frozen according the methods described above or were expanded into additional mice (either for LDA or future use).

2.2.3 Tumour Measurement

Tumour measurements were made every 72 hours using electronic calipers. Tumour volume was approximated using the formula: volume = length x width² x 0.52.

2.2.4 Carboplatin Administration

Carboplatin 10mg/mL (Hospira) was purchased from the PM pharmacy. Based on the mouse-to-human drug translation formula (Section 2.1.1), and assuming an average human adult weight of 60kg, I estimated that an appropriate Carboplatin dose for mice to be approximately 125 mg/kg. Given the published Carboplatin LD₁₀ of 100mg/kg, and published literature using smaller (<125mg/kg) doses, I performed an MTD assay (Figure 2.1), on healthy, 6-8 week female NSG mice. From this, I concluded that 75mg/kg would be an appropriate dose. As such, experimental mice were given 75mg/kg Carboplatin IP (in a total volume of 0.35 cc normal saline) once weekly (day 0 & day 7) for 2 doses (dosing based on Table 2.1). Control mice received 0.35 cc of normal saline IP.

2.2.5 CA125 ELISA & Immunohistochemistry

Serum was collected from all xenografts corresponding to specimens 3654 and 3670 at four times: baseline (prior to inoculation with tumour cells), 6 weeks after inoculation with tumour, pre-chemotherapy dose 1 and post-mortem (one week after 2nd chemotherapy dose). To do so, mice were warmed with a heating lamp for approximately 10 minutes. The dorsal limbs were shaved, and blood was drawn from the saphenous vein and collected into Eppendorf tubes. Post-mortem blood was collected via cardiac puncture. Whole blood was stored on ice until it was centrifuged at 16,000 rotations per minute. The serum layer was collected and stored at -20°C for future use. A CA125 ELISA kit was purchased from Alpha Diagnostic International (Cat. No. 1820). Assays were performed according to the kit instructions. As most serum samples were
less than 25 μL, typically 20μL of standards, controls and serum was pipetted into each well. Patient serum samples (obtained with consent) were used as positive controls, with known CA125 values of 500, 50 and 5 U/mL.

To evaluate CA125 by immunohistochemistry (IHC), paraffin-embedded tissue sections were stained with antibodies against CA125 (Leica, NCL-CA125; 1:50), according to the UHN Pathology Research Program protocol.

2.2.6 Limiting Dilution Assays

**Figure 2.0. Diagram showing LDA method and sample preparation.** At the completion of A) Carboplatin treatment or B) control treatment (3 weeks), mice with palpable tumours were sacrificed and tumours were excised. Each tumour was dissociated into a single cell suspension. Cells were counted and bilaterally injected into the fat pads (FP) of NSG mice.
Upon reaching the termination of Carboplatin treatment, all mice were sacrificed in accordance with the protocol procedures. Tumours were excised and processed as described previously. If there were sufficient cells, LDAs were performed by injecting three cell doses into the mammary fat pads of NSG mice (Figure 2.0). The number of fat pads injected for each dose was case- and cell number-dependent and ranged from 2 to 8. TIC frequencies were calculated using ELDA software (http://bioinf.wehi.edu.au/software/elda/) (Hu and Smyth, 2009).

2.3 Results

2.3.1 Engraftment

A total of 27 patient tumours were implanted between January 2012 and March 2013. Of these, 17 were classified as platinum-resistant, 6 platinum-sensitive, and 4 prospective (Table 2.2). Eighteen out of 27 (67%) tumours (corresponding to 16 unique patients) engrafted successfully: 13/17 (76%) resistant cases, 3/6 sensitive cases (50%) and 2/4 (50%) prospective cases. Two cases represent the same patient with samples obtained at different points in the patient’s clinical course (3654/3875 and 2462/2803). Of the 18 successfully engrafted cases, 10/18 (56%) were from ascites and 8/18 (44%) from solid tissue samples. One mouse had lymphoid proliferation, without evidence of an epithelial tumour was found (6259) and was discarded. Median time from cell injection to tumor treatment (at ~200mm³) was 15 weeks (range 3-21 weeks).

2.3.2 Patient Demographics

Patient demographics were analyzed for the 18 cases (corresponding to 16 patients) and are summarized in Table 2.3. The mean age of all patients was 60.3 years. The mean age of platinum-resistant, platinum-sensitive and prospective patients was 63.2, 53.6 and 54.5 years, respectively. One patient had a known BRCA1 mutation (3444) and one patient was a BRCA1 variant of undefined significance (3748). The majority of patients (12/16; 75%) had either not been tested or were negative. All patients in the ‘resistant’ category received their initial course of Carboplatin/Taxol intravenously, whereas the ‘sensitive’ and ‘prospective’ patients received either IP + IV or IP only. In addition, all sensitive and prospective patients were optimally
debulked. Patient 61382 was ‘optimally’ debulked, but there was a question of a liver lesion post-operatively. In the resistant group, 4/11 (36%) were known to be optimally debulked. All patients had stage 3C or greater, high-grade disease.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sample Type</th>
<th>Sample Chemo Status</th>
<th>Xenograft Formed</th>
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<tbody>
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2.3.3 Mouse Carboplatin MTD

The maximum tolerated dose is defined as the dose that causes no more than a 10% decrease in body weight and does not produce mortality or toxicity. Based on a known mouse LD$_{10}$ value of 100mg/kg (data not shown) and published experiments (Table 2.1), I performed an MTD assay using a dose ranging from 20-80mg/kg to assess whether a target dose of 75mg/kg was appropriate. Mouse body weight was measured weekly and compared to original (starting) body weight (Figure 2.1). Based on this assay, no mouse had a >10% weight loss at any of the tested doses. As a result, I proceeded with using 75mg/kg Carboplatin IP for our experiment.

![Figure 2.1 Establishment of Carboplatin maximum tolerated dose (MTD) in NSG mice.](image)

Treatment was administered once weekly (d0, d7; black arrows) and body weights were measured weekly. Points are an average of two mice represented as percent of original starting weight.
2.3.4 PDXs from Platinum-Sensitive Patients Show a Near-Complete Response to Platinum Therapy

To define response, I used the RECIST criteria definition of at least a 30% decrease in tumour size (Eisenhauer et al., 2009). In total, PDXs were derived from 3 platinum-sensitive patients: 3670, 3748 and 61382. The clinical history of each patient will be described in detail. PDXs derived from chemo-sensitive patients showed 77-91% (average 83%) reduction in tumour volume, compared with the initial tumour.

Figure 2.2A shows the clinical history for patient 3670 based on her CA125 graph and Figure 2.2B shows the PDXs’ response to platinum therapy. At the time of diagnosis, patient 3670 was a 68-year-old woman with a history of an abdominal mass and pain. This pain persisted over 3 months, until finally in October of 2009, she presented to the emergency room. A CT scan showed a large left lower quadrant mass and evidence of bowel ischemia. She underwent urgent surgery, which consisted of a TAH-BSO, omentectomy, debulking and sub-total colectomy. She had stage 3C disease and was optimally debulked. Post-operatively, she received one cycle of IV Carboplatin/Taxol, followed by 6 cycles of IP Cisplatin+IV Taxol. In November of 2011, she had ultrasound imaging that confirmed the presence of multiple solid pelvic masses consistent with recurrent carcinoma, as well as metastatic adenopathy in the peri-aortic region. She continued further chemotherapy in Barrie, Ontario. As of August 2013, the patient continues to be alive.
Figure 2.2. Clinical course of patient 3670 and PDXs’ response to platinum. A) Clinical history of patient based on serum CA125 (U/mL) over time (months). Patient 3670 recurred 18 months after completion of initial therapy. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #3670 PDXs (n=13) to platinum therapy ± s.e. PDXs received initial dose of Carboplatin when the tumours averaged 200 mm³ (black arrow indicates time of treatment). Note 91% percent decrease in tumour volume of Carboplatin-treated PDXs, compared with initial tumour volume (paired t-test one-tailed P value = 0.0005).
Figure 2.3A shows the clinical history for patient 3748 based on her CA125 values, and Figure 2.3B shows the PDXs’ response to platinum therapy. Patient 3748 was a 40-year-old women referred to the GYN oncology clinic in October 2009 with bilateral pelvic masses. The patient had experienced abdominal pain since August 2009, and had an ultrasound through her family physician that revealed a vascular, 8 x 7 x 5.9 cm predominantly solid mass in the posterior cul-de-sac, and a small 2.5 cm mass in the inferior aspect of the sigmoid thought to arise from the left ovary. Her CA125 was 3533. In November, she underwent primary debulking surgery (TAH/BSO, omentectomy, LAR, right diaphragmatic stripping, peri-aortic lymph node dissection, pelvic peritonectomy) and was found to have stage 3C disease. Post-operatively, she received 2 cycles of IV Carboplatin/Taxol, followed by 4 cycles of IP Cisplatin/IV Taxol, which finished in May 2010. Following routine examination in October 2011, the patient was thought to have a local recurrence at the rectovaginal septum, which was confirmed by MRI. The patient was offered Carboplatin/Taxol but preferred to delay treatment until May 2012. She completed 6 cycles of Carboplatin/Taxol in July 2012. In July 2013, she was diagnosed with a second recurrence with a nodule in the rectovaginal septum and a CA125 of 2000U/mL. As of August 2013, she is alive and may be re-starting Carboplatin/Taxol therapy in the near future.
Figure 2.3. Clinical course of patient 3748 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Patient 3748 recurred 17 months after initial therapy. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #3748 PDXs (n=5) to platinum therapy± s.e. PDXs received initial dose of Carboplatin when the tumours averaged 300 mm³ (black arrow indicates time of treatment). Note, 77% decrease in tumour volume of Carboplatin-treated PDXs, compared with initial tumour volume (paired t-test one-tailed P value is 0.04).
Figure 2.4A shows the clinical history for patient 61382 based on her CA125 values and Figure 2.4B shows the PDXs’ response to platinum therapy. At the time of initial diagnosis, patient #61382 was a 52 year-old, post-menopausal female. She had an ultrasound to investigate symptoms of abdominal pain and vaginal discharge, which revealed bilateral adnexal masses, with mixed solid and cystic components and no ascites or extra-ovarian disease. Her CA125 at the time was 341. In January 2008, she underwent a TAH, BSO, pelvic peritonectomy, appendectomy, resection of sigmoid colon, right partial diaphragmatic resection, and omentectomy. She had stage 3C disease, which was optimally debulked. She began with one cycle of IV chemotherapy (Carboplatin + Taxol) and switched to IP chemotherapy for five additional cycles. Following completion of chemotherapy, the patient had a CT scan to confirm remission; However, this scan demonstrated a ~1cm lesion on the serosa of the liver, which was suggestive of disease and called into question whether the patient was optimally debulked. This lesion stayed relatively stable in size over the course of several months. However, a repeat scan in November 2009 showed a new lesion in the hilum of the spleen. In September 2010, she underwent a debulking splenectomy with no adjuvant chemotherapy. In December 2011, the patient had a PET scan, which showed two lesions in the liver and one peri-aortic node suggestive of recurrent disease. The patient completed 6 cycles of second-line IV Carboplatin/Taxol in July 2012 and continues to have no evidence of disease on CT scan.
Figure 2.4. Clinical course for patient 61382 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Patient 61382 recurred 18 months after initial therapy. Dashed line represents normal serum CA125 (35 U/mL) B) Response of #61382 PDXs (n=4) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 150 mm³ (black arrow indicates time of treatment). Note 81% reduction in tumour volume of Carboplatin-treated PDXs, compared with initial tumour volume (paired t-test one-tailed P value is 0.001).
2.3.5 PDXs from Platinum-Resistant Patients do not Respond to Platinum Therapy

In total, PDXs were derived from 11 platinum-resistant patients: 3654/3875, 6447, 2261, 2462/2803, 2489, 3444, 2555, 2903, 2753, 2028 and 2685. PDXs response ranged from 29% (#6447) reduction in average tumour volume to 307% (#2555) increase in tumour volume, despite platinum therapy. Six of the 11 cases showed some degree of reduction in tumour volume, 1 case showed no response and 4 cases progressed in spite of platinum treatment. None of the cases met the RECIST response criteria (30% reduction). The clinical histories of each patient are described in detail.

Figure 2.5A shows the clinical history for patient 3654/3875 based on her CA125 values, and Figures 2.5B and 2.5C show the response of PDXs 3654 and 3875 to platinum therapy, respectively. At initial diagnosis, the patient was a 77-year-old referred from a transitional hospital due to extensive abdominal carcinomatosis and elevated CA-125 (8500U/mL). She experienced bloating, constipation, and urinary difficulties, with a 20 pound weight loss and significant fatigue. Her past medical history was significant for a colonic adenocarcinoma treated with colectomy 37 years prior and a renal cell carcinoma, treated by nephrectomy 9 years earlier. For these reasons, in October 2008, she was seen in a peripheral hospital where a CT scan showed ascites, peritoneal carcinomatosis, and a possible recurrence at her rectal stump.

Prior to commencing chemotherapy, the patient had an omental biopsy, which revealed a high-grade metastatic carcinoma. She began to receive Carboplatin (20% reduction due to patient’s frailty) and Taxol. She received 4 cycles prior to surgery. March 2009, she underwent a delayed debulking surgery, consisting of a BSO, omentectomy. She was found to have stage 3C disease, and went on to receive 4 additional cycles of chemotherapy. Unfortunately, in July 2009, she had increasing abdominal pain and bloating, and a CT scan done in the emergency room confirmed disease progression. Given her platinum-refractory disease, the patient was started on Caelyx chemotherapy in September 2009, then Gemcitabine in October. Minimal response was observed and the patient continued to decline. She had several palliative paracentheses and died in March 2010.
Figure 2.5. Clinical course for patient 3654/3875’s and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. The patient recurred 3 months after initial therapy. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #3654 PDXs (n=7) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 200 mm³ (black arrow indicates time of treatment). C) Response of #3875 PDXs (n=5) to platinum therapy± s.e. Carboplatin-treated PDXs 3654 showed an average 21% increase in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.2). PDXs 3875 showed an average 6% reduction in tumour volume of Carboplatin-treated PDXs, compared with initial tumour volume (paired t-test one-tailed P value is 0.1).

Figure 2.6A shows the clinical history for patient 6447 based on her serum CA125 values and Figure 2.6B shows PDXs’ response to platinum therapy. Patient 6447 was a 62-year-old woman with a 1-month history of bloating, abdominal pain and fatigue. Ultrasound imaging revealed free fluid in the cul-de-sac and a mass measuring 3.8 x 2.5 cm, with a CA125 of 1204 U/mL. She received single agent Carboplatin in December 2009, followed by 2 cycles of Carboplatin/Taxol. She had interval debulking (TAH/BSO, omentectomy) surgery in April 2010, with stage 3c disease that was optimally debulked. Post-operatively, she completed a further 3 cycles of Carboplatin/Taxol, ending in June 2010. CT scan done February 2011 showed peritoneal nodules and ascites. She received one cycle of IV Taxol, and then 7 more cycles of Carboplatin/Taxol, completed in October 2011. Nevertheless, she had significant progression of disease based on
CT imaging. She started Gemcitabine in April 2012, but continued to decline in function. She died in May 2012.

Figure 2.6 Clinical course for patient 6447 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Patient 6447 recurred 9 months after initial therapy. Dashed line represents normal serum CA125 (35 U/mL). B. Response of #6447 PDXs (n=9) to platinum therapy± s.e. PDXs received first
dose of Carboplatin when the tumours averaged ~300 mm$^3$ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed an average 29% reduction in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.004).

Figure 2.7A shows the clinical history for patient 2261 based on her CA125 values, and Figure 2.7B shows the PDXs’ response to platinum therapy. This patient was seen in May 2007 as a 47 year-old, who had been diagnosed previously with stage 3c disease in July 2005 at a peripheral hospital. At that time, she had a subtotal hysterectomy, BSO and omentectomy, followed by 6 cycles of Carboplatin/Taxol. CT imaging in May 2007 showed a rectovaginal nodule, in combination with a rising CA125. The patient opted to have her recurrence treated in Hungary, with naturopathic measures (vitamin-B17 IV) and paracenthesis. The patient was counseled to receive traditional chemotherapy but continued to refuse until July 2008, when she received one dose of Carboplatin/Taxol. She died in September 2008.
Figure 2.7 Clinical course of patient 2261 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on CA125 serum measurements over time. The patient recurred 18 months after initial therapy. B) Response of #2261 PDXs (n=3) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 150 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed an average 21% increase in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.08).

Figure 2.8A shows the clinical history for patient 2028 based on her CA125 levels and Figure 2.8B shows the PDXs’ response to platinum therapy. Patient 2028 was seen in the gynecologic oncology clinic in October 2007, as a 76-year-old with presumed ovarian cancer. She had a month-long-history of abdominal distention, anorexia, early satiety, and constipation, which prompted her to go to the emergency room, where an ultrasound showed ascites and bilateral hydronephrosis from a solid left adnexal lesion, measuring 2.7x2.2x2.4 cm. Her CA125 was 1971 U/mL. She received five courses of neoadjuvant Carboplatin/Taxol and proceeded to interval debulking (TAH/BSO, omentectomy, peritoneectomy), which was sub-optimal, in February 2008. She then completed 2 additional cycles of Carboplatin/Taxol in April 2008. Unfortunately, her CA125 never fell below 136, and in July, it was 862, with continued disease progression. In January 2009, she was treated with single agent Carboplatin. She was admitted to the palliative care floor in March 2009 and died in April 2009.
Figure 2.8 Clinical course of patient 2028 and PDX’s response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Patient 2028 recurred 3 months after initial therapy. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #2028 PDXs (n=6) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 150 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed an average 15.5% reduction in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.03).
Figure 2.9A shows the clinical history for patient 2803 based on her CA125 levels and Figure 2.8B shows the PDXs’ response to platinum therapy. This patient was initially seen in 2004, as a 57-year-old with abdominal pain. An ultrasound demonstrated a large adnexal mass, and the patient’s CA125 was elevated at 313U/mL. This patient received 4 cycles of neoadjuvant chemotherapy, followed by interval debulking surgery (BSO, omentectomy). She had stage 3C disease, and received 4 adjuvant courses of chemotherapy, completed in September 2004. In October 2004, a CT showed minimal residual lesions in the pelvis and omentum. For this reason, the patient started second line Caelyx in November 2004. She received 6 doses of Caelyx, ending March 2005. In March 2007, she began to experience flank pain, and a CT showed lymphadenopathy and moderate ascites. In July 2007, she was started on gemcitabine, but due to the development of a rash, was switched to Topotecan for 8 cycles, ending in December 2007. The patient was then re-started on cisplatin for 6 cycles, ending July 2008 due to ototoxicity. As a consequence of her persistent disease, the patient was started on etoposide in September 2008, but stopped in October due to disease progression. She died in August 2009.
Figure 2.9 Clinical course of patient 2803 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on CA125 serum measurements over time. The patient progressed on therapy. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #2803 PDXs (n=6) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 100 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed an average 6% increase in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.3).

Figure 2.10A shows the clinical history for patient 2489 based on her CA125 level and Figure 2.10B shows the PDXs’ response to platinum therapy. Patient 2489 was seen in clinic in January 2008. At the time, she was 48-years-old, with a biopsy-proven papillary serous carcinoma, thought to be ovarian in origin. She presented in December of 2007 with shortness of breath. A chest x-ray showed a right pleural effusion, which was tapped and showed malignant cells. She also had an omental biopsy, which showed papillary serous carcinoma and an elevated CA125 (2107 U/mL). She was given seven courses of Carboplatin/Taxol, ending in June 2008. Due to disease progression, she began avastin and cyclophosphamide in September 2008. Unfortunately, a breast lump confirmed to be a synchronous breast primary carcinoma was discovered in November 2008. In March 2009, the patient began third line therapy in the form of Caelyx, but continued to have ascites and a right pleural effusion. She was admitted to the palliative care unit in July 2009 and died in August.
Figure 2.10 Clinical course of patient 2489 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. The patient progressed on therapy. B. Response of #2489 PDXs (n=4) to platinum therapy ± s.e. PDXs received first dose of Carboplatin when the tumours averaged ~150 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed an average 11% reduction in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.04).
Figure 2.11A shows the clinical history for patient 3444 based on her CA125 levels and Figure 2.11B shows the PDX’s response to platinum therapy. Patient 3444 was initially treated for serous ovarian cancer in 2007. At that time, she received 3 cycles of neoadjuvant Carboplatin/Taxol, followed by interval debulking surgery, and a further six cycles of Carboplatin/Taxol, completed in February 2008. A routine CT scan performed in September 2008 showed an irregular mass adjacent to the rectosigmoid, which increased in size on a follow-up scan. This mass received radiotherapy in 15 fractions completed in April 2009. The patient’s past medical history was significant for a T1N2 receptor-positive breast cancer, treated in 1994 with a lumpectomy followed by chemotherapy, radiation and five years of tamoxifen treatment. Given the breast and ovarian cancers, the patient underwent genetic testing and was found to be BRCA1 mutation carrier. It was felt that the best way to deal with this recurrent pelvic mass would be to perform a secondary surgery, which occurred in July 2009. The patient completed another six courses of Carboplatin/Taxol, which finished in 2011. In November 2012, she was found to have enlarging para-aortic nodes, and was started back on chemotherapy, which she finished in March 2013. The patient had a third surgery in March 2013, in the form of a bilateral para-aortic lymphadenectomy and common iliac node dissection. All nodes were positive for disease. She resumed chemotherapy at her local hospital and is alive as of August 2013.
Figure 2.11 Clinical course of patient 3444 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. The patient progressed on therapy. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #3444 PDXs (n=7) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 150 mm³ (black arrow indicates time of treatment). 3444 PDXs showed no reduction in tumour volume when treated with Carboplatin, compared with initial tumour volume (paired t-test one-tailed P value is 0.5).
Figure 2.12A shows the clinical history for patient 2555 based on her serum CA125 levels and Figure 2.12B shows the PDXs’s response to platinum therapy. Patient 2555 was seen in June 2008 at age 77. Her history dated back to August 2007 when she first noted a hard right-sided inguinal mass. The mass gradually increased in size but the patient did not seek medical attention until a year later. She went on to have a CT scan in April 2008, which showed several masses in both the left and right inguinal regions. In addition, there were enlarged nodes along the left and right common iliac arteries. Biopsy of the groin node was performed in May 2008, and showed metastatic carcinoma, consistent with an ovarian origin. CA125 was 1289 U/mL. The patient remained largely asymptomatic, aside from some leg swelling as a result of the nodal compression. She received one cycle of single agent Carboplatin in August, then presented to the ER shortly thereafter with abdominal pain from advanced disease. She died in September 2008.
Figure 2.12 Clinical course of patient 2555 and PDX's response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Dashed line represents normal serum CA125 (35 U/mL). B) Response of #2555 PDXs (n=6) to platinum therapy ± s.e. PDXs received first dose of Carboplatin when the tumours averaged 250 mm³ (black arrow indicates time of treatment). 2555 PDXs showed a 307% increase in tumour volume when treated with Carboplatin, compared with initial tumour volume (paired t-test one-tailed P value is 0.02).

Figure 2.13A shows the clinical history for patient 2903 based on her CA125 levels and Figure 2.13B shows the PDXs’ response to platinum therapy. This patient was referred from an outside hospital. She was 57-years-old with stage 4 disease, and had previously received three lines of chemotherapy. She was initially diagnosed in 2004 with ascites, a pelvic mass and a CA125 of 3344. She received 3 cycles of neoadjuvant Carboplatin/Taxol, followed by a TAH/BSO/omentectomy in April 2005. She received 3 additional cycles of Carboplatin/Taxol. Her CA125 was undetectable at the completion of treatment in August 2005. One year later, in July 2006, she re-developed ascites and had a CA125 of 2500U/mL. She was then treated with 6 cycles of liposomal doxorubicin, which ended in December 2006. In April 2007, she developed ascites again, with a CA125 of 1483U/mL and was treated with cisplatin plus VP-16 for 2 cycles. In January 2008, she was started on Letrozole, but her CA125 continued to rise and CT imaging
showed peritoneal metastases. In July 2008, she started on a clinical trial of AMG 386 (an anti-angiopoietin 1 and 2 drug) plus Taxol, but progressed on this and was started on Etoposide in mid-January 2009. She continued on Etoposide treatment for a very short period and was enrolled in a PARP-inhibitor trial, but had progression of disease. She died shortly thereafter, in May 2010.
Figure 2.13 Clinical course of patient 2903 and PDX’s response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Dashed line represents normal serum CA125 (35 U/mL). B) Response of PDXs (n=4) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 150 mm$^3$ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed a 10% reduction in tumour volume when treated with Carboplatin, compared to initial tumour volume (paired t-test one-tailed P value is 0.1).

Figure 2.14A shows the clinical history for patient 2753 based on her CA125 levels and Figure 2.14B shows the PDXs’ response to platinum therapy. Patient 2753 was first seen in the fall of 2008 as a 58-year-old referred for a pelvic mass and extensive ascites. She had surgery in late October 2008, consisting of TAH/BSO/omentectomy and debulking, but was sub-optimally debulked due to diffuse disease. The patient received six cycles of adjuvant Carboplatin/Taxol and two cycles of single agent Carboplatin, but continued to have residual pelvic disease at the completion of treatment in May 2009. The patient pursued some travel prior to initiating second line treatment with Caelyx in November. She had continued disease progression and started third line therapy (Gemcitabine) in May 2010 and fourth line therapy (Topotecan) in August 2010. The patient is alive as of August 2013.
Figure 2.14 Clinical course of patient 2753 and PDX’s response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Dashed line represents normal serum CA125 (35 U/mL). B) Response of xenografts (n=4) to platinum therapy ± s.e. PDXs received first dose of Carboplatin when the tumours averaged 400 mm³ (black arrow indicates time of treatment). Experiment had to be terminated prematurely as tumour volume was approaching endpoint. After one dose, PDXs did not respond to Carboplatin therapy and continued to grow at a rate similar to that of controls.
Figure 2.15A shows the clinical history for patient 2685 based on her CA125 levels and Figure 2.15B shows the PDX’s response to platinum therapy. This patient was initially seen in June 2008, at the age of 72, when her family physician found clinical and radiological evidence of ovarian cancer. The patient had symptoms of bloating and abdominal discomfort. An ultrasound showed a moderate amount of ascites, and blood work revealed a CA125 of 4759 U/mL. The patient had a previous history of breast cancer, treated with surgery in 2001 and tamoxifen, ending in 2006. She had a paracenthesis confirming serous adenocarcinoma, consistent with an ovarian origin. She completed four cycles of IV neoadjuvant chemotherapy in September 2008, prior to her surgical procedure in October 2008 (BSO, omentectomy). She was sub-optimally debulked, with residual disease on the diaphragm, large bowel and cul-de-sac. She received four additional cycles of Carboplatin/Taxol, completed in January 2009. CT scan performed shortly after the completion of chemotherapy showed increased peritoneal disease and an enlarging pelvic mass. The patient died in March 2009.
Figure 2.15 Clinical course of patient 2685 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on serum CA125 measurements over time. Dashed line represents normal serum CA125 (35 U/mL). B) Response of xenografts (n=4) to platinum therapy± s.e. PDXs received first dose of Carboplatin when the tumours averaged 200 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed a 37% increase in tumour volume compared with initial tumour volume (paired t-test one-tailed P value is 0.03).

2.3.6 ‘Prospective’ PDXs Predict Chemo-Responsiveness

PDXs from two patient samples (67199 and 67326) were derived and treated prospectively (i.e., prior to knowing the clinical outcomes of the patients). Xenografts derived from both patients showed a marked reduction in tumour volume (90% and 63% for 67199 and 67326, respectively), suggesting that these patients would be platinum-sensitive. After following their clinical course for over 1 year, both patients have not recurred (>12 months), confirming that both patients have platinum-sensitive disease.

Figure 2.16A shows the clinical course of patient 67199. The patient was 51-years-old when initially seen in the gynecologic-oncology clinic with CT scan results showing a solid mass with hyper-vascularity, as well as ascites and omental caking. At the end of March, she received a TAH/BSO, peritonectomy, diaphragm resection, and para-aortic lymph node sampling. She was
optimally debulked and had stage 4 disease. She received one cycle of IV Carboplatin/Taxol, followed by 5 cycles of IP cisplatin/IV Taxol. In June, her CA125 rose to 63 (from 36 in March), suggesting an early indication of recurrence; however, a CT scan in July showed no evidence of disease. Her current PFI is 17 months. The PDXs had a near complete response to platinum therapy. Given that the patient’s PFI is >17 months, the PDXs correctly predicted the platinum-response of this patient.
Figure 2.16 Clinical course of patient 67199 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on CA125 serum measurements over time. Dashed line represents normal serum CA125 (35 U/mL). B) Response of PDXs (n=5) to platinum therapy ± s.e. PDXs received first dose of Carboplatin when the tumours averaged 150 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed a 90% reduction in tumour volume, compared with initial tumour volume (paired $t$-test one-tailed $P$ value is 0.03).

Similarly, patient 67326 was initially seen in the gynecologic oncology clinic in March 2012, as a 58-year-old female with a left adnexal mass and a CA125 of 92 U/mL. The patient had been feeling otherwise asymptomatic, aside from some mild abdominal distention. In April 2012, she underwent a TAH/BSO, LAR, omentectomy, pelvic and para-aortic lymph node dissection. She had stage 3c disease and was optimally debulked. Post-operatively, she received 6 cycles of IP cisplatin and IV Taxol day 1 and IP Taxol day 8. Last seen in April 2013, she had no evidence of disease. Her current PFI is 16 months. Figure 2.17 shows the patient’s clinical course and the PDXs’ response to platinum.
Figure 2.17 Clinical course of patient 67326 and PDXs’ response to platinum. A) CA125 graph showing clinical history of patient based on CA125 serum measurements over time. Dashed line represents normal serum CA125 (35 U/mL). B) Response of PDXs (n=4) to platinum therapy ± s.e. PDXs received first dose of Carboplatin when the tumours averaged 400 mm$^3$ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed a 63% reduction in tumour volume, compared with initial tumour volume (paired t-test one-tailed P value is 0.05).
2.3.7 PDX ‘Recurrence’ also Parallels Patient Clinical Course

PDX 3670 was derived from a platinum-sensitive patient, and these PDXs showed a 90% reduction in average tumour volume upon platinum treatment (Figure 2.2B). At the completion of the experiment, several mice (n=4) without palpable tumours were not sacrificed in order to see if the tumours would re-grow. One-hundred forty-four days after the completion of treatment, tumours in these mice became palpable again, and they were subjected to a second round of Carboplatin therapy. Figure 2.18 shows the response of the ‘re-grown’ xenografts to platinum treatment. Again, the xenografts showed a complete response to platinum therapy, parallels the patient’s response.

![Graph showing response of PDXs to platinum treatment.](image)

**Figure 2.18 #3670 PDXs response to second course of treatment with platinum.** PDXs re-formed tumours 144 days after completion of initial therapy. PDXs (n=2) received ‘third’ dose of Carboplatin when the tumours averaged 150 mm³ (black arrow indicates time of treatment). Carboplatin-treated PDXs showed a 90% reduction in tumour volume, compared with initial tumour volume.
2.3.8 Summary of PDXs' Response to Carboplatin

Figure 2.19 summarizes the results for all PDXs tested. Cases classified as ‘resistant’ showed a range in response to Carboplatin treatment, from a 29% reduction (#6447) to a 305% increase (#2555). The sensitive cases and prospective cases (which turned out to be sensitive), showed a 60-90% reduction in tumour volume. Figure 2.19B shows that when the treatment groups are divided between ‘sensitive’ and ‘resistant’ cases, those classified as platinum-sensitive show a significant reduction in average tumour volume, compared to resistant cases. There is no difference in final average tumour volume between sensitive and resistant cases among the untreated controls.
2.3.9 Primary Tumour Histology is Maintained in PDX Tissue

All PDX tissue was sent for histologic examination and review by a gynecologic pathologist. All cases were confirmed to be HGSC, reflecting the histology of the primary tumour.

2.3.10 PDXs’ Serum CA125 Cannot be Used to Monitor Disease Progression and/or Response

Using two cases (3670 and 3654), blood was collected from control (n=3) and treatment mice (n=7) at 4 various times: baseline (prior to tumour inoculation); at 6 weeks; prior to first dose of chemo (12 weeks); at endpoint (14 weeks). Patient serum CA125 at sample acquisition was 50
U/mL and 3725 U/mL for cases #3670 and #3654, respectively (Table 2.4). Although there was seemingly a temporal change in mouse serum CA125 as demonstrated by absolute absorbance values (Figure 2.20A), this corresponded to a clinically irrelevant CA125 value of ~10 U/mL when plotted on the standard curve (Figure 2.20B). Furthermore, temporal changes seen in Figure 2.20A were seen in both treated and control mice, suggesting normal fluctuations not related to treatment.

A)

B)

Figure 2.20. PDX 3670 and 3654 CA125 levels A) CA125 ELISA showing absolute absorbance values measured over time ± s.e. Serum was collected at four time points, one of which corresponds to prior to administration of chemotherapy (black arrow). For both cases, the control group consisted of n=3, and treatment group, n=7. B. Absorbance values from graph (A) plotted on CA125 standard curve. All values correspond to CA125 < 10 U/mL (red triangle).
2.3.11 PDXs CA125 Immunohistochemistry Parallels Patient Serum CA125 Value

CA125 IHC staining was obtained on 15 PDX samples and compared with patient serum values from the time of sample acquisition (Table 2.4). PDXs’ tissue obtained from patients with a low serum CA125 (<100 U/mL) had minimal positive cell staining (cases: 3670, 2803, 2261, 2803). Conversely, PDXs from patients with high serum CA125 (>500 U/mL) have considerably greater CA125 staining (cases: 2903, 3654, 6447, 2489, 2028, 67199). However, there does not appear to be a strong positive correlation, as some PDXs from high CA125 patients showed only a moderate amount of staining (cases: 2555, 3748).

Table 2.4. Patient serum CA125 value and PDX CA125 immunohistochemistry.

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2.2.12 Effect of Carboplatin Treatment on TICs

Six cases were used for LDAs; however, one was subsequently found to be a mouse lymphoma and results were discarded. Four of the 5 cases were from platinum-resistant patients and one was from a ‘prospective’ patient, who was ultimately found to be platinum-sensitive. Cases 3654, 3875, 2555 and 6447 are all ascites specimens, whereas 67326 was a solid tumour sample. Additional clinical details can be found in Table 2.2. In addition, LDA PDXs were passage 4 (x4) for case 3654, x3 for case 3875, x5 for case 2555, and x2 for cases 6447 and 67326.

Only one case (3654) showed a significant enrichment for CSC’s after in vivo treatment with chemotherapy (Figure 2.21, Table 2.5). At the time of the LDA, treated mice had an average tumour volume of 300 mm$^3$ and control mice of 600 mm$^3$. This case was derived from a chemoresistant patient. TIC frequency increased from 1/18,000 to 1/3,000 following Carboplatin treatment, with a p-value of 0.03.

Three cases (3875, 6447 and 2555), all derived from platinum-resistant patients, yielded insignificant results (Tables 2.6-2.7). Both control and treated PDXs from case 3875 had an average tumour volume of ~250 mm$^3$ (no difference between groups) at the time of the LDA (Figure 2.22). For case 3875, two PDXs (one mouse) died prior to tumour formation, making the results difficult to interpret.
Control PDXs from case 6447 had an average tumour volume of 800 mm$^3$, compared with 200 mm$^3$ in the treatment group (Figure 2.24). This case had the largest number of pooled tumours (n=5 in control group; n=9 in treatment group) and showed the most response to chemotherapy of all of the platinum-resistant cases (29% reduction in tumour volume). There was a trend toward enrichment in the chemo-treated PDXs (1/700 versus 1/1,400), but this was not statistically significant.

At the time of the LDA, PDXs from case 2555, for both treatment and control, had the largest average tumour volume of all cases (1800 mm$^3$ and 500 mm$^3$, respectively) (Figure 2.23). This case grew very rapidly (from implantation to palpable tumours = 3 weeks) and aggressively, as indicated by a TIC frequency of 1/1 in the treated group and 1/36 in the control group. The TIC frequency in both control and treated groups was much higher than in any other case.

The LDA for one case (67326) showed significant enrichment (p<0.005) for CSCs in the control arm of the experiment. These PDXs were derived from a platinum-sensitive patient (Figure 2.24). The tumour volume of the control and treated groups averaged 750 mm$^3$ and 200 mm$^3$, respectively.
Figure 2.21 Carboplatin sensitivity experiment for case #3654. Carboplatin administration occurred at day 0 and day 7 (168 hours) (black arrows). Tumours from all mice were pooled for LDA experiments on day 14 (red arrow). Table 2.5 summarizes the results of the LDA experiments (xenografts formed/xenografts injected).
Figure 2.22 Carboplatin sensitivity experiment for case #3875. Carboplatin administration occurred at day 0 and day 7 (168 hours) (black arrows). Tumours from all mice were pooled for LDA experiments on day 14 (red arrow). Table 2.6 summarizes the results of the LDA experiments (xenografts formed/xenografts injected).

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C.I. = Confidence Interval

\[ p = 0.2 \]

a= Two xenografts died prior to tumor formation
Figure 2.23 Carboplatin sensitivity experiment for case #2555. Carboplatin administration occurred at day 0 and day 7 (168 hours) (black arrows). Tumours from all mice were pooled for LDA experiments on day 14 (red arrow). Table 2.7 summarizes the results of the LDA experiments (xenografts formed/xenografts injected).
Figure 2.24 Carboplatin sensitivity experiment for case #6447. Carboplatin administration occurred at day 0 and day 7 (168 hours) (black arrows). Tumours from all mice were pooled for LDA experiments on day 14 (red arrow). Table 2.8 summarizes the results of the LDA experiments (xenografts formed/xenografts injected).

Table 2.8 LDA results for case 6447

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TIC Frequency

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C.I. = Confidence Interval
Figure 2.25 Carboplatin sensitivity experiment for case #67326. Carboplatin administration occurred at day 0 and day 7 (168 hours) (black arrows). Tumours from all mice were pooled for LDA experiments on day 14 (red arrow). Table 2.9 summarizes the results of the LDA experiments (xenografts formed/xenografts injected).
2.4 Discussion

The value of an *in vivo* model depends on the extent to which its characteristics reflect the properties of the cancer. The aim of this study was to establish a preclinical model of ovarian high-grade serous cancer that maintains a correlation with the characteristics of the clinical disease. My findings suggest that, despite the tremendous cellular heterogeneity of the primary tumour and the diverse clinical course of each patient, PDXs correctly reproduce the patient response to platinum therapy. The PDXs’ response (or lack thereof) to platinum therapy correlated with sample status (i.e., platinum-resistant or platinum-sensitive) in 100% of the cases.

I established and treated 18 cases, corresponding to 16 unique patients. For patients in whom Carboplatin was ineffective, irrespective of whether this was primary or acquired resistance, the corresponding PDXs showed no response when treated with platinum. Conversely, treatment of PDXs derived from platinum-sensitive patients showed near-eradication of the tumour. Furthermore, after completion of therapy, tumours for one platinum sensitive case (3670) regrew. Upon re-treatment with Carboplatin, this tumour was once again sensitive to the effects of platinum. This also is in keeping with the patient response: the patient was re-treated with platinum at her recurrence 18 months after completing initial therapy (data not shown). Four years after her initial diagnosis, the patient continues to be alive, suggesting that her tumour had a good response to platinum re-treatment.

Most impressively, the PDXs were able to predict the chemo-responsiveness of two patients for who the clinical course of disease was not known at the time of the experiments. Cases #67199 and #67326 were primary, chemo-naïve samples engrafted at the time of the patients’ primary surgery. PDXs derived from both patients showed a nearly complete response to platinum therapy. Based on my PDX results, I predicted that each patient’s respective PFI would be >12 months, in keeping with platinum sensitivity. To date, both patients have a PFI of >17 months.

Three elements of this model that are worth discussing in detail: first, is the choice and dose of chemotherapy administered; second, is the method used to determine PDX response; and third, is the correlation with patients’ clinical outcomes.
2.4.1 Chemotherapy

Standard chemotherapy treatment for patients with ovarian cancer is platinum based using either Carboplatin or Cisplatin. Originally cisplatin was the agent of choice, however, data from numerous clinical trials of advanced ovarian cancer have now documented that IV Carboplatin is equivalent to IV Cisplatin in activity, but causes considerably fewer side effects, including ototoxicity, neurotoxicity, and nephrotoxicity. Standard IV treatment with Carboplatin and paclitaxel is administered every 21 days for six cycles (Hennessy et al., 2009). More recently, the preferred timing and route of administration have come into question. For optimally debulked patients, IP chemotherapy has shown to have an advantage over standard IV chemotherapy, with Cisplatin being the preferred analog (Armstrong et al., 2006). Despite some of the current debates surrounding chemotherapy administration, platinum-based chemotherapy remains the cornerstone of treatment of ovarian HGSC.

For my PDX experiments, mice were treated with IP Carboplatin, using a dose of 75mg/kg/dose. Carboplatin was selected over Cisplatin because 15/16 patients in my cohort received Carboplatin during their clinical course, whereas only one received exclusively Cisplatin (#67326). Because Cisplatin and Carboplatin exert the same effects on DNA, I expect that the results observed in this study would be very similar if IP Cisplatin (at the appropriate dose) had been used instead. IP administration was used because attempts at mouse tail (IV) administration were unsuccessful. These treatments were difficult and imprecise, particularly when administering the second dose. After receiving the first chemotherapy dose, mice were vasoconstricted, causing drug to spill out of the puncture site. This same phenomenon was observed when attempting to collect blood for serum CA125 analysis, and as such, could only be done post-mortem. In humans, it has been shown that through IP administration there is a high IV drug concentration, therefore, I am confident that the IP mode of drug administration in the PDXs is adequate in delivering the drug to the tumour.

As previously stated, Carboplatin is administered based on the Calvert formula, so that each patient receives an individualized dose (Section 2.1.1). Because of this, there is no ‘standard dose’; however, the average dose ranges between 600-900mg/treatment. Converting this to a mg/kg dose (~10mg/kg), and then using the $K_m$ factor to determine the mg/m$^2$ dose, I estimated
that the approximate equivalent Carboplatin dose for mice is 125 mg/kg. Although using the \( Km \) factor provides a reasonable approach for estimating human-equivalent/mouse-equivalent doses, this factor does not take into account the mouse strain. Compared with other strains, NSG mice are more sensitive to DNA damage. As such, I searched the literature to determine previously published Carboplatin doses, administered specifically to immunocompromised mice. Because no study utilized NSG mice, I performed an MTD assay to ascertain an appropriate drug dose (Figure 2.0). Based on these results, I decided to use a conservative Carboplatin dose of 75 mg/kg/mouse, appreciating the fact that I could be under dosing the mice, and thereby having less chemotherapeutic effect than expected. However, the results demonstrate that this dose was appropriate to discriminate between platinum-resistant versus platinum-sensitive cases.

2.4.2 Assessment of Response

This study showed that PDXs predict response to platinum-therapy. However, in order to draw this conclusion, several methods of assessing 'response' were evaluated. First, I attempted to validate the use of PDX serum CA125 as a surrogate marker for chemo-responsiveness. Second, I examined the use of BLI and other imaging modalities, and finally I examined actual tumour size, which was possible due to the MFP tumour site. Ultimately, tumour size measurement proved to be the most robust method.

In patients, CA125 levels at the time of diagnosis are of limited prognostic significance; however, changes in CA125 in response to treatment have proven useful. Decreasing levels of CA125 after cytoreductive surgery and during initial courses of chemotherapy have been used as an indicator of clinical outcome (Gupta and Lis, 2009). Patients with advanced disease have a significantly lower OS when CA125 is persistently elevated after three courses of neoadjuvant chemotherapy (Van Dalen et al., 2000a; van Dalen et al., 2000b). Considering the success of CA125 in determining response to chemotherapy in humans, I wanted to see if this method could be used in PDXs.

In my assay, I used patient serum samples as positive controls (500, 50 and 5 U/mL), and these mapped appropriately to the standard curve, suggesting that the ELISA was sensitive enough to detect low CA125 levels. However, PDXs’ serum CA125 value was equivalent to that of the negative control (un-injected NSG mice). Additionally, CA125 levels did not decrease with
chemotherapy administration, and there was no link to a clinically significant value. There are multiple potential reasons for this discrepancy. It is possible that mouse factors do not allow the protein to enter circulation (e.g. due to large protein size). Second, it could be that CA125 undergoes additional modifications in the mouse that render it undetectable by the ELISA assay. Also, it could be that the ectopic mammary fat pad model does not allow CA125 to be cleaved into the serum and perhaps, an orthotopic model using the peritoneal cavity would resolve this issue. Other groups have successfully quantified serum CA125 using IP PDXs (unpublished results, Paul Haluska; Ronny Drapkin). Considering these data, and the fact that PDXs tumours did show positive CA125 IHC staining, means that CA125 could have a role in future models; however, for this project, CA125 could not be used as a corollary of platinum-response.

The next step was to evaluate the ability to monitor tumour load longitudinally by using BLI. This method provides sensitive, quantitative, and non-invasive detection of luciferase-positive cells. I attempted to generate luciferase-expressing PDXs to track tumour growth and metastasis (Appendix 1). Although these PDXs initially displayed a luciferase signal, ultimately, no tumour was seen histologically. This method might become useful in monitoring disease progression and response to treatment, but requires further development and optimization, and as such, could not be used in this study.

Although using BLI was unsuccessful in my study, I also evaluated the utility of other imaging modalities. Previously, the Neel laboratory assessed several sites for xenotransplantation, and found that tumours were most readily and reliably detected in the MFP (Stewart, 2013). Moreover, xenotransplantation into the ovarian bursa generally resulted in the development of ascites, without formation of solid tumours (unpublished results, Jocelyn Stewart). To use an IB PDX model and monitor disease via longitudinal measurements of ascites (by CT, MRI and/or U/S) is not practical for many reasons. First, the ability to accurately quantify disease has not been optimized at our institution. Furthermore, the requirement of an experienced operator, combined with the high cost and low throughput of the technology decreases its practicality and affordability.
These combined observations provided the rationale for using the MFP site, where tumor measurements can easily be done with calipers. ‘Response’ was evaluated according to the RECIST definition. These criteria were developed to assess the change in tumour burden following the use of therapeutics, and is considered the standard for assessing response in a clinical trial setting. According to RECIST, a 30%, or greater, reduction in the target lesions constitutes a partial response. Based on this, a reduction in PDXs tumour volume of >30% was interpreted as ‘response’, whereas anything below 30% was characterized as ‘no response’ (i.e., progressive/stable disease).

2.4.3 Patients’ Clinical Outcomes

Clinical response to therapy in patients is typically assessed by changes in serum CA125 levels, which are inexpensive, reproducible, and quantitative measurements. CA125 is elevated in ~90% of ovarian HGSC, and in these patients, CA125 is the most useful serum marker for monitoring response to chemotherapy and detecting disease recurrence (Bast et al., 2005). For these reasons, I used serum CA125 levels as the primary mode of assessment of tumor response in patients included in this study. One patient in the study (#3444) was a CA125 non-producer, and in this case, imaging was used to determine response and to diagnose recurrence.

Standard definitions of platinum-resistance and sensitivity in ovarian cancer are based on time-to-recurrence after completion of platinum treatment. This is a practical definition that has been used to guide clinical management; however, results obtained in this project question this definition. For example, there is a group of patients who have an immediate, complete decline in CA125 in response to platinum therapy (primary and/or recurrent tumours). Riedinger et al. showed that CA125 normalization after the first course of chemotherapy is an independent prognostic factor for achieving a complete response and for improving OS, indicating that these tumours are dramatically sensitive to platinum-based therapy (Riedinger et al., 2007). By contrast, in another group, CA125 normalizes slowly and gradually over the course of chemotherapy (corrected for tumour burden). If patients from both groups recur within 6 months of completion of treatment, they are both treated uniformly and considered platinum-resistant. The limitation of this definition is highlighted by one case (#6447) tested in my cohort.

Case #6447 showed a 29% reduction in tumour volume, which I classified as ‘no response’.
However, given the standard error, this represents a borderline partial response according to the RECIST definition. Looking closely at the clinical course for this patient, she had a good response to Carboplatin/Taxol therapy, but recurred 8 months following treatment. Because she was classified as ‘platinum sensitive’ (>6 months to recurrence), she was re-treated with platinum/Taxol, and again, showed a good response as demonstrated by a complete and immediate normalization of her CA125. Six-months later, the patient had a rise in CA125 and was treated with Gemcitabine, to which she did not respond. The PDXs’ response (29% tumour reduction) suggests that this patient might have had some response to 3rd line Carboplatin/Taxol treatment, and this could have been more effective than treatment with Gemcitabine.

Case 6447 highlights the challenges that clinicians face in the monitoring and treatment of patients. The 6-month cutoff used to classifying patients as platinum-resistant or sensitive is clearly imperfect and based on a few small, old studies (Gore et al., 1990; Markman et al., 1991). Certainly, platinum refractory patients are unlikely to benefit from platinum therapy; however, some patients characterized as ‘platinum-resistant’ might still display a clinical benefit in receiving second-line platinum. Although this response might be small, it is likely comparable, and perhaps, better than other second-line agents.

Another problem with the current definition of platinum-resistance is that it can often be related to the frequency of clinical surveillance and not necessarily the biology of the tumour. For example, two patients (A and B) might recur within 6 months of completion of therapy; Patient A is seen at the time of recurrence (6 months) but Patient B’s recurrence is diagnosed at 8 months, because she is only seen every 4 months. Due to the arbitrary cut-off of 6 months to classify platinum resistance, Patient A will likely receive second-line agents, whereas Patient B will be re-treated with platinum (provided there are no contra-indications), and might have a clinical outcome different from Patient A. Case 6447 illustrates this scenario; the patient’s first recurrence was documented 8 months following the completion of chemotherapy. However, she did not have any investigations (e.g., blood work, imaging) performed during those 8 months, and her ‘real’ recurrence time could have been months earlier. Because she was diagnosed at 8 months, she was eligible to receive platinum therapy.

As platinum-based therapy is the most successful chemotherapy option for patients, the current
classification based on time-to-recurrence might be doing patients a disservice. The response to platinum is likely to be unique to each recurrence and should be identified longitudinally through the course of the patient’s disease, therefore providing patients with the chance to gain some additional benefits from this agent. To return to the example of case 6447, according to the clinical record, this patient’s 3rd recurrence (<6 months) was characterized as platinum-resistant. However, my study demonstrates that the cells from 6447, when grown as PDXs, show borderline-response. This suggests that this patient might have benefitted from a third course of platinum therapy, despite being classified as platinum-resistant. In the context of this study, PDXs derived from multiple recurrences of the same patient, or treated with several rounds of platinum, might serve to assess the responsiveness of the tumour to platinum and other second-line and/or investigational drugs, in order to determine which treatment(s) would be most effective.

As experimental models, PDXs are not without limitations. First, they require ample amounts of fresh tumour material. Typically, in ovarian cancer, this is not a constraint, but might be in the context of patients who have received neoadjuvant chemotherapy. Second, PDXs are costly to maintain compared to traditional cell lines. In addition, their genomic stability with increasing passage remains to be defined. However, these limitations also apply to cell lines, which although cheaper to maintain, have ultimately cost billions of dollars in failed drug development projects. Unlike cell lines and cell-line derived xenografts, PDXs maintain the architecture of the original tumour. Third, in addition, there is drift of stromal components from human to mouse, genetic drift away from the primary tumour after numerous transplantations, loss of the immune microenvironment, and difficulties with orthotopic transplantation (Sausville and Burger, 2006). Last, PDXs’ engraftment rates are variable (~60% in this study) and those that engraft can take up to 4-6 months (3-21 weeks in our study) to be ready for treatment. That time period can correspond to recurrence time for some patients. Nevertheless, because multiple therapies, including platinum, can be tested in parallel, PDXs could have the capacity to predict the next most effective drug for recurrences.

The PFI is one of the strongest predictors of overall survival: the longer this interval lasts, the better the response rate to subsequent chemotherapy (Huang et al., 2012). The ability of PDXs to predict a long versus short PFI could have clinical implications, particularly for patients who are
likely to have a short PFI. Patients with short PFIs (i.e., platinum refractoriness/resistance) typically have poor response rates to subsequent therapy and these are the patients whose treatment options are very limited. Identifying these patients early in the course of their disease can potentially alter their clinical course. First, it could determine how frequently these patients are monitored. Currently, there is no standard protocol for how to assess patients once they have completed initial therapy. Many clinicians monitor their patients every 3-6 months, but this can only capture advanced recurrent disease. Because PDXs provide evidence regarding patients’ chemo-responsiveness, it may be beneficial to monitor patients identified as platinum-resistant (based on their respective PDXs) more closely and rigorously for evidence of disease recurrence. Second, PDXs could allow novel drugs and chemotherapeutic options to be evaluated for each patient. Platinum-resistant patients require targeted or novel therapies for their disease, and PDXs might resolve which therapies are best suited for each specific patient. Current knowledge regarding the clinical management of ovarian cancer suggests that targeted agents must be used in combination to select the right drugs for the right patient at the right time (Romero and Bast, 2012). ‘Personalized cancer therapy’ rather than ‘one size fits all’ is required to increase survival in ovarian cancer patients, and PDXs may serve to resolve this issue.

2.4.4 Tumour-Initiating Cells

The majority of patients with ovarian HGSC have resistant and/or recurrent disease, and as such, interest into whether CSCs are responsible for drug-resistance in ovarian cancer has grown. In this scenario, although bulk tumour shrinkage occurs with chemotherapeutic treatment, recurrence and metastasis arise from failure of chemotherapy to eradicate CSCs (O’Brien et al., 2009b). Several studies examining other cancer sites, have reported that current chemotherapeutics are unable to target CSCs; consequently, treatment with chemotherapy might enrich for tumourigenic cells.

Because I was using a chemotherapy-based PDX model, it seemed reasonable to examine TIC frequency following the chemotherapy-response experiments. Specifically, I wanted to address whether the pool of TICs was enriched following chemotherapy compared to untreated tumours. Unfortunately, partly due to small sample size, my results yielded inconclusive data: one case enriched for TICs following chemotherapy, three cases gave statistically insignificant data, and
one case enriched for TICs in the control group. If I assume that ovarian HGSC conforms to the CSC model, important limitations and considerations obtained from these experiments could improve future projects of this kind.

Some limitations of this study arise in the methods. For successful LDA studies, total cell number is important and inadequate numbers may explain my inconclusive results. At the completion of chemotherapy treatment, many cases had an insufficient number of viable cells to perform an LDA. Consequently, I could only use samples that generated many PDXs simultaneously (in order to pool all tumour cells) or were relatively refractory to platinum treatment (so that tumours at the completion of treatment had high cell numbers). A consideration for future work would be to interrogate the effect of various smaller doses of chemotherapy over time, as opposed to the administering the near-MTD that I utilized. Perhaps using a lower chemotherapy dose (or one dose) and sacrificing mice at earlier, incremental time periods, would still allow for a gradual enrichment of TICs. Doing so would allow more PDXs to be generated per cell dose (this study had 4 PDXs/dose for the majority of cases), which was a major limitation. For this assay to be robust, the experiment requires 8-12 PDXs per cell dose, at a minimum.

TICs are relatively rare in many solid tumours and this number can be misestimated depending on the nature of the PDX-assay. Tumourigenicity assays performed using NSG mice (compared with NOD/SCID) and/or heavily passaged PDXs considerably increase the TIC frequency in some cancers (Ishizawa et al., 2010; Magee et al., 2012). In ovarian HGSC, the Neel laboratory found that the ability to enrich for TICs deteriorated upon PDXs passage. They also found that TIC frequency was at most ~10-fold higher in NSG versus NOD/SCID mice, and even then, it represented ≤0.02% of the CD45-depleted ovarian HGSC cells. In addition, they showed that the median TIC frequency in ascites was ~1/10,000 and ~1/90,000 for solid tumours, but that this ranged considerably between various tumours (Stewart, 2013; Stewart et al., 2011). Given this tremendous range, it would be useful to know in advance the approximate TIC frequency to ensure that the appropriate cell doses are used. This would avoid scenarios, such as those seen in my study, where cell doses are too small or too large to provide meaningful results.
In this study, as in others that use PDXs, one cannot exclude the possibility that the mouse microenvironment causes a misestimation of the TIC frequency. Incompatibilities between mouse ligands and human receptors may impair survival and proliferation of human cells. In addition, because the human cells are ectopically transplanted into mice, the differences in the environment may modify the engraftment of cells and tumourigenic potential (Shackleton et al., 2009). Tumour stroma has been shown to be critical in the development of a TIC niche, where TICs can be maintained and allowed to proliferate and future experiments in this area should explore the role stromal compartments play in maintaining tumour growth.

Furthermore, it would be extremely valuable to identify a marker (or marker combination) of TICs. The CSC model has been carefully tested in a small subset of cancers and is often assumed to apply widely to other cancer types (Shackleton et al., 2009). In conjunction with functional assays, cell markers could help in understanding tumourigenesis, particularly in the context of post-treatment with chemotherapy. Specifically, provided the marker expression was not affected by chemotherapy (an experimental variable that would have to be tested), one could assay TIC markers in the post-chemotreated PDXs, providing a correlative surrogate for TIC enrichment after chemotherapy.

Despite the limitations described, variability in these results might be due to the known inter-patient heterogeneity of this disease. It was shown by the TCGA (The Cancer Genome Atlas Research Network, 2011) that patient tumours could exhibit expression profiles that reflect different subtypes of HGSC. It is possible that the PDXs that I used in this study might fall into different subtypes and that each subtype might have a differential sensitivity of TIC to chemotherapy. In addition, enrichment in the control group might simply reflect the healthiness of the tissues after treatment. It is possible that, despite cells “scoring” as alive by trypan blue exclusion, the chemotherapy-treated cells are actually much less healthy than the control group. Again, this might be PDXs-dependent.

Finally, the lack of significant enrichment of TIC after treatment of some cases might suggest that conventional chemotherapy equally targets tumour-initiating and non-initiating populations – this might suggest that there is no correlation between the treatment-resistant populations of cells and the TIC population. Alternatively, it could suggest that, given the proposed level of
clonal evolution at the time of diagnosis (Bashashati et al., 2013), there are multiple populations (at similar frequencies) that have tumour-initiating ability, but that are differentially resistant to chemotherapy. Consistent with these data, no study has shown that a prospectively phenotypically identifiable population of cells has the unique ability to initiate HGSC. Based on my limited results, I could not make any conclusions about the nature of TIC dynamics in ovarian HGSC PDXs in response to chemotherapy.

Reliable, predictive models are required when a treatment decision must be made, which is of greatest importance for resistant and/or recurrent patients. I conclude that ovarian high-grade serous patient-derived PDXs closely reflect the histology and platinum sensitivity of original donor tissue. This newly validated model represents an effective tool for the identification of platinum-resistant and platinum-sensitive patients. Furthermore, this model may serve as a predictive tool for the investigation and development of novel and targeted therapeutics.
Chapter 3:
Conclusions & Future Directions

Ovarian HGSC is the leading cause of mortality amongst all gynecologic malignancies. Due to a lack of specific symptoms, patients typically present with advanced stage disease. Treatment for all patients follows a similar formula: all patients receive debulking surgery and platinum-based chemotherapy. Most patients have a good response to initial therapy, but nearly all recur. If the recurrence occurs within 6 months of completion of initial therapy or during the course of initial therapy, these patients are classified as ‘platinum-resistant’ or ‘platinum-refractory’, respectively. Second-line treatment for patients with platinum resistant/refractory disease is expected to produce low levels of response and is aimed at extending the symptom-free interval and improving quality of life (Fung Kee Fung, 2011). In these situations, patients are offered non-platinum-based mono-therapy (often with paclitaxel, topotecan, doxorubicin or gemcitabine) and the opportunity to participate in clinical trials.

Patients that recur more than 6 months after completing initial therapy are considered ‘platinum sensitive’. Upon recurrence, they are offered re-treatment with Carboplatin/Taxol (provided there are no contraindications), or platinum in combination with another drug (usually gemcitabine or liposomal doxorubicin). Response to re-treatment with platinum is dependent on the length of the PFI: the longer the interval, the greater the likelihood of response to second-line platinum therapy (Huang et al., 2012).

This treatment ‘protocol’ and the arbitrary use of six months to distinguish platinum-resistant from platinum-sensitive patients, has resulted in a stagnant OS of ~30% since the 1970s. To date, we have no methods to identify platinum-sensitive patients from those that are platinum resistant/refractory. Furthermore, despite strategies that have included the use of chemosensitivity assays, we are unable to optimize second-line treatment, as we cannot predict which second-line therapy will yield a response in recurrent disease. Early identification of platinum-resistant/refractory patients and the personalization of cancer treatment might improve survival in ovarian HGSC patients.
The value and utility of any *in vivo* cancer model rests on its capacity to accurately reflect the properties of the disease. The aim of my study was to establish a pre-clinical model of ovarian high-grade serous cancer that maintains a high correlation with the characteristics of the clinical disease. The Neel laboratory previously established conditions to generate PDXs form primary tumours and ascites with high efficiency. These PDXs recapitulate the primary tumours with respect to their histological appearance and expression of markers by flow cytometry and immunohistochemistry (Stewart, 2013; Stewart *et al.*, 2011).

To further validate these PDXs as clinically relevant pre-clinical models of ovarian HGSC, I assessed their response to Carboplatin treatment (Chapter 2). To do so, I retrospectively identified platinum-resistant and platinum-sensitive ovarian HGSC patients, engrafted these into NSG mice, and tested the PDXs’ response to platinum therapy. Using the RECIST criteria to define response, PDXs derived from platinum resistant patients showed no response (i.e. stable or progressive disease) in response to Carboplatin treatment. Conversely, PDXs derived from platinum sensitive patients showed a striking response to platinum therapy, as seen by marked reductions in tumour volumes.

Furthermore, I generated PDXs from ‘prospective’ patients (n=2). These were patients from whom I generated PDXs from primary tumours obtained at the time of their primary surgery, when their clinical course was yet unknown. I followed these patients prospectively, predicting that based on the PDXs’ response to platinum therapy, these patients would be sensitive to platinum treatment corresponding to a PFI > 6 months. Remarkably, both patients are platinum-sensitive, with PFI’s >12 months and neither has yet recurred. These findings support the capacity of the PDX model to predict the patient’s clinical course.

Interestingly, following treatment and elimination of palpable tumours, one PDX case re-grew tumours 3.5 months after completion of therapy. Upon re-treatment with platinum, this case proved to be, once again, sensitive to platinum, paralleling the patient’s response. Although I only have one case from which to draw conclusions, this may suggest that not only can PDXs predict initial response to platinum treatment, but also upon re-treatment, can further predict how the patient’s *recurrence* will respond to second-line platinum therapy. This finding highlights that perhaps following treatment of PDXs, we are selecting for cellular clones that are clinically
relevant to the patient’s recurrent disease.

My findings suggest that despite the tremendous cellular heterogeneity of the primary tumour and the diverse clinical course of each patient, patient-derived PDXs reproduce patient response to platinum therapy. Combined with the PDX profiling performed by Stewart, this PDX system has proven to be a valid pre-clinical model that may be useful for defining personalized approaches to ovarian HGSC therapy (Stewart, 2013; Stewart et al., 2011).

Along these lines, I explored whether PDXs could be used to investigate combination drug studies. To do so, I tested the effects of Carboplatin in combination with Disulfiram (DSF), a drug used to treat alcohol abuse, which has shown anti-cancer effects in several cancer models (Appendix 2). Although exact drug doses require optimization, mice treated with both DSF and Carboplatin show a trend toward reduced tumor growth rates. Repurposing DSF for ovarian cancer therapy might represent a novel method to enhance the cytotoxic effects of platinum therapy. Future work should focus on elucidating the possible mechanisms responsible for DSF’s effects.

To examine the role of tumourigenicity following chemotherapy, I used PDXs to assess the nature of TICs following platinum-treatment. As mentioned previously, over 85% of patients will recur following initial therapy and often, the recurrences tend to be platinum-resistant. Understanding how chemoresistant tumour cells develop and potentially cause disease resurgence could provide a strategy for modification of the current therapeutic management of both primary and recurrent ovarian cancer. A possible explanation for recurrence is that following treatment, there are remaining cancer cells, which comprise a mixed tumour cell population. Within this mixed population is hypothesized to exist a distinct subpopulation of cells (CSCs) that escape the effects of chemotherapy, and as a result, treatment with chemotherapy is thought to actually enrich for tumourigenic cells.

Limited results do not allow me to derive conclusions about the nature of TIC dynamics in ovarian high-grade serous cancer PDXs following chemotherapy. Despite the limitations of the assay (described in Chapter 2), variability in these results might be due to the known inter-patient heterogeneity of this disease or could suggest that conventional chemotherapy equally targets tumour-initiating and non-initiating cells. There might even be multiple populations with
tumour-initiating abilities (at similar frequencies) that are differentially resistant to chemotherapy. Future work should focus on identification and isolation of ovarian HGSC cells with tumourigenic capacity in order to determine their responses to therapy.

Overall this thesis demonstrated that PDXs are a useful, pre-clinical model of ovarian HGSC with the capacity to predict response to platinum therapy. Ideally, in the future, every patient with ovarian HGSC should have a cohort of PDXs created at the time of diagnosis. This cohort would enable: 1) assessment of the response of the tumour to platinum therapy (i.e. predict platinum-resistance or sensitivity); 2) assessment of the response of the tumour to additional targeted and/or novel therapeutics; and 3) assessment of the response of the recurrent tumour to re-treatment with platinum and/or targeted therapeutics. This model also allows the assessment of TIC dynamics and drug-combination studies. Together, my findings positively impact ovarian cancer research and ultimately, the patients suffering from this disease.
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Appendix 1: Establishment of an Orthotopic Bioluminescent Patient-Derived Xenograft Model of Ovarian Cancer
A1 Introduction

To elucidate key determinants in ovarian cancer initiation, progression and response to treatment, models that recapitulate the disease are required. This demands both in vitro and, more importantly, in vivo models that reflect the cell biology and heterogeneity of the disease. Most studies of ovarian carcinogenesis and drug response utilize immortalized cancer cell lines as in vitro tumour models; however, many of these ‘ovarian HGSC’ cell lines are either not actually serous histology, do not reproduce serous histology when implanted into immune-compromised mice or do not give rise to xenografts at all (Domcke et al., 2013).

Few animal models develop spontaneous ovarian tumours, with the exception being hens and macaques; however, using these for drug development is not feasible given their cost and/or lack of appropriate reagents (Ricci et al., 2013). As a consequence, mice have become the primary mammalian model in cancer research due to their short generation time, ability to bear large litters, relative ease of breeding, and advances in mouse genomics (Edinger et al., 2002). Two main types of mouse have been utilized for cancer research: xenografts and genetically engineered mouse models (GEMMs).

Engrafting human tumour cells in immunodeficient mice to generate xenografts has been established for over 30 years (Kung, 2007). Typically, these models have used immortalized cell lines that are then injected subcutaneously (SC) into mice. To complement xenografts, GEMMs were developed to provide an efficient system in which to analyze the specific roles of oncogenes and tumour suppressor genes in tumourigenesis in vivo (Mullany and Richards, 2012; Talmadge et al., 2007). The extent to which either of these systems represent the heterogeneity of HGSC is unclear, and their role in drug development has proven limited (Talmadge et al., 2007).

Optimal preclinical models are those that reduce or eliminate the inconsistency between the original patient tumour and the mouse (Jin et al., 2010). Patient derived xenografts (PDXs) do so by utilizing patient tissue obtained at the time of surgery, as opposed to cells or cell lines grown in culture. As a result, PDX models maintain the original tumour architecture, and histological characteristics, as well as key genes and signaling pathways (Gray et al., 2004).
shown in lung, colorectal, and pancreatic cancer PDX models, where a high degree of genetic similarity between the primary cancer and the corresponding PDX tumour was seen (Daniel et al., 2009; Fichtner et al., 2008; Jones et al., 2008; Tentler et al., 2012).

A limitation of many PDX models is the location of tumour formation. The majority of xenograft studies inoculate cells or implant tumours ectopically into the subcutaneous layer or into the mammary fat pad (i.e., not in their anatomical site of origin). These animal models typically rely on caliper measurements, which are relatively rapid and simple to perform and allow easy monitoring of disease progression. Nevertheless, there are limitations to this approach; experiments based on caliper data do not account for areas of necrosis and edema, and so do not necessarily assess the effect of treatment on the number of viable cells (Jenkins et al., 2003a; Jenkins et al., 2003b). Although SC/MFP PDXs recapitulate many aspects of the tumour, orthotopic implantation of tumour cells greatly enhances metastasis, which some argue is required to make PDXs most predictive of clinical response (Kerbel, 2003).

Orthotopic ovarian xenografts (injected into the rodent ovarian bursa) provide the appropriate microenvironment to influence cell behavior, these xenografts provide a challenge with respect to monitoring disease initiation, progression and recurrence (Shaw et al., 2004). Unlike orthotopic xenografts derived from cell lines, orthotopic PDXs produce diffuse ascites and lack ovarian tumours. Furthermore, similar to patients, mice do not show signs of disease prior to the development of discernable ascites, which represents advanced stage (Stewart, unpublished results). This makes the ovarian orthotopic PDXs difficult to use for monitoring response to therapy.

A newer method for in vivo assessment involves the use of bioluminescent reporter genes and bioluminescence imaging (BLI). This method allows sensitive and quantitative detection of cells non-invasively in small research animals (Edinger et al., 2002). Bioluminescence refers to the enzymatic generation of visible light and the firefly luciferase gene is the most commonly used in animal models. Luciferase oxidizes luciferin in the presence of ATP and oxygen to form an electronically excited oxy-luciferin (Figure 4.0). Light is emitted following relaxation of the excited oxy-luciferin to its ground state, and BLI relies on external detection of this signal (O'Neill et al., 2010). Light emission is proportional to tumour volume, increases as the cell
population multiplies and is derived solely from metabolically active, transformed tumour cells (Rehemtulla et al., 2000). The main obstacle with BLI is the scattering of light by tissues, which make quantification of photons more difficult. Additionally, there is reduced luciferase activity in large tumours due to hypoxia and necrosis (Soling and Rainov, 2003).

**Figure 4.0 The luciferin reaction.**

<table>
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<tr>
<th>Luciferin</th>
<th>ATP</th>
<th>Oxyluciferin + Light</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Luciferase</td>
</tr>
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</table>

One group was able to generate a *luc*-expressing primary breast cancer PDX model and was able to detect early metastases (Harrison et al., 2010). Several groups have done this with a variety of other cancers, including ovarian, but all used immortalized cell lines (Cordero et al., 2010; van der Horst et al., 2011; Zabala et al., 2009). I wanted to exploit this technology to derive *luc*-expressing ovarian HGSC cells.
A2 Methods

Virus Preparation

Approximately 8 hours before transfection, 293T cells were seeded and incubated in a 15 cm dish at 18 x 10⁶ cells. Media was changed prior to transfection with IMDM, 10% FBS, Penicillin (100U/ml), Streptomycin (100U/ml) and Glutamine (22.5 ml final volume). A plasmid DNA mix was prepared by adding: ENV plasmid (VSV-G), packaging plasmid (CMV ΔR8.74), REV plasmid, and transfer vector plasmid (courtesy of Erno Weinholds). Finally, 125μl of 2.5M CaCl₂ was added and incubated at room temperature for 5 minutes. The GFP-Luciferase plasmid was generously provided by Dr. Joseph Wu (Stanford University). The precipitate was formed by the drop-wise addition of 1.25ml of 2X HBS solution to the DNA-TE-CaCl₂ mixture while vortexing at full speed. The CaPi-precipitated plasmid DNA was allowed to stay on the cells for 14 hours, after which the media was replaced with fresh media for virus collection to begin. The cell supernatant was collected 30 hours after changing the media.

Ascites Samples Preparation

Human ascites specimens were obtained with informed consent from patients undergoing surgery at PMH, following a protocol approved by the University Health Network Research Ethics Board (REB #06-0903T). Primary, bulk, chemo-naive ascites was plated in 75mL vented flasks, at a ratio of 1:1 with media (RPMI with 20% FBS, Penicillin (100U/ml), Streptomycin (100U/ml) and amphotericin B (2 μg/mL) (Qiagen). Cells were allowed to adhere for 48-72 hours, after which the media was changed every 48 hours. Once the cells were actively proliferating (20-40 days after plating), high titer virus was added for 24 hours. Cells were then washed, and collected by centrifugation. A small aliquot was removed for fluorescence-activated cell sorting assessment.

Orthotopic Xenograft Establishment

All experiments involving mice were carried out under the UHN animal protocol #1239, and
utilized 6-8 week-old female NSG mice. Aliquots of $10^5$ luc-ascites tumour cells were mixed 1:1 by volume with Matrigel for mouse injections. Mice were anaesthetized using isoflorane vapor. The skin was disinfected using an alcohol swab. A dorsolateral 1 cm incision was made on the top of the spleen. Using curved forceps, the fat pad surrounding the ovary was isolated and the cell suspension (10μL) was injected into the ovarian bursa. An absorbable suture was used to suture the skin. Sterile saline (300cc) was administered SC, and the animals were given oral antibiotics post-operatively for one week.

*In vivo* Imaging

72 hours after orthotopic injection, *in vivo* imaging was performed using the Xenogen IVIS Imaging system. Mice were injected IP with 250 μL of 5mg/mL d-luciferin dissolved in PBS (50mg/kg), placed in the anesthesia chamber and imaged 20 minutes following injection (based on a previously determined luciferin kinetics curve). Imaging was repeated at 7, 30, 60, 90 and 120 days, at which point the mice were sacrificed. Total photon flux (in regions of interest) was measured in photons/second (p/s), using IVIS Living Image ® software.

**Histology**

Tumours were collected, formalin fixed and paraffin-embedded. Slides were obtained and stained for H&E. Immunohistochemistry using anti-GFP antibody (Abcam, cat. No. #6556) was performed. All slides were reviewed by a gynecologic pathologist.
A3 Results

Cultured ascites cells show epithelial morphology and are readily transduced

Sample 2844 was grown in culture for 40 days prior to the addition of high-titre virus. At the time virus was added, the cells were rapidly proliferating and showed epithelial morphology (Figure 4.1A) Transduction with lenti-virus generated 85.4%-GFP positive cells (Figure 4.1B). Sample 3393 was cultured for 20 days prior to lenti-virus, was rapidly proliferating and showed epithelial morphology (Figure 4.1C). Viral transduction yielded 89.3% GFP-positive cells (Figure 4.1D). Once cells were GPF-positive, they were no longer maintained in culture and were injected immediately into mice.

PDXs Show a Luciferin Signal 72 Hours after Inoculation

Mouse imaging was performed 72 hours after inoculation. Figure 4.2 shows that both mice generated from samples 2844 and 3393 showed a positive luciferase signal, however the region of interest (ROI) measurement was considerably stronger for 3393 PDXs (maximum total photon flux of $10^6$ p/s in 2844 compared with $2 \times 10^8$ p/s in 3393). Similarly, the signal was stronger for luc-3393 IP PDXs, compared with luc-2844 ($10^9$ versus $2 \times 10^8$ p/s) (Figure 4.3).
Figure 4.1. Morphology of cultured ascites cells and flow cytometer plot showing percent GFP transduction. A) 2844 ascites after 40 days of culture, B) Flow cytometer plot showing 85.4% transduction of sample 2844. C) Phase contrast of sample 3393 showing after 20 days in culture showing epithelial morphology and D) FACS graph showing 89.3% transfection efficiency.

Figure 4.2 Intrabursal PDXs 72 hours after inoculation with luc- ascites cells. A) Luc-2844 IB PDXs showing regions of interest measuring $5 \times 10^5$, $1 \times 10^6$ and $3 \times 10^5$ p/s, respectively. B) Luc-3393 IB PDXs showing regions of interest. Mouse 3393-1 ROI measures $2 \times 10^5$ and mouse 3393-2 measures $6 \times 10^7$ p/s.
Figure 4.3 Intraperitoneal PDXs 72 hours after inoculation with luc-ascites cells. *Luc*-2844 IP PDXs showing regions of interest measuring A) $2 \times 10^8$ p/s and B) $8 \times 10^6$ p/s. *Luc*-3393 IP PDXs showing regions of interest measuring C) $1 \times 10^9$ p/s and D) $3 \times 10^7$.

Figure 4.4 Luciferase signal measured over time in *luc*-2844 IP PDXs. Images were taken at A) 30 days, B) 60 days, C) 90 days and D) 120 days (experiment termination).
Figure 4.5 Luciferase signal measured over time in *luc*-3393 IP PDXs. Images were taken at A) 30 days, B) 60 days, C) 90 days and D) 120 days (experiment termination).

<table>
<thead>
<tr>
<th>IP PDXs</th>
<th>ROI initial (p/s)</th>
<th>ROI final (p/s)</th>
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<tbody>
<tr>
<td>2844 -1</td>
<td>2.0e⁸</td>
<td>3.3e⁵</td>
</tr>
<tr>
<td>2844 -2</td>
<td>8.5e⁶</td>
<td>4.2e⁵</td>
</tr>
<tr>
<td>3393 -1</td>
<td>1.2e⁹</td>
<td>3.1e⁵</td>
</tr>
<tr>
<td>3393 -2</td>
<td>3.2e⁷</td>
<td>2.8e⁵</td>
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Table 4.1 Initial and final ROI values for 2844 and 3393 IP PDXs

Decreased luciferase signal over time in IP and IB PDXs

Mouse imaging was performed every 30 days. Figures 4.4 and 4.5 show the progressive weakening of the luciferase signal over time for both 2844 and 3393 IP PDXs. Initial and final ROI measurements are listed in table 4.1. Although 2844 and 3393 IP PDXs started out with
considerably different initial ROI measurements, at the termination of the experiment, their respective ROIs were all similar (~3e^5 p/s).

Similar to the IP PDxs, IB PDXs showed a strong signal at 72 hours (Figures 4.1A,B); however this signal faded over time (Figures 4.6 and 4.7). At the completion of the experiment, no luc-2844 PDXs generated a luciferase signal. A weak signal could be seen in two luc-3933 IB PDXs (Figure 4.7), corresponding to an ROI of 1-2e^5.

Figure 4.9 shows a DLIT image of PDXs luc-3393-1 at 72 hours (panels A-D) and at endpoint (panels E-H). Diffuse Light Imaging Tomography (DLIT) utilizes the data obtained from a filtered 2D bioluminescent sequence (by measuring the effects of tissue absorption at different wavelengths to determine depth and flux) in combination with a surface topography to represent the bioluminescent source in a 3D tissue.

Figure 4.6 Luciferase signal measured over time in luc-2844 IB PDXs. Images were taken at A) 30 days, B) 60 days, C) 90 days and D) 120 days (experiment termination)
Figure 4.7 Luciferase signal measured over time in *luc-3393 IB* PDXs. Images were taken at A) 30 days, B) 60 days, C) 90 days and D) 120 days (experiment termination). ROIs remained largely unchanged from 30 days to 120 days.
Figure 4.8 Luciferase signal measured over time in luc-3393-1 IB PDX. Images were taken at A) 72 hours after inoculation and B) 120 days (experiment termination). The initial ROI 72 hours after inoculation was $1.7 \times 10^8$ and faded to $3.8 \times 10^5$ by 4 months.
Figure 4.9. 3D-Reconstruction of bioluminescence images (DLIT) of luc-3393-1 IB PDX. A), B), C) are coronal, sagittal and transaxial cuts, respectively, of the PDX taken at 72 hours after inoculation. D) Represents a 3D reconstructive image of luc-3393-1 at 72 hours. E-G) are coronal, sagittal and transaxial cuts, respectively, of the PDX taken at endpoint 4 months. H) 3D reconstruction of luc-3393-1 at 4 months with luciferase signal in the area of the adnexa. Digital mouse ovaries are registered onto the image by the software, and are meant to represent the location of ovaries in a typical mouse.
IB PDXs have an ‘Ovarian Mass’ at Necropsy

All IP and IB xenografts were carefully dissected looking for evidence of adnexal and extra-ovarian disease. No IP xenograft showed evidence of visible disease. Conversely, all IB xenografts, even those that did not have a luciferase signal, had an ovarian/adnexal mass on the left side (where cells were inoculated) (representative image shown of luc-3393-1, Figure 4.10). All other abdominal organs were examined and did not show gross evidence of disease. No xenografts had ascites.

Figure 4.10 Necroscopic examination of luc-3393-1 IB xenograft. At initial dissection (A) a large ovarian mass could be seen. All other organs appeared normal. Comparison of normal (B) right ovary and fallopian tube (FT) compared to ‘ovarian mass’ arising from inoculated left ovary (C).
Histology

Despite the ‘ovarian mass’ seen at necropsy, histological examination by H&E (Figure 4.11) showed no abnormal or tumour cells within the ‘mass’, ovary, or fallopian tube.

Figure 4.11 Histologic examination of PDX luc-3393-1 IB. H&E stain at A) 4x and B) 10x show a normal fallopian tube and ovary with no evidence of high-grade serous histology. GFP staining of xenograft luc-3393-1 IB fallopian tube and ovary at C) 4x and D) 10x magnifications show no positive GFP cells.
A4 Discussion

Nearly all primary and recurrent ovarian HGSC presents at advanced stage. Knowledge of the mechanisms of metastasis, and the development of technologies to detect them are critical. Pre-clinical models that more closely mirror human disease have an important role in evaluating putative cancer therapies (Kung, 2007). Clinically relevant animal models that incorporate: disease heterogeneity and allow monitoring of cancer growth/metastasis and treatment efficacy continue to be lacking.

The high sensitivity of BLI has been demonstrated in several studies and its main strength is the ability to monitor tumour load longitudinally. However, it is poorly suited for determination of absolute tumour mass and the exact locations of tumours due to its limited spatial resolution (Klerk et al., 2007). The sensitivity of BLI is dependent on several factors, such as the number of cells expressing the reporter gene, the efficiency of the promoter and the stability of luciferase expression (Sato et al., 2004). Although the relationship between the bioluminescence signal and viable cancer cell load has been validated in several cancer cell line studies, a strong correlation in orthotopic models, particularly those with larger tumours, warrants further investigation (Zabala et al., 2009). Zabala et al., showed that larger tumours tend to have less copies of luciferase plasmid per genome, suggesting that stromal cells are more abundant in these tumours thereby making the BLI signal not representative of true tumour burden (Zabala et al., 2009).

Several barriers in obtaining reproducible quantification of BLI have been identified. Cui et al., found that the concentration of luciferin injected, the kinetics of luciferin (from time of injection to maximal signal) and light scattering inside the animal (due to anatomical factors, tissue depth, signal impedance, etc.) can all vary the efficiency of light transmission. In addition, animal positioning (prone versus supine) and even the type of anaesthetic used can generate significant variability in the BLI signal (Cui et al., 2008; Sato et al., 2004).

Perhaps the major disadvantage of BLI is that cells must be genetically modified to carry the luciferase gene. The effects of gene transfection on cancer cell health, behavior, and tumourogenicity are not well defined, and as such, this makes a direct translation of data difficult (Klerk et al., 2007). Tiffen et al., found that a high level of luciferase expression did not have
detrimental effects on cancer cell growth in vitro or in vivo; however this experiment used cell lines and not primary cells (Tiffen et al., 2010). Other reports suggest that production of intracellular light is cytotoxic to cancer cells (Brutkiewicz et al., 2007; Theodossiou et al., 2003).

Furthermore, there could also be effects of serum-containing media on ascites cells. I attempted to expose the cells minimally to culture medium, so virus was added at various times to multiple ascites samples (data not shown). Short periods of culture (< 1 week), resulted in no transduction; high transduction was observed only when cells were in culture for 2-4 weeks. Effects of prolonged culture on primary cells is undefined, but may affect tumourigenicity. This might explain why no tumour cells were seen by histology. This is supported by the control mice, which were inoculated with cells grown in culture but without virus. No tumours were seen in these mice suggesting perhaps that the in vitro culture conditions reduce and/or eliminate tumourigenicity.

In our study, a strong luciferase signal was generated in both intrabursal and intraperitoneal luc-2844 and luc-3393 PDXs 72 hours after inoculation, however this signal dissipated over time, and at the endpoint of my study, I only had two xenografts that maintained a positive, albeit weak, luciferin signal (intrabursal luc-3393-1 and luc-3393-2). These mice appeared to have an ‘ovarian mass’ at the time of necropsy, however, histologically, there was no evidence of HGSC or any abnormal cells. Its possible that the initial strong signal was generated from a small population of cells with multiple viral genome integrations. Over time, it appears that these cells, although viable, are unable to cause tumours. Optimization of this protocol may allow for monitoring of tumour burden over time and in response to various treatments.
Appendix 2: Disulfiram-Induced Toxicity in Ovarian Cancer
A2.1 Introduction

Ovarian cancer is the sixth most common cancer in women and the most lethal gynecologic malignancy (Institute, 2013). Approximately 80% of ovarian cancers have high-grade serous histology: these appear in the absence of a well-defined precursor, present at an advanced stage and are highly aggressive (Kurman and Shih Ie, 2010). They metastasize early in the disease course, with tumour generally found on the ovarian surface, fallopian tubes and widely disseminated throughout the peritoneal cavity (Crum et al., 2007b; Shih Ie and Kurman, 2004). Despite improvements in debulking surgery and generally good initial responses to chemotherapy, five-year survival for these patients has remained largely unchanged.

Following initial therapy with Carboplatin/Cisplatin and Taxol, more than 85% of patients will recur. The challenge in treating ovarian HGSC is that recurrences tend to be drug-resistant, thereby making treatment options more limited and less effective (Kulkarni-Datar et al., 2013). Thus, novel therapies are needed for patients with both newly diagnosed and relapsed ovarian HGSC; however, the development of new drugs is lengthy and costly. It has been estimated that it takes an average of 15 years and US$800 million to bring a single drug to market and only 20-30 new compounds are approved annually, many of which are not chemotherapeutics (Chong and Sullivan, 2007). Therefore, repurposing existing drugs prescribed for treating other diseases is a potentially useful approach that circumvents this lengthy and costly FDA process. Combining currently employed chemotherapeutics with existing compounds could be an important strategy in enhancing drug efficacy.

Disulfiram (Tetraethylthiuram disulfide; Antabuse®) is a commercially available drug that has been used for decades as aversion therapy in the treatment of alcohol abuse (Sauna et al., 2005). Ethanol is converted to acetaldehyde (AA) by alcohol dehydrogenase, but is further metabolized to acetate by aldehyde dehydrogenase (ALDH) (Figure 5.0). While many other members of the aldehyde dehydrogenase superfamily are capable of catalyzing the oxidation of AA to acetic acid, ALDH2 is thought to be the most important (Sladek, 2003). Disulfiram (DSF) reportedly has numerous enzymatic targets, but it works primarily through irreversibly inhibiting ALDH2 (Eneanya et al., 1981). The presence of DSF and ethanol causes an accumulation of acetate,
yielding an unpleasant physical response (flushing, tachycardia, nausea) and thus, an aversion reaction (Gaval-Cruz and Weinshenker, 2009).

Figure 3.0 Ethanol metabolism and disulfiram mechanism of action.

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>ADH</th>
<th>Disulfiram</th>
<th>ALDH</th>
<th>Acetaldehyde</th>
<th>Acetate</th>
<th>DNA Damage</th>
</tr>
</thead>
</table>

Given its long history, the toxicity and pharmacological properties of DSF have been well described. DSF is absorbed after oral ingestion and rapidly reduced to its corresponding thiol metabolite, diethyl(diethiocarbamic acid (DDC). It is believed that both DDC and DSF are effective at inhibiting ALDH (Eneanya et al., 1981). In addition, DDC is a potent copper chelator and can affect the activity of copper-dependent enzymes, such as cytochrome oxidases (Gaval-Cruz and Weinshenker, 2009). Several studies have shown that there is marked intersubject variability in plasma concentrations of DSF and its metabolites, likely caused by its high lipid solubility (Faiman et al., 1984).

Numerous studies have shown that DSF has antitumour activity against a number of cancer cell lines, including melanoma, colorectal, prostate and breast (Brar et al., 2004; Cen et al., 2002; Daniel et al., 2005; Wang et al., 2003). Although the exact mechanisms of action of DSF have not been well characterized, it is believed to exert anti-tumour effects through via a multitude of mechanisms, including reduction of angiogenesis, inhibition of DNA topoisomerases and NFκB and mitochondrial membrane permeabilization (Table 5.1). Lovborg et al., found DSF to be very active against ovarian cancer and breast cancer cell lines and patient samples in vitro and claimed that concentrations needed to induce cytotoxicity in patients can be safely reached (Lovborg et al., 2006). Furthermore, several groups have investigated these effects in vivo. Using
MDA-MB-231 breast cancer xenografts, Chen et al. showed that DSF inhibited tumour growth by up to 74% compared with controls (Chen et al., 2006). In addition, several studies demonstrated that DSF and its metabolites potentiate the effects of existing anticancer drugs, including cyclophosphamide, colchicine and 5-FU (Wang et al., 2003). Most recently, Liu et al. showed that DSF may reverse chemo-resistance and have a direct effect on CSC (Liu et al., 2013).

In light of the evidence of DSF’s anti-cancer activity, a phase I study was initiated in 1987 in an effort to increase the therapeutic index of cisplatin in a variety of cancers (prostate, lung, thyroid, adrenal, pharynx, melanoma, adenocarcinoma of unknown primary). Acute, reversible confusion was the only noted side effect of DSF, and this was only seen in patients treated with high doses (3,000mg/m²). Furthermore, no toxic effects of cisplatin were observed by the addition of DSF. Based on these promising results, further studies were to be initiated (Stewart et al., 1987).

**Table 3.1. Published effects of disulfiram.**

<table>
<thead>
<tr>
<th>Cell type (Author)</th>
<th>Reported Mechanisms of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal Cancer Cell lines (Wang et al., 2003)</td>
<td>Inhibits activation of NFkB</td>
</tr>
<tr>
<td>Melanoma cell lines (Cen et al., 2002; Morrison et al., 2010)</td>
<td>Induces apoptosis via redox related mitochondrial membrane permeabilization; production of ROS</td>
</tr>
<tr>
<td>Lung &amp; bladder cancers cell lines (Shian et al., 2003)</td>
<td>Inhibits matrix metalloproteinases</td>
</tr>
<tr>
<td>Prostate cancer cell lines (Lin et al., 2011)</td>
<td>DNA methyltransferase inhibitor</td>
</tr>
<tr>
<td>Embryonic Kidney Cells (Loo and Clarke, 2000)</td>
<td>Blocks maturation of p-glycoprotein membrane pump</td>
</tr>
<tr>
<td>Breast Cancer cell lines (Chen et al., 2006)</td>
<td>Inhibits proteosomal activity</td>
</tr>
<tr>
<td>Endothelial cells (Marikovsky et al., 2003)</td>
<td>Reduces angiogenesis</td>
</tr>
</tbody>
</table>

Aldehydes are not only the main product of the disulfiram+ethanol reaction, but are also produced endogenously as a byproduct of cellular metabolism. Numerous studies have shown
that cells exposed to AA accumulate DNA damage. AA generates several DNA adducts that can block DNA replication and cause DNA interstrand crosslinks (ICLs), which might play a role in the pathogenesis of some cancers (Brooks et al., 2009; Abraham et al., 2011). The Fanconi Anemia-BRCA (FA-BRCA) DNA repair pathway has a crucial role in counteracting AA induced genotoxicity and is required for cellular resistance to the toxic effects of AA (Langevin et al., 2011). The FA-BRCA DNA repair pathway comprises 13 proteins that act together to repair DNA ICLs and respond to stalled replication forks (Abraham et al., 2011). It is also essential to counteract aceteldehyde-induced genotoxicity in mice, as shown by Langevin et al. In their study, Aldh2+/−Fancd2−/− mouse embryos were extremely sensitive to ethanol exposure in utero and rapidly developed bone marrow failure post-natally (Langevin et al., 2011).

The effects seen in the absence of a functional FA-BRCA pathway, combined with ALDH2 deficiency, as demonstrated by Langevin et al., provided the rationale for this study. One of the FA proteins frequently mutated in ovarian cancer is BRCA2 (FANCD1), and it is important in repair through homologous recombination. Similarly, BRCA1 also complexes with three FA proteins to coordinate HR repair (D'Andrea, 2010; Kee and D'Andrea, 2010). Combining germline and somatic, BRCA1 and BRCA2 mutations are found in approximately 20-50% of ovarian HGSCs (Castellarin et al., 2013; The Cancer Genome Atlas Research Network, 2011; Pal et al., 2005; Press et al., 2008a). Therefore, I reasoned that cells defective in homologous recombination (BRCA2null: PEO1 cell line) would be more susceptible to the toxic effects of increased levels of acetaldehyde, which could be achieved by inhibiting ALDH2, via DSF. In combination with platinum, DSF causes increased DNA adducts and should enhance the effects of platinum, particularly in the BRCA2null cells. In this study, I investigate the actions of DSF in combination with Carboplatin in a panel of ovarian cancer cell lines and in PDXs.
A2.2 Methods

A2.2.1 Cell Culture

The established human ovarian cancer cell lines OV 90, OV 1946, OV 1369, TOV 1369, PEO1, PEO4, PEO23, TOV 2223g were purchased from ATCC (or obtained from R. Rottapel) and used for the studies. OV90 in DMEM with 10% FBS, Penicillin (100U/ml), Streptomycin (100U/ml); PEO1, PEO4, PEO23 were cultured in RPMI containing 10% FBS, Penicillin (100U/ml), Streptomycin (100U/ml), and OV 1369, TOV 1369, OV 1946, TOV 2223G were cultured in DMEM 10% FBS, Penicillin (100U/ml), Streptomycin (100U/ml). Cells were incubated at 37°C and 5% CO2 tissue culture incubator.

For drug testing, 5-10 x10^3 cells/well were culture in 96-well plates overnight and treated with indicated doses of Carboplatin or DSF for 72 hours. Carboplatin was dissolved directly in media; Tetraethylthiuram disulfide (DSF) (Sigma Aldrich) was dissolved in DMSO (1000x) and added to media (1:1000). IC_{50} values were determined by Alamar Blue assay (Invitrogen, Burlington, ON, Canada). At 72 hours, Alamar Blue was added (10% of total volume) and incubated for 4 hours. The fluorescence was measured using a spectrophotometer with an excitation setting of 530 nm and emission at 590 nm (Spectra MAX Gemini EM, Molecular Devices).

A2.2.2 Combination Studies

For combination treatment, cells cultured overnight were treated with various concentrations of Carboplatin/DSF at a ratio of 1:1, based on IC_{50} data generated from previous experiments. After 72 hours of incubation, cells were subjected to an Alamar Blue assay. The combination index (CI) was calculated using CalcuSyn Software (Biosoft, Cambridge, UK) CI<1 and CI>1 indicates synergism or antagonism, respectively, and CI=1 indicates an additive effect (Chou and Talalay, 1984).

A2.2.3 Cell Imaging

Immunofluorescence: After 24 hours of drug exposure, PEO1 and PEO4 cells were fixed with 4% paraformaldehyde in PBS, permeabilized with PBS containing 0.4% Triton-100 and blocked with 100% ADB. Fixed and permeabilized cells were incubated with primary anti-\(\gamma\)H2AX
antibody (Upstate Biotechnology) at 4C overnight, followed by a 1-hour incubation at room temperature with the secondary antibody (Alexa Fluor 488 goat anti-mouse; Invitrogen). Irradiation with 4 Gy was used as the positive control. Images were obtained using Axio-vision software and nuclear counts were quantified using Foci-Counter (http://focounter.sourceforge.net).

For IncuCyte imaging, cells were seeded in 96-well plates at a density of 10,000 cells/well and cultured overnight. Carboplatin, DSF and Carboplatin+DSF were serially diluted in growth medium containing YOYO-1 (Life Technologies) at a final concentration of 0.1 µM in media, as described above. Cells were placed in an IncuCyte™ FLR (Essen Bioscience) with a 10X objective in a standard cell culture incubator at 37°C and 5% CO2. Two images per well were collected every 2-3 hours in both phase contrast and fluorescence-modes for 72 hours.

A2.2.4 PDX Studies

Patient case #2555 was thawed and prepared as described previously. 10^6 cells in 1:1 HBSS:growth factor-reduced Matrigel (BD Biosciences) were injected into the right mammary fat pad NOD/SCID mice. When xenografts reached ~100mm³, mice were treated with either DSF 200mg/kg PO by oral gavage (Sigma, Israel) dissolved in DMSO/corn oil; Carboplatin 75mg/kg IP; both Carboplatin 75mg/kg IP and DSF 200mg/kg PO (gavage) or vehicle control (DMSO/corn oil PO and 300 cc saline IP). DSF was prepared as described: 5mg of DSF was dissolved in 20µL DMSO, then mixed in 180µL corn oil (Brar et al., 2004). DSF was intended to be administered by oral gavage every Monday, Wednesday, Friday; however, because it was felt that the mice had significant weight loss at this dose, only the Monday and Wednesday doses were administered. Carboplatin was administered every Monday. Mice were sacrificed when tumours were greater 1500mm³ (within 2 weeks of initiation of treatment).
A2.3 Results

A2.3.1 Combination Treatment with DSF/Carboplatin shows Synergistic Effects in PEO4 cells

To begin to assess the effect of DSF and Carboplatin on BRCA2 mutant ovarian cancer cells, I treated two isogenic cancer cell lines: PEO1 (BRCA2\textsuperscript{null}) and PEO4 (BRCA2\textsuperscript{wt}) (Sakai \textit{et al.}, 2009) with increasing doses of DSF and Carboplatin to determine their respective half-maximal inhibitory concentration (IC\textsubscript{50}). Alamar Blue was used as an indicator of metabolic activity and cellular health (Figure 5.1A, 5.1B). The Carboplatin and DSF IC\textsubscript{50}s for PEO1 were \(~60\) µM and 20 µM, respectively. The Carboplatin and DSF IC\textsubscript{50}s for PEO4 were \(~120\) µM and 25 µM, respectively. I next asked whether combination treatment with DSF and Carboplatin would demonstrate synergy. I expected the PEO1 cell line (BRCA2\textsuperscript{null}) to be more sensitive to the combined effects of Carboplatin and DSF. Surprisingly, the PEO4 cells, with a functional BRCA2 protein, were found to be more sensitive in combination drug studies (Figure 5.2). This effect was synergistic based on the combination index (CI) as determined by the Chou-Talalay method (Figure 3) (Chou and Talalay, 1984). The CI for PEO1 cells was >1 (not synergistic), whereas the CI for PEO4 cells was <1 (synergistic) at all effective dose (ED) levels (Table 5.2).

![Figure 5.1. A) DSF and B) Carboplatin IC\textsubscript{50} dose-response curves for PEO1 and PEO4 cell lines. PEO1 (light grey diamonds) and PEO4 cells (dark grey squares) were treated with indicated concentrations of a) DSF and b) Carboplatin, and effects on cell number were inferred by an Alamar Blue assay. Results represent the average of three experiments expressed as a mean percentage of untreated control cells. The Carboplatin and DSF IC\textsubscript{50}s for PEO1 are \(~60\) µM and 20 µM, respectively. The Carboplatin and DSF IC\textsubscript{50}s for PEO4 are \(~120\) µM and 25 µM, respectively.](image-url)
Figure 5.2. **Effects of DSF and Carboplatin combinations on A) PEO1 and B) PEO4 cell lines.** PEO1 and PEO4 cells were exposed to graded concentrations of Carboplatin or DSF, either alone or in combination at a ratio of 1:1 of for 72 h, followed by analysis of viability by an Alamar Blue assay. Each data point represents the mean ± SD of at least three determinations. Carboplatin (black circles), disulfiram (diamond), Carboplatin + disulfiram (square, dotted line).

Figure 5.3. **Isobolograms of DSF/Carboplatin combinations.** Isobologram analysis of the combination of DSF/Carboplatin in A) PEO1 and B) PEO4 cells. The individual doses of Carboplatin and DSF to achieve 90% (blue line), 75% (green line) and 50% (red line) growth inhibition were plotted on the x- and y-axes. Combination index (CI) values calculated using CalcuSyn software are represented by points above (indicate antagonism) or below the lines (indicate synergy). (X symbol) ED$_{50}$, (plus sign) ED$_{75}$ and (open dotted circle) ED$_{90}$. 
Table 5.2. Combination Index (CI) values for PEO1 and PEO4 cell lines.

<table>
<thead>
<tr>
<th>ED</th>
<th>CI PEO1</th>
<th>CI PEO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>2.2</td>
<td>0.27</td>
</tr>
<tr>
<td>75</td>
<td>1.5</td>
<td>0.40</td>
</tr>
<tr>
<td>90</td>
<td>1.1</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Next, I performed an analysis of γ-H2AX foci formation. PEO1 and PEO4 cells were incubated in 61µM of Carboplatin and 25µM of DSF for 24 hours, after which γ-H2AX immunofluorescent staining was performed (Figures 5.4 & 5.5) and quantified (Figure 5.6). Again, DSF in combination with Carboplatin exerts a synergistic effect on PEO4 cells and an additive effect in PEO1 cells, as seen by γ-H2AX foci formation. Treatment with DSF or Carboplatin produced an average of 1.5 and 4.8 γ-H2AX foci, respectively (Figure 5.6). In combination drug treatment, an average number of 8.38 γ-H2AX foci were seen, representing synergistic effects.

Furthermore, I used YoYo-1 staining to quantify cell death. YoYo-1 is a cell impermeant cyanine dimer nucleic acid stain that binds to dsDNA. When added to the culture medium, YoYo-1 fluorescently stains the nuclear DNA of cells that have lost plasma membrane integrity. With Incucyte technology, I visualized and quantified cell death as a result of combination drug treatment (Figures 5.8 and 5.9). There was a greater amount of cell death seen in the PEO4 cell line with combination drug treatment (object counts >8000) than would be expected based on either drug alone (object count = 2000 for both DSF and Carboplatin at 72hr). Conversely, although the PEO1 cell line had a similar total amount of cell death (object count = 8000), the effects of Carboplatin and DSF at 72hr were 3500, and 3800 respectively and were not synergistic.
Figure 5.4. Formation of γ-H2AX foci in drug treated PEO1 (BRCA2<sup>null</sup>) cells. Each panel shows the DAPI-stained nucleus (blue) and anti-γ-H2AX antibody (green). Panels: A) negative control; B) positive control (4Gy); C) DSF 25µM; D) Carboplatin 61µM; E) combination of DSF and Carboplatin.
Figure 5.5. Formation of γ-H2AX foci in drug treated PEO4 (BRCA2<sup>−/−</sup>) cells. Each panel shows the DAPI-stained nucleus (blue) and anti-γ-H2AX antibody (green). Panels: A) negative control; B) positive control (4Gy); C) Disulfiram 25µM; D) Carboplatin 61µM; E) combination of DSF and Carboplatin.
Figure 5.6. Quantification of γ-H2AX foci in drug-treated PEO1 and PEO4 cells. Data shown represent the average number of foci formed ± s.e.
Figure 5.7. Effect of DSF and Carboplatin treatment on PEO1 cell proliferation. YoYo-1 fluorescent nuclear counts in PEO1 cells at t= 2 hours and termination of experiment (t=72 hours). DSF 25µM, Carboplatin 61.25µM and drug combination.
Figure 5.8. Effect of DSF and Carboplatin treatment on PEO4 cell proliferation. YoYo-1 fluorescent nuclear counts in PEO1 cells at t= 2 hours and termination of experiment (t=72 hours). DSF 25µM, Carboplatin 61.25µM and drug combination.
Figure 5.9. Quantification of YoYo-1 fluorescence in A) PEO1 and B) PEO4 cell lines. Negative control (yellow square with cross); DSF (light grey diamond), Carboplatin (dark grey circle), combination treatment (black square).
A2.3.2 Many Ovarian Cancer Cell Lines show Synergistic Effects of DSF/Carboplatin *in vitro*

Given the above results, I wanted to explore whether similar synergy between DSF and Carboplatin could be observed in additional ovarian cancer cell lines. To do so, I first determined the IC50 values for DSF and Carboplatin for the following cell lines: OV 1369, TOV 1369, OV 1946, PEO23, TOV2223G, A2780, OV 90, and SKOV3 (data not shown). Next, I performed combination studies to assess whether the combined effects of DSF and Carboplatin (Figure 5.10). I excluded SKOV3 and A2780 as a recent publication identified these cell lines as ‘unlikely to be high-grade serous’ (Domcke *et al.*, 2013). Of the six additional cell lines tested, synergy between DSF and Carboplatin was observed in 5 cell lines (Figure 5.11): OV 1369, TOV 1369, TOV 2223G, PEO23 and OV 1946.
Figure 5.10: Effects of Carboplatin and DSF on human ovarian cancer cell lines. OV 1369, TOV 1369, OV 1946, PEO23, TOV2223G, and OV90 cells were exposed to graded concentrations of Carboplatin or DSF, alone or in combination, at a ratio of 1:1 of for 72 hr. Viability was analyzed by Alamar Blue assay. Each data point represents the mean ± SD of at least three determinations. Carboplatin (black circles), disulfiram (diamond), Carboplatin + disulfiram (square, dotted line).
Figure 5.11. **Isobolograms of drug combinations.** Isobologram analysis of the combination of Carboplatin and DSF in OV 1369, TOV 1369, OV 1946, TOV 2223G, PEO23, and OV 90 cells. The individual doses of Carboplatin and DSF to achieve 90% (blue line), 75% (green line) and 50% (red line) growth inhibition are plotted on the x- and y-axes. Combination index (CI) values, calculated using Calcusyn software, are represented by points above (indicate antagonism) or below the lines (indicate synergy). (X symbol) ED$_{50}$, (plus sign) ED$_{75}$ and (open dotted circle) ED$_{90}$. 
Table 5.3. Combination Index (CI) values for all cell lines evaluated.

<table>
<thead>
<tr>
<th>ED</th>
<th>PEO1</th>
<th>PEO4</th>
<th>OV1369</th>
<th>TOV1369</th>
<th>TOV2223G</th>
<th>PEO23</th>
<th>OV1946</th>
<th>OV90</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>2.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>4.2</td>
</tr>
<tr>
<td>75</td>
<td>1.5</td>
<td>0.4</td>
<td>0.2</td>
<td>0.5</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>90</td>
<td>1.1</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
<td>2.4</td>
</tr>
</tbody>
</table>

A2.3.3 DSF/Carboplatin Combination Slows Tumour Growth

The doses of DSF required to inhibit ovarian cancer growth in short term assays *in vitro* were higher than those predicted to be achievable *in vivo*. However, with 6/8 evaluated ovarian cancer cell lines displayed synergy in combination drug treatments of DSF and Carboplatin, I proceeded to evaluate the effects of these drugs *in vivo*. I decided to use PDXs, as opposed to cell line-derived xenografts for reasons previously discussed (Section 1.14.2). The clinical characteristics of patient 2555 are listed in Table 2.3.

When tumours reached ~200mm³ (Figure 5.12.A), DSF/Carboplatin treatment was initiated at doses similar to those used to treat adult humans. Mice treated with both drugs demonstrated slower growth, compared with control mice (Figure 5.12C). The mean tumour volume of DSF/Carboplatin-treated group was lower than the control group on day 9: 218 mm³ versus 998 mm³ (unpaired t-test p = 0.04) (Figure 5.12B).

Although mice treated with DSF+Carboplatin had the lowest tumour volume, they also had the lowest percent survival (Figure 5.12.D; 5/9 mice died within 24 hours of drug administration. I believe this result to be the product of two main factors: a non-optimized disulfiram dose and/or poor oral gavage drug delivery. I did not perform an MTD analysis during this study given that an oral dose had previously been published for another strain of mice. In retrospect, the oral dose for NSG mice should have been optimized, particularly in the context of drug combinations.
Figure 5.12 DSF/Carboplatin inhibit tumour growth in vivo. Patient 2555 NOD/SCID PDXs were treated with DSF (200 mg/kg PO, q48 hr x 2 doses), Carboplatin (75 mg/kg IP x 1 dose), vehicle (10% DMSO in corn oil PO, saline IP) or combination of drugs (DSF 200mg/kg PO + Carboplatin 75 mg/kg IP). A) Pre-treatment tumour volume. B) Post-treatment tumour volume. P values (two tailed) were calculated using students t-test. C) Average tumour volume versus time for each treatment group ± s.e. DSF/Carboplatin group showed slower tumour growth and smaller tumour volume at endpoint. D) Kaplan-Meier survival curve of NOD/SCID mice from control (black line; n=5), Carboplatin IP (red line, n=5), DSF PO (green dashed line, n=5) or combination drug (grey dashed line, n=9).
A2.4 Discussion

Approximately 30-50% of ovarian HGSC have defective homologous recombination. I undertook this study to examine whether cells lacking a functional BRCA2 would be more sensitive to the combined cytotoxic effects of Carboplatin and DSF drug treatment. Using two isogenic cell lines, one with a functional BRCA2 protein (PEO4) and one without (PEO1) (Sakai et al., 2009), I evaluated the effects of combining DSF with Carboplatin. In contrast to expectations, a synergistic response was observed in the PEO4 cells, which harbor a functional homologous repair pathway. This effect was seen by increased γ-H2AX foci formation, as well as increased nuclear fluorescence using a marker of cell death. This prompted us to explore the effects of Carboplatin with DSF in 8 additional cell lines (2 were ultimately excluded given recent evidence suggesting they are not ovarian high-grade serous). In total, 8 cell lines were tested and 6 showed in vitro synergy (as indicated by the CI) between Carboplatin and DSF.

Concentrations of DSF in man have been reported to range between 0.5 µg/mL to 10 µg/mL, depending on dosing schedules, body weight and renal function. This corresponds to DSF plasma concentrations ranging from 1 – 35 µM and 10- 600nM for its metabolites (Faiman et al., 1978; Johansson, 1992). This makes concentrations needed to achieve cytotoxicity in vitro nearly achievable in vivo.

Based on this data, I assessed the effects observed in vitro in an in vivo PDX model, using a case known to be highly aggressive. A 200mg/kg/d oral dose of DSF has been previously published in NOD/SCID mice and corresponds to what would be expected based on a human-equivalent drug dose translation (Reagan-Shaw et al., 2008) However, although this dose seemed appropriate, this study did not use DSF in combination with other drugs (Brar et al., 2004). When used in combination with Carboplatin, this DSF dose was too toxic and resulted in the death of >50% of mice in the combined-treatment group. An MTD study using DSF/Carboplatin needs to be performed to elucidate the optimal DSF dose.

A wide range of anti-cancer effects have been described in a multitude of cancers (Table 5.1) and it was outside the scope of this project to elucidate the mechanism(s) of DSF’s activity against
ovarian cancer cells. Future projects should focus on assessing how DSF is able to potentiate the effects of Carboplatin, both in vitro and in vivo.

Numerous studies further support a role for the use of DSF in the treatment of a number of cancers. For example, phase II clinical trials are underway examining the effects of DSF for the treatment of prostate and lung cancers (ClinicalTrials.gov identifiers: NCT01118741 and NCT00312810). Our results suggest that targeting ovarian cancer cells with DSF, in addition to standard chemotherapeutics, may be an effective novel method to treat ovarian cancer. Our findings suggest that clinical trials incorporating DSF and platinum therapy should be pursued in ovarian cancer.