Identification And Characterization of Potential Drug Targets In
Serous Ovarian Cancer

BY

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THESIS
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer susceptibility gene</td>
</tr>
<tr>
<td>CA-125</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>CAS9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CCC</td>
<td>Clear cell carcinoma</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>EC</td>
<td>Endometrial carcinoma</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal transition</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</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>FIGO</td>
<td>Federation of Gynecological and Obstetrics</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>FTE</td>
<td>Fallopian tube epithelium</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain of function</td>
</tr>
<tr>
<td>gRNA</td>
<td>guide RNA</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HGSC</td>
<td>High grade serous ovarian cancer</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screen</td>
</tr>
<tr>
<td>IARC</td>
<td>International agency for research on cancer</td>
</tr>
<tr>
<td>LGSC</td>
<td>Low grade serous carcinoma</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss of function</td>
</tr>
<tr>
<td>MC</td>
<td>Mucinous carcinoma</td>
</tr>
<tr>
<td>MLH</td>
<td>MutL homolog</td>
</tr>
<tr>
<td>MOE</td>
<td>Murine oviductal epithelium</td>
</tr>
<tr>
<td>MOSE</td>
<td>Mouse ovarian surface epithelium</td>
</tr>
<tr>
<td>MTEC</td>
<td>Mouse tubal epithelial cells</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OGCT</td>
<td>Ovarian germ cell tumors</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian surface epithelum</td>
</tr>
<tr>
<td>OVCA</td>
<td>Ovarian cancer</td>
</tr>
<tr>
<td>OVCAR</td>
<td>Ovarian cancer resistance</td>
</tr>
<tr>
<td>P₄</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP-ribose polymerase</td>
</tr>
<tr>
<td>PAX8</td>
<td>Paired box family 8</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCST</td>
<td>Sex cord stromal tumors</td>
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### LIST OF ABBREVIATIONS (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SMAD</td>
<td>Mothers against decapentaplegic (transcription factor)</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TCGA</td>
<td>The cancer genome atlas</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIC</td>
<td>Tubal intraepithelial carcinoma</td>
</tr>
<tr>
<td>TVS</td>
<td>Transvaginal ultrasound</td>
</tr>
<tr>
<td>VGEF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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Chapter I - Introduction

Ovarian cancer and classification

Ovarian cancer is the fifth leading cause of cancer related death among women, and the most lethal gynecological disease in the U.S. In 2015, approximately 21,290 new cases of ovarian cancer will be diagnosed with 14,180 patients succumbing to their disease (66% mortality rate) (1-11). The five-year survival rate for ovarian cancer is 92% when the disease is localized and diagnosed at an early stage. However, only 15% of ovarian cancers are diagnosed at an early stage. The majority of ovarian cancers, 61%, are diagnosed once the disease has metastasized, and five-year survival rates are as low as 27%. This means that over 60% of all ovarian cancer in the U.S. have a five-year survival rate of under 30% (6, 8, 12-18). This high mortality rate is due to the lack of understanding of the early stage disease, and to a lack of specific biomarkers to detect the disease. Many ovarian cancers are diagnosed once they have reached stage III-IV and metastasized to other organs in the body (4, 8, 19).

The most common form of ovarian cancer is epithelial ovarian cancer (EOC), which accounts for over 95% of ovarian cancers. Other forms of ovarian cancer include ovarian germ cell tumors (OGCT) and sex cord-stromal tumors (SCST), which account for less than 5% of all ovarian tumors (8, 17, 19-21). EOC can be categorized into five different histotypes: mucinous, endometrioid, clear cell, low-grade serous and high grade serous. Each histotype is named after the gynecologic reproductive tissue that it most morphologically resembles. Serous ovarian cancer (both low and high) resembles the fallopian tube, endometrioid forms glands similar to those found in the normal endometrium, mucinous resembles endocervical epithelial cells and clear cell resembles epithelium of the gastrointestinal tract (16, 22). EOC is designated
a grade based on the architecture of the tumor (19, 23, 24). Grade 1 is when there is less than 5% of solid tumor compared to papillary/glandular structures (23, 25). This is often called low grade. Grade 3 is when there is over 50% solid tumor compared to papillary/glandular structures. This is called high grade cancer (23, 26, 27). High grade serous ovarian cancer (HGSC) accounts for 70% of all EOCs, with endometrioid and clear cell making up 10%. Mucinous occurs in about 3% of all cases, and low-grade makes up <5% (9, 26, 28).

Risk factors

Age: The main risk factor for ovarian cancer is age (3, 9, 29). Ovarian cancer incidences are rare in women under the age of 40; however, they dramatically increase in post-menopausal women (3, 5, 29). Nearly half of all ovarian cancers occur in women over the age of 60.

Ovulation: An increase in the number of lifetime ovulations correlates with the risk of developing ovarian cancer. This phenomenon is associated with the “tear and repair” hypothesis (30). Additionally, it has been shown that some of the reactive oxygen species (ROS) released during ovulation can damage DNA in the OSE cells, leading to increased proliferation and activation of oncogenes (31). This incessant ovulation, accompanied by increase levels of DNA damaging agents, may lead to clonal selection of cells with high levels of genomic instability that are resistant to apoptosis, thus driving carcinogenesis (31, 32).

Oral contraception: The use of oral contraception has been associated with a reduced risk for ovarian cancer. Many studies correlate oral contraceptive use and a decreased incidence of ovarian cancer (33, 34). A recent study highlighted the use of high levels of progestins in contraceptives as a potential cause for the reduced risk of ovarian cancer (33). Progesterone and estrogen are two hormones use to control ovulation. While estrogen may increase ovarian cancer risk, progesterone (P4) is thought to be protective (4). Oral contraceptives that contain high
levels of progestins, have been linked with a decrease incidence (35). Additionally, oral contraceptives inhibit ovulation, which may account for their protective mechanism (33).

**Parity:** The number of pregnancies experienced by women in her lifetime has shown to be protective against ovarian cancer (9, 36, 37). This effect has been attributed to a decrease in lifetime ovulations and hormonal changes associated with pregnancy (9, 31). During pregnancy, ovulation does not occur and the OSE do not undergo tear and repair. Moreover, levels of progesterone are high during pregnancy, while levels of estrogen are low (4). The corpus luteum maintains high levels of progesterone during implantation (38). These high levels of progesterone are continued after implantation to maintain uterine dormancy (9). Furthermore, progesterone may induce cell death in some OSE cell models, and thereby protects against the transformation of OSE cells (9).

**Hormone replacement therapy (HRT):** Many women undergo HRT during menopause to relieve some of the symptoms. HRT is comprised of an estrogen and a progestin. Artificially high levels of estrogen in the postmenopausal woman are associated with an increased risk of developing ovarian cancer (4). HRT also increases the risk of many other cancers including breast (5), and endometrial (39).

**Hereditary:** Roughly 10% of ovarian tumors are hereditary (40). Of these hereditary ovarian cancers, 90% are due to germline mutations in the *BRCA1* and *BRCA2* genes (40). Germline mutations in these genes are also linked with increased risk of breast cancer (41). Inheritance of these germline mutations leads to a 40-50% increase in lifetime risk for ovarian cancer, and 80% increase lifetime risk for breast cancer (40, 41). The BRCA genes are involved in DNA repair, and mutations in them lead to genomic instability and incorporation of mutations (41, 42).
While 90% of hereditary ovarian cancers are related to alterations in the BRCA genes, the remaining 10% can be attributed to germline mutations in MLH1 and MSH2 genes (40, 43). These genes are involved in the mismatch repair (MMR) system and control genomic stability. Lifetime risk of developing ovarian cancer is 12% with alterations in MLH1 and MSH2 genes (43).

**Treatment**

Ovarian cancer is a notoriously difficult disease to treat. This is due to the heterogeneity of the disease, genomic instability and acute metastatic ability of the tumors (19, 21, 44). The epithelial cells of ovarian tumors often slough off into the surrounding area, and colonize many different organs and tissues (19, 23, 24). Due to the location of the ovaries within the peritoneal cavity, tumor cells usually metastasize into the peritoneal cavity and omentum (24). The omentum is highly vascularized, and can facilitate intravasation and further spread of the cancer throughout the body (19). In addition, ovarian tumor cells posses the ability to circumvent the immune system, further inhibiting treatment of the disease (25). First line therapy includes tumor debulking by surgical means (9, 23, 26-28). If tumor burden is localized to one or both ovaries, total tumor debulking can be achieved (3, 9, 26, 28, 29). Surgery is performed to remove all visible tumor burdens, and to determine the extent, grade and histotype of the ovarian tumor (3, 5, 9, 29). Classification of ovarian malignancies follow the International Federation of Gynecological and Obstetrics (FIGO) and World Health Organization (WHO) grading systems (3, 5, 26, 29, 39, 45). Due to the spread of the disease through the highly accessible peritoneal cavity and difficulty in locating all metastatic tumors, cytoreductive surgery is often performed to remove as many of the tumors as possible (26, 39, 40, 45-48). After surgical debulking of tumors, many patients will undergo some form of treatment to reduce the likelihood of recurrent
tumors. Over the past 30 years, treatment for ovarian cancer has not improved. The gold standard is a combination of platinum based drugs, such as carboplatin and paclitaxel (4, 40, 46-48). Platinum based agents were first used to treat ovarian cancer in 1973 (4, 34, 40, 46). The first platinum based therapeutic used for ovarian cancer was cisplatin. However, cisplatin has very strong side effects including cytotoxicity to normal cells, leukemia, nervous system issues and frequent nausea and vomiting (4, 34, 46). Carboplatin is a variant of cisplatin that was first synthesized in the early 1980s (4, 45-47, 49, 50). Carboplatin gained considerable attention due to the decreased side effects compared to cisplatin, and increased efficacy in certain tumor types (8, 9, 31, 45-47, 49-51). A large meta-analysis study in 1997 of a large cohort of ovarian cancers found that there was “no good evidence that cisplatin is more or less effective than carboplatin in any particular subgroup of patients (51).” Since carboplatin proved as effective as cisplatin, but without many of the side effects, carboplatin became the gold standard platinum-based therapeutic. In 2003, the Gynecologic Oncology Group (GOG) further demonstrated less toxicity in patients receiving carboplatin/paclitaxel over cisplatin/paclitaxel, with a slight increase in overall survival of patients on the carboplatin regime (52). Some patients receive chemotherapeutics before cytoreductive surgery in a regime called neoadjuvant therapy (4, 8, 17, 20, 21, 26, 46, 47).

Most patients receiving first-line therapy respond well to treatment. Unfortunately, the majority of patients will relapse and the disease will return. Treatment options for recurrent tumors are relatively ineffective and the patients usually succumb to the disease (9, 17, 20, 21, 26, 28, 47, 53). Recurrent tumors are either chemo-sensitive or chemo-resistant (2-4, 6-11, 28, 47, 53). Chemo-sensitive tumors are retreated with carboplatin/paclitaxel. Chemo-resistant tumors undergo treatment with second line therapeutics. These therapeutics include doxorubicin (1, 3, 5-
9, 12-18), gemcitabine (1, 3, 5, 26, 39, 45, 47) and topotecan (7, 26, 39, 40, 45, 47, 48). Doxubicin is an antracycline, which had a median survival rate of 11 months in relapsed tumors. Side effects were well tolerated, with minimal toxicity (1, 4, 7, 40, 48). Gemcitabine is a nucleoside analogue, showed well tolerance with a median survival of 6.7 months (1, 4, 40, 41, 54). Topotecan is a topoisomerase inhibitor, however it shows considerable toxicity and side effects in clinical trials (40-42, 54). Once tumors relapse occurs, death is an inevitable. New therapeutics are in clinical trials, with drugs such as bevacizumab (VEGF monoclonal antibody), sorafenib (small molecule targeting VEGF), EGF inhibitors and PARP inhibitors.

**Origins of ovarian cancer**

The term “ovarian cancer” is used to describe all four histotypes of the epithelial derived tumors. The expression “ovarian cancer” arose due to the fact that the majority of the epithelial tumors were found in, on, or around the ovary, leading many to believe that these tumors were of ovarian origin. Of all the cells that make up the ovary, the OSE was postulated to be the progenitor cell of ovarian cancer. However, in more recent time, the cell of origin has been brought into question. The epithelium of the fallopian tube (FTE) has been shown to give rise to ovarian cancer. While research has been focused on the FTE as a progenitor cell, there is ample evidence in support of both cell types as potential progenitor cells.

**OSE** – The OSE was the first potential progenitor cell postulated to give rise to ovarian cancer (2, 10, 29, 41, 42). OSE cells are mesothelial like, and form a single layer that surrounds the ovary (2, 10, 29, 55-57). Mesothelial cells are epithelial cells that possess mesenchymal characteristics, such as expression of vimentin (2, 10, 11, 29, 55-59). These mesothelial cells are less differentiated than other epithelial cells of the gynecological tract. This is evident by the lack of expression of epithelial markers CA-125 and E-cadherin, and the expression of mesenchymal
markers N-cadherin, vimentin and presence of other epithelial markers such as keratins (2, 6, 10, 11, 58, 59). The biggest issue regarding the OSE is that it does not resemble any of the histotypes of epithelial ovarian cancer (6, 12, 14, 16, 60, 61). Thus, it was hypothesized that the OSE can differentiate to form all histotypes of the disease, depending on the mutations that occur.

The OSE as a potential source of ovarian cancer was first touted in 1872 (42, 60-63), however, it was not until 1971 that Fathalla et al. came up with the first incessant ovulation hypothesis (42, 59, 62-66). Fathalla et al worked on the laying hen. The laying hen is the only non-human animal that spontaneously develops ovarian cancer (42, 59, 64-66). As is in humans, hens develop a high rate of ovarian cancer over time (31, 42, 65-67). They ovulate profusely, with little or no breaks in between ovulation. This follows the theory that more lifetime ovulations leads to an increase in ovarian cancer risk (31, 32, 67, 68). As previously mentioned, incessant ovulation leads to constant rupturing of the OSE and release of the oocyte (2, 32, 68). After damage, the OSE exhibits fibroblastic characteristics and it proliferates and migrates over the wound (2, 18, 68). This process happens immediately post-ovulation (2, 18, 32, 69). Over a woman’s reproductive lifetime, this constant insult to the OSE, followed by rapid proliferation and migration results in an increased risk of DNA damage and instability (18, 32, 69). Evidence in support of this theory has been validated in multiple different animal models (70-72). In gonadotropin-induced superovulated mice, the OSE had higher levels of proliferation compare to unstimulated mice (70). In addition, the increase in proliferation was not accompanied by apoptosis, a mechanism that could clear the ovary of damaged OSE cells (70). Furthermore, another study in western ewes found that ovulation induced DNA damage in OSE cells, both in vivo and in vitro (72). This evidence of OSE involvement in epithelial ovarian cancer is further
enhanced by the fact that EOC is not common in non-primate animals. This is due to the lower number of total ovulations experienced by these mammals in comparison to humans (32).

While there is a causative link between ovulation, proliferation and DNA damage, there are also other factors during ovulation that can aid in the damage of OSE cells. Ovulation is induced by gonadotropins in humans, which also induces an inflammatory response (32). Pro-inflammatory cytokines are released upon OSE rupture (32). Many of these pro-inflammatory cytokines actually facilitate OSE rupture by upregulating enzymes involved in breaking down the OSE structure (2). Luteinizing hormone (LH), a gonadotropin, stimulates the release of TNFα and IL-1β, which promotes the production of nitric oxide (NO). LH also stimulates production of prostaglandins, which can upregulate collagenase enzymes to break down OSE structure (73). This immune response leads to leukocyte recruitment and generation of oxidative stress. Studies have shown that oxidative stress can induce DNA damage in OSE cells (73). Using H2O2 as an oxidative stress mimetic, King et al showed that OSE cells had increased proliferation due to activation of Akt, and significant levels of DNA damage (48). The combination of rapid proliferation, increased levels of DNA damage, and gonadotropin stimulation may lead to transformation of OSE cells and the initiation of tumorigenesis.

The final piece of evidence in support of the OSE as a progenitor cell comes in the form of epithelial-lined inclusion cysts within the ovary (32). Inclusion cysts in the ovary are fluid filled invagination of epithelial cells into the ovary (32, 55). These fluid filled sacs are lined by a single layer of epithelial cells (55). For many years, it was hypothesized that these epithelial cells may be derived from the OSE (55). As mentioned, after oocyte release during ovulation, the OSE repairs the wounded surface. The OSE may get pinched off from the ovarian surface and become trapped inside the ovarian cortex (2). Given the plasticity of the OSE to transform from
mesothelial-like cells in response to microenvironment cues, the OSE lined inclusion cells might undergo metasplasia and start to resemble serous epithelium of the fallopian tube (74). The metaplastic OSE cells within the cysts have been shown to express epithelial markers such as oviductal glycoprotein 1, E-cadherin, TAG-72 and Ber-EP4 (55, 75, 76), and lose mesenchymal markers such as calretinin (74, 77). In addition to serous metaplasia, the OSE have exhibited endometrioid metaplasia when growth in culture with endometrioid stromal cells and in the presence of estrogen (2, 78, 79). All this evidence has led many to believe that epithelial ovarian cancer is derived from the OSE.

**Fallopian tube fimbria** – While there is plenty of evidence in support of the OSE as the cell of origin of ovarian cancer, the one major draw back to the theory is that it lacks a well defined precursor lesion (55). While it is widely believed that OSE inclusion cysts transform into malignant tumors, definitive experimental evidence of this transition from epithelial cysts to high grade serous ovarian cancer has not been reported (12). In addition to this, many of the other histotypes of ovarian cancer have been attributed to other cells of origin (80). Malignant transformation of endometriosis into both endometrioid and clear cell ovarian cancers has led many to believe that there are many origins to epithelial ovarian cancer (80). Furthermore, mouse models deleting BRCA in the OSE have not supported that this mutation enhances spontaneous tumor formation (81). Also, the notion that an epithelium would gain E-cadherin as part of malignant progression is counter to most other tumors. And to date, only one spontaneous OSE-derived cell model was able to form serous-like tumors, while all others produced dedifferentiated tumors (82-84).

The fallopian tube fimbria epithelium (FTE) has recently been suggested as a potential progenitor cell of serous ovarian cancer, owing in part to the similarities in both morphology and
cellular markers (21). The fimbria are finger-like projections at the distal end of the fallopian tube next to the ovary (55, 85). During ovulation, the fimbria swoop over the OSE to capture the released oocyte and push it towards the uterus (77). The fallopian tube is derived from the Müllerian duct (59). While the OSE and FTE do share a common cellular origin in the coelomic epithelium, the OSE maintains many mesothelial characteristics, whereas the fallopian tube progenitors continue to differentiate to form the Müllerian duct (86). The Müllerian duct gives rise to the fallopian tube, endometrium and cervix (16). The OSE and FTE share common epithelial markers, such as cytokeratins (86). The FTE has expression of other epithelial markers like OVGP-1, E-cadherin and PAX8 (55, 58, 86, 87).

The first clinical evidence in support of the FTE as a precursor cell for ovarian cancer came in 2001 (88). Piek et al examined tubal segments of women with a familial history of ovarian cancer, or carriers of the BRCA mutation, after undergoing salpingal-oophorectomy in a risk reducing surgery (59, 88). It was found that 6 of the 12 patients exhibited dysplasia, with one patient exhibiting “severe dysplasia” in the fallopian tube epithelium while the ovaries remained normal (88). Five patients had hyperplastic lesion in the fimbriae fingers, with no hyperplastic or dysplastic lesions found in control women (88). Lesions showed increased Ki-67 staining, indicating an increase in proliferation, accompanied by lower levels of p21 and p27. Accumulation of p53 was observed in highly dysplastic lesions, indicating that it may have a stabilizing mutation in the p53 gene (55, 88). Mutations in p53 are seen in 96-100% of HGSC (44).

This first clinical evidence of a precursor lesion led to a larger study on these tubal lesions. One study found that between 1-5% of all BRCA mutant patients had lesions in the fallopian tube, with 100% occurring in the distal fimbria (89). A second study found similar
results, with dysplasia occurring in the distal end of the fallopian tube. Heightened p53 staining was only present in malignant or dysplastic lesions (90). These areas of dysplasia have been renamed “p53 signature” and are usually accompanied by high expression levels of p53 (59). These areas of heightened p53 staining were more frequent in patients containing TICs, with 80-100% occurring the distal fimbriae. Upon analysis of the mutational status of p53, Lee et al found that 8 out of 14 “p53 signatures” contained a mutation in p53, while 12 TICs had mutations in p53. In one case, the same p53 mutation was found in both the p53 signature and TIC from the same patient (91). The authors finally concluded that these p53 signatures are benign, and precede TICs as a possible precursor lesion to HGSC (91).

The first studies on the fallopian tube as a potential progenitor cell to HGSC were performed in patients carrying the BRCA susceptibility mutations (59, 88-91). However, only 5-10% of all ovarian cancers are due to hereditary mutations (40, 92). Therefore, the question of whether these precursor lesions are specific to BRCA+ women or common amongst all HGSC patients remained. Kindelberger et al studied a cohort of 55 women with late stage HGSC, and found that 75% of patients had TICs (93). Tumors were classified as either tubal carcinomas, peritoneal carcinomas or ovarian carcinomas (93). 5 out of 5 tubal carcinomas, 4 out of 6 peritoneal carcinomas and 20 out of 30 ovarian carcinomas presented with TICs. 93% of TICs were in the distal fimbriae (93). In 5 of the cases classified as ovarian carcinoma, laser capture microdissection of the DNA from at least one TIC and one tumor was used to sequence the p53 mutation (93). Identical mutations in p53 were identified in both the TICs and tumors from the same patient in all 5 cases (93). This was the first evidence to strongly suggest a clonal progression of HGSC from the fallopian tube to peritoneal and ovarian metastasis.
In support of this theory, multiple models of HGSC have been created derived from the FTE (94, 95). Karst et al showed that fallopian tube epithelium immortalized with Large and small T antigen SV40 and human telomerase reverse transcriptase (hTERT) could be transformed by the addition of either oncogenic Ras or c-Myc (94). This cellular model gave rise to high-grade Müllerian carcinoma that resemble that of HGSC (94). In addition, Perets et al, developed a murine model of HGSC derived from the oviduct using a PAX8 driven Cre-recombinase (95). The PAX8 is expressed in the oviduct, but not the OSE. The PAX8-Cre animals were used to knockout BRCA 1 or 2, PTEN and introduce a p53 mutation in the oviductal epithelium. These animals produced tumors derived from the oviduct that stained positive for HGSC markers. The authors also identified serous TICs in the model (95). This data informs us that HGSC can be derived from the fallopian tube.

**Dual model for the origin of HGSC** – While it is evident that not all HGSC cases had TICs or a p53 signature, the vast majority did express these newly defined precursor lesions (93, 96). Thus, a theory of multiple origins to ovarian cancer has been proposed (16, 59). Most cases of HGSC begin in the fallopian tube with a p53 signature, which progresses to dysplasia and TICs (59). TICs can then become malignant and metastasis to the ovary or the peritoneal cavity, giving rise to the name ovarian cancer. However, the remaining cases of HGSC that do not present with p53 signatures or TICs, may be of ovarian origin (59). The FTE cells may exfoliate onto the ovary, and become stimulated by the presence of reproductive hormones (93, 96). Implantation onto the ovary has been proven experimentally (31). Hormones and cytokines release from the ovary during ovulation attract the malignant FTE cells to the ovary, where the primary tumors form (31).
Potential cancer stem cell niches have also been identified for both the fallopian tube and OSE (97, 98). Flesken-Nikitin et al identified a stem cell niche in the hilum region of the ovary, at a junction between the OSE, mesothelium and murine oviduct that stained positive for 5 different stem cell markers and are slow cycling (97). In addition, the authors showed increased transformation potential of these stem cells when specific tumor suppressors, such as p53 and retinoblastoma protein (Rb), were inactivated (97). Auersperg et al identified a similar region in the distal fimbria of the oviduct (98). While these stem cell markers were seen throughout the oviduct, they were most prevalent and concentrated in the distal end, next to the OSE (98). It is at the distal end of the fallopian tube were TICs occur, and this region is believed to give rise to the precursors to HGSC due to the presence of stem cells. This connection of OSE and fallopian tube gives even more evidence for s dualistic model of HGSC.

**Pathways in ovarian cancer**

As previously mentioned, many of the histotypes of epithelial ovarian cancer are not only morphologically different, but also molecularly distinct. Much research has suggested that each histotype of EOC are so distinct, that it is not one disease but many diseases originating from similar cells (16). Each histotype has unique precursor lesions, unique molecular profiling, and unique responses to treatment. Information on the altered pathways and mutations in the various histotypes is provided in the table below (Table 1).

**Serous ovarian cancer** – Serous ovarian cancer is the most lethal histotype of epithelial ovarian cancer, and accounts for about 70% of all EOCs (99). This histotype derives it name from its similarities to the epithelium of the fallopian tube (2). Serous ovarian cancer comes in two forms, low-grade serous and high grade serous.
Low grade serous ovarian cancer (LGSC) makes up about 10% of all serous ovarian
cancers, and 2% of all EOCs (100). LGSC is characterized by dual mutations in \textit{KRAS} and \textit{BRAF}
in 68% of cases (100, 101). In addition, mutations in \textit{ERBB2} and \textit{PIK3CA} are significantly
associated with LGSC (102). Unlike high grade serous ovarian cancer, LGSC usually presents
with wild-type p53, and rarely have mutations in the \textit{BRCA} genes (100). Histological staining of
LGSC shows expression of PAX8, WT-1 and ER, similar to high grade, and aid in differential
diagnoses of serous tumors from other EOC histotypes (103). However, due to the presence of
wild-type p53, LGSC does not stain positively (103). Moreover, the proliferation index of
LGSC, as determined by Ki67 staining, is considerably lower than that seen in high grade (103).
LGSC does not respond well to first line therapy of paclitaxel and carboplatin, and thus is in
need of novel therapeutics (100, 103)

High grade serous ovarian cancer (HGSC) is the most lethal and aggressive of all EOCs
and accounts for 70% of all deaths from EOC (44). HGSC is heterogenetic, possessing many
different mutations, with few common alterations between patients (44, 104). The most
associated mutations found in this disease are either germline mutations in the \textit{BRCA} genes and
mutations in \textit{TP53}. Immunohistological staining of HGSC samples often stain positive for
PAX8, WT-1, heightened p53 and CA-125, while staining negative for p21 and PR (55). HGSC
initially responds well to conventional treatment, due in part to the aggressive nature of the
disease and high proliferation index (Ki67 > 50%) (103). However, disease often reoccurs and
becomes resistant to therapy (46, 47, 103). The high mortality rate and lack of effective therapies
has, in part, been due to a lack of understanding of the molecular aspects of the disease. To
rectify this issue, the cancer genome atlas network group (TCGA) (44) performed a pan-omics
study where they analyzed over 600 provisional tumor samples from chemoresistant patients.
They performed genomics, transcriptomics, proteomics, and epigenomics on all tumor samples (44). Of all mutations found in HGSC, only one mutation was consistent. These were mutations in the TP53 gene (44). Certain mutations in p53 can lead to stabilization of the protein, a phenomenon heavily linked to the p53 signatures proceeding TICs as a precursor lesion to HGSC (105).

While TCGA only found one common set of mutations in HGSC, they also concluded that HGSC was a disease of altered pathways, and not of single mutations (44). This means that many different mutations in many different genes could be present in HGSC, as long as the pathway in question was dysfunctional in a similar manner. For instance, the PI3K/RAS pathway is altered in 45% of HGSCs, however, Ras is amplified in only 11% of cases, while PTEN is deleted in 7% (Fig 1-1) (44). The main pathways altered in HGSC were PI3K/Ras pathway, RB signaling, p53 pathway, FoxM1 signaling and Notch signaling (44). These alterations make it difficult to design targeted therapeutics to any part of the pathway, since no one part is consistently altered.
Figure 1-1: Pathways altered in HGSC
Schematic of RB signaling pathway, PI3K/RAS pathway, and NOTCH signaling in HGSC, and illustration of the frequency of alteration to each member of the pathway. Figure extracted from TCGA (44)
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http://www.nature.com/nature/index.html
**TABLE I: CHARACTERISTICS OF EPITHELIAL OVARIAN CANCER HISTOTYPES**

<table>
<thead>
<tr>
<th></th>
<th>HGSC</th>
<th>LGSC</th>
<th>MC</th>
<th>EC</th>
<th>CCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor Lesion</td>
<td>TICs</td>
<td>SBT</td>
<td>Cystadenomas</td>
<td>Endometriosis</td>
<td>Endometriosis</td>
</tr>
<tr>
<td>Common mutations</td>
<td>BRCA1, BRCA2, TP53</td>
<td>BRAF, KRAS, PIK3CA, ERBB2</td>
<td>KRAS, HER2</td>
<td>CTNNB1, PTEN, MMR pathway</td>
<td>ARID1A, KRAS, PTEN, PIK3CA</td>
</tr>
<tr>
<td>Biomarkers upregulated</td>
<td>WT1, TP53, PAX8, CA-125,</td>
<td>ER, WT1, CA-125, PAX8</td>
<td>HER2, CK7</td>
<td>ER, CA-125, HE4</td>
<td>HNF1B</td>
</tr>
<tr>
<td>Biomarkers downregulated</td>
<td>p21, PR</td>
<td>TP53</td>
<td>WT1, TP53, PAX8, CK20</td>
<td>TP53, WT1, PAX8, Mesothelin</td>
<td>ER, PR, TP53, WT1</td>
</tr>
<tr>
<td>Therapeutic response</td>
<td>First line: Good Recurrent: Poor</td>
<td>Mixed</td>
<td>Intermediate</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Grade (% Ki67)</td>
<td>High Ki67: &gt;22%</td>
<td>Low Ki67: &lt; 3%</td>
<td>Ki 67: 12.5%</td>
<td>High and Low Ki 67: 8.2%</td>
<td>No grade Ki 67: 7.6%</td>
</tr>
</tbody>
</table>

*Table 1: Information on all epithelial ovarian cancer histotypes. Precursor lesions, common mutations, immunohistochemical biomarkers, response to therapy and grade for all histotypes. High grade serous ovarian cancer (HGSC), Low grade serous ovarian cancer (LGSC), Mucinous ovarian cancer (MC), Endometrioid ovarian cancer (EC), Clear cell ovarian cancer (CCC), Tubal intraepithelial carcinoma (TIC), Serous boardline tumor (SBT). (101, 103, 104, 106-113)*
TP53 and its mutations

Tumor protein 53 (TP53) encodes for the protein p53. p53 is a tumor suppressor transcription factor involved in many cellular processes including cell cycle control, DNA repair, genomic stability, apoptosis and metabolic pathways (114-116). It has been termed “the guardian of the genome” due to its importance in DNA stability (117). p53 is a transcription factor that binds to its canonical binding sequence motif of 5’-RRRCWWGYYY-3’ (R= purine, W= A or T, Y= pyrimidine) (115). It forms a tetramer with itself using the tetramerization domain located near the C-terminal of the proteins (Fig 2) (114, 118). The p53 complex can then bind to its response elements in DNA using the DNA binding domain, and either induce or repress transcription of a target gene.

Due to the complexity and importance of p53 in many cellular homeostatic processes, loss of p53 is seen in many different cancers and is often one of the drivers of carcinogenesis. Somatic mutations, usually missense mutations, are found in around 50% of all cancers, with alteration of the p53 pathway presenting in nearly all solid tumors (119). Several years after the discovery of p53, it was found that some p53 missense mutations may possess oncogenic functions (120). These are specific mutations that do not lead to a non-functioning p53 protein (121). They are usually single base pair mutations occurring within the DNA binding domain of the protein (120). These single base pair mutations may alter the structure of the protein, alter the DNA-protein interaction, or do both (122, 123). Examples of such mutations include arginine to histidine at amino acid 273 (R273H), which is a DNA contact mutation (124). A similar mutation occurs in the DNA binding domain at R175H, which causes global conformational changes in the protein structure (124). These novel mutations in p53 that tend to exhibit a pro-oncogenic function were termed “gain-of-function” (GOF) mutations (125). While it is unclear exactly how these mutations change this tumor suppressor into a tumor promoter, it has been
hypothesized that both the structural changes and DNA binding deficiencies alter the ability of p53 to induce or repress transcription of certain genes (123, 125). Many GOF p53 mutations also have a dominant negative effect over wild-type p53, meaning that only one allele of p53 needs to be mutated to have pro-oncogenic effects (125). In GOF p53 mutations, the tumor cell may still posses some wild-type p53 on certain genes, but also “gains” a pro-migratory and pro-invasive phenotype (124). This new migratory ability has been well documented in many different cancer types (126-128). GOF p53 mutations also help confer chemoresistance to cancer cells (129). This may be in part due to GOF mutations retaining some wild-type p53 activity, as it has been shown that cancers harboring wild-type p53 may be more resistant to traditional chemotherapy (130)
**HGSC and TP53 mutations**

As mentioned previously, TCGA performed a pan-omics analyses of over 300 HGSC samples, and found that the only common mutation was in the gene *TP53*. In fact, they concluded that mutations in p53 were seen in between 96-100% of HGSCs (44). They found many different types of mutations in this gene, including missense mutations, splice variants, truncating mutations and in frame deletions (44). Over 50% of the mutations were missense mutations, leading to a single amino acid change. These missense mutations were almost solely clustered within the DNA binding domain of the protein, with only one or two occurring outside the DNA binding domain (fig 2) (44). Splice variants, frame shift deletions, and truncations were spread across the whole gene, with most clustering within the DNA binding domain. The most common mutation in p53 was arginine to histidine/Leucine/cysteine/proline at amino acid residue 273 (R273H/L/C/P) (44). This concurred with previous data generated by The International Agency for Research on Cancer (IARC) in ovarian cancer, with amino acid 273 being the most frequently mutated by missense mutations (131).
Figure 1-2: Schematic of p53 mutations in HGSC:

Each lollipop represents a different mutation found in the TP53 gene in HGSC. The longer the lollipop, the more tumors with that mutation in the patient population. The red block represents the DNA binding domain of p53, the green block represents the transactivation domain, and the blue block represents the tetramerization domain. Most mutations occur within the DNA binding domain of p53, with R273 being the most common mutation, followed by R248, G245 and I195. Red lollipops are nonsense mutations, and frameshift mutations (insert and deletions). Black lollipops are in frame deletions, while green lollipops are missense mutations. Data and figure derived from TCGA (44)
The fact that nearly 100% of HGSCs have mutations in p53 gave weight to the idea that the FTE was the precursor lesion for serous cancers. Both the p53 signature and TICs had heightened expression of p53, a characteristic seen in GOF p53 mutations due to a lack of MDM2 and p21 transcription (105). Since the same mutations in p53 were found in patient-matched HGSC and TICs, and given the high percentage of tumors that harbor these mutations, many hypothesize that p53 may be a driver of HGSC. Indeed, a mouse models of HGSC derived from the oviduct (mouse equivalent of fallopian tube) expressed these same GOF p53 mutations (95). In addition, ovarian cancer cell lines that have been genetically validated as being HGSC, harbor mutations in p53 (86). However, while p53 may be required for transformation, it is not sufficient on its own (132, 133). Therefore, a second hit in other genes may drive HGSC (133). While much has been done to study the mechanism of GOF p53 mutations in various different cancers, the role of mutant p53 in HGSC remains relatively elusive.

TGFβ signaling in EOC

Alternations in TGFβ signaling is one of the main phenotypes associated with chemoresistance in ovarian cancer (134). Transforming growth factor beta (TGFβ) pathway is a signaling pathway involved in many processes including differentiation, migration, cell cycle control and immune response (135). The TGFβ receptors are a family of serine/threonine kinases, that bind many members of the TGFβ polypeptide superfamily, including TGFβ ligands, Activin ligands and BMP ligands (136). Upon binding of the TGFβ polypeptides, the serine/threonine TGFβ receptors phosphorylate intracellular proteins called receptor regulated SMADs (R-SMAD) (135, 136). The phosphorylated R-SMADs then travel into the nucleus, where they bind with more SMAD proteins, called Co-SMADS to form a transcriptional
complex (136). This SMAD transcriptional complex can then bind with different co-activators or co-repressor on the DNA to regulated transcription of various genes (136).

As previously mentioned, several studies have eluded to the role that TGFβ plays in chemoresistance (135, 137). One such study found that pathway analysis of gene signatures in chemotherapy treated ovarian cancers, linked TGFβ and p53 as the most significantly associated pathways with chemoresistance (138). This data suggests a role for both TGFβ and p53 in chemoresistance, and potentially in ovarian cancer development

**TGFβ and p53 crosstalk**

There is significant crosstalk between TGFβ and wild-type p53 in normal somatic epithelium cells (139). In somatic cells, TGFβ controls cell growth by inducing transcription of key cell cycle regulator genes, p21 and p15INK4B (139). However, while there are SMAD binding elements within these promoters, SMAD mediated transcription alone is not sufficient to induce transcription of these anti-proliferation genes. Indeed, transcription from many genes cannot be mediated by SMADs alone, and usually require co-activators/repressors (140, 141). In 2003, Piccolo et al found that one of co-factors required by SMADs to induce TGFβ mediated growth regulation, was p53(140). They showed that in the presence of p53, TGFβ induced cell cycle arrest in normal somatic cells. When p53 was knocked out, TGFβ lost its ability to induce cellular arrest(140). Furthermore, induction of p21 protein by TGFβ was lost in the absence of p53, showing that p53 activity was required for some of TGFβ induced actions (140). Piccolo et al also showed that p53 and SMADs bind to their respective responsive elements on the DNA to alter transcription (140). Since then, intense investigation has been performed on p53 and TGFβ, with over 200 genes potentially co-regulated by both transcription factors (142)
Wild-type p53 is required for TGFβ induced transcriptional response on certain genes, and loss of p53 abrogates certain TGFβ induced functions. In 2009, Piccolo et al tested the effect of GOF mutations on TGFβ signaling in breast cancer (128). They found that p53 R273H mediated TGFβ stimulated migration in breast cancer cells. In the presence of oncogenic Ras, p53 R273H formed a transcriptional complex with p63 and SMAD2 to repress p63 regulated genes, and induce invasion and migration (128). Two genes downstream of p63 that were repressed only in the presence of the p53 R273H/p63/SMAD complex were uncovered (128). Therefore, one of the aspects of this thesis was to investigate whether a similar phenomenon occurred in HGSC due to the high rate of p53 mutation and the amplification of TGFβ in chemoresistant tumors.

**PAX8 and HGSC**

Paired-box (PAX) genes are a family of transcription factors that are expressed in a cell specific manner, and are involved in development of many different cell types (143). PAX genes regulate and promote cell lineage differentiation and migration in embryos, cell proliferation, migration and cell survival (144). There are 9 members in the PAX family, PAX 1-9 (143). PAX genes get their name from the homologous paired box domain that all 9 family members contain (143-145). These paired domains possess DNA binding capability (145). PAX genes are expressed in a wide range of cell types in a specific manner, including in the pancreas (PAX4 and PAX6), lung (PAX7), Thyroid (PAX8) and Müllerian duct (PAX8 and PAX2) (144). While most PAX expression subsides after development, some PAX genes remain expressed in somatic, terminally differentiated tissue to promote repair and proliferation of cells, such as PAX2 and PAX8 in the Müllerian duct (146).
Due to the developmental role, many PAX genes play in proliferation and migration, and may become co-opted by tumors (146, 147). PAX8 is expressed in the thyroid, kidney and Müllerian duct (148). In fact, PAX8 is essential for both thyroid and Müllerian organogenesis (149, 150). Mice with a double knockout of PAX8 (PAX8 <sup>−/−</sup>) fail to develop a functioning thyroid gland, and need supplementation with thyroid hormones in order to survive past weaning (149). Additionally, PAX8 <sup>−/−</sup> female mice are infertile due to a lack of development of Müllerian derived organs (150). Organs derived from the Müllerian duct include the uterus, fallopian tube (oviduct in mice), cervix and upper part of the vagina (151). The ovary and lower half of the vagina are not derived from the Müllerian duct. Therefore, PAX8 is expressed in the uterus, fallopian tube and cervix, but not expressed in the ovary.

The most interesting aspect of PAX8 expression, is that it is detected in 80-96% of HGSC, and is often used to histologically diagnose HGSC (152-154). The combination of PAX8 and Wilms-tumor protein 1 (WT-1) is useful in diagnosing serous ovarian cancers from other EOCs, with up to 92% of serous carcinomas staining positive for both proteins (155). In addition, one study found that loss of PAX8 in HGSC cell lines induced apoptosis, leading the researchers to conclude that PAX8 is essential for HGSC growth and survival (156). This data has given weight to the idea that HGSC originates in the fallopian tube, since the normal fallopian tube expresses PAX8, and the OSE cells and ovary do not. However, recent models of HGSC derived from the OSE show high levels of PAX8 expression (84, 157). In a mouse model of HGSC, the tumor suppressors Pten and Lkb1 are knocked out (157). These mice develop papillary serous ovarian cancer with expression of PAX8 seen in the OSE (157). In addition, a spontaneous model of HGSC obtained by continuous passaging of mouse OSE cells in culture, leading to
HGSC like pathology in xenografts, which demonstrated PAX8 expression (84). These data suggest that PAX8 may play a role in HGSC regardless of the cell of origin.

**Aims of this study**

In chapter III, the goal of the study was to investigate the effect of the gain-of-function mutation in p53 (p53^{R273H}) on TGFβ in ovarian cancer progression and metastasis. In chapter IV, the goal of the study was to elucidate the function of PAX8 in the progression of high-grade serous ovarian cancer from both the OSE and FTE, and determined whether targeting PAX8 or its transcriptional targets could be exploited as potential therapeutic targets. In chapter V, the aim was to use bioassay-guided fractionation to identify novel anti-cancer drugs from aquatic actinomycetes in collaboration with Dr. Brian Murphy’s lab.
Chapter II: Materials and Methods:

Cell culture:

“All reagents were obtained from Life Technologies (Carlsbad, CA) unless otherwise indicated. OVCA 420, OVCA 429, and OVCA 432 are cell lines that have been previously published (158, 159), while OVCAR5 cells are available through the national cancer institute (NCI) as part of the NCI60 tumor cell line anticancer drug screen (160). The OVCA 420, OVCA 429, OVCA 432, and OVCAR5 cells (gifts from Dr. Gustavo Rodriguez and Dr. Teresa Woodruff at Northwestern University) were maintained in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin/streptomycin. OVCAR3 cells were obtained from ATCC (Manassas, VA) and maintained in the same media as above, with the exception of supplementation with 20% FBS. SKOV3 cells were acquired from ATCC and maintained in McCoy’s 5A supplemented with 2.3 g/L sodium carbonate, 10% FBS, and 1% penicillin/streptomycin.

Normal immortalized human ovarian surface epithelial cells (IOSE 80) were a gift from Dr. Nelly Auersperg at the University of Vancouver and were maintained in 50% v/v Medium 199 and 50% v/v MCDB (Sigma-Aldrich, St. Louis, MO), 15% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.055% epithelial growth factor (EGF, PeproTech Inc, Rocky Hill, NJ)(161). Normal human fallopian tube secretory epithelial cells (FTSEC) were a gift from Dr. Ronny Drapkin at Harvard University and were maintained in 50% v/v DMEM and 50% v/v F-12 (Mediatech, Manassas, VA), 1% L-glutamine, 1% penicillin/streptomycin, and 2% Ultroser G (Pall Corporation, Port Washington, NY) (94). Mouse ovarian surface epithelial cells (MOSE) were isolated from C57BL/6 mice and mouse tubal epithelial cells (MTEC) were isolated from
CD1 mice as previously described (48). Cultured cells were maintained at 37°C in a 5% CO₂ incubator” (121).

**Cloning of CRISPR/Cas9 plasmids**

pX260-U6-DR-BB-DR-Cbh-NLS-hSpCas9-NLS-H1-shorttracr-PGK-puro was a gift from Dr. Feng Zhang (Addgene plasmid # 42229). PAX8 gRNA sequences were designed using the CRISPR design tool provided by Dr. Zhang (http://crispr.mit.edu/). An exonic sequence of DNA from within the PAX8 gene was obtained from the NCBI database (ID: 18510) and blasted through the CRISPR design tool. The resulting single stranded 20nt gRNA sequence obtained was extended by 10bps using the NCBI database for PAX8 on the 5’ end, and complimentary sequence determined using ApE software. The gRNA overhangs were as follows: Leading strand 5’-AAAC-30nt-GT-3’, lagging strand 5’-TAAAC-30nt- 3’, with the 30nt representing the aforementioned PAX8 exonic sequence. The gRNA sequence was cloned into the pX260 vector according to previously published protocols (162). Positive clones were confirmed by colony PCR using primers corresponding to the gRNA sequence of interest and a sequence within the plasmid (5’-CCTCGACCATGGTAATAGCGA-3’). The positive clones were then sequenced using the human U6 promoter forward primer 5’-ACTATCATATGCTTACCCTGAAC- 3’. Sequences of gRNAs were as follows. PAX8 gRNA exon 2 forward 5’-aacACCATTGATCTGGTGAGTTGCAGCgt -3’, PAX8 gRNA exon 2 reverse 5’-taaacGCGATGCTTCAACTCGATCGATCCCGT-3’. PAX8 gRNA exon 4 forward 5’-aacAGACTGGCCCATCCGCTTGAGTGTGATTAGTgt-3’, PAX8 gRNA exon 4 reverse 5’-taaaacCTATCACTCAGGCGCGCGAATGCTGCGGCTC-3’. PAX8 gRNA exon 7 forward 5’-aacAGATGGTGCTGGCTGAAGGATCTGCTACGAggt-3’, PAX8 gRNA exon 7 reverse 5’-
taaaacTCGTACGGACACCTTCAGCCAGCACCATCT-3’. Lower case letters designate cloning overhangs, with uppercase letters designating PAX8 exonic sequence.

**Generation of stable cell lines:**

“Stable cell lines were selected using antibiotic resistant plasmids containing the gene of interest. SKOV3 cells stably expressing mutant p53 R273H (163) (Addgene, plasmid: 16439, donated by Dr. Vogelstein, Johns Hopkins University school of Medicine, Baltimore, MD) were selected using 500 µg/mL G418 (Gemini bio-products, West Sacramento, CA) and maintained in SKOV3 media containing 200 µg/mL G418. OVCA 420 cells expressing p53 shRNA or scrambled shRNA (Sigma-Aldrich, St. Louis, MO) were selected using 4µg/mL puromycin (Sigma-Aldrich) and maintained with 1 µg/mL puromycin. P53 wild-type plasmid (163) was purchased from Addgene (Addgene plasmid: 16434, donated by Dr. Vogelstein, Johns Hopkins University school of Medicine, Baltimore, MD)” (121).

Mouse ovarian epithelial (MOE) cells stably expressing mouse PAX8 shRNA (Catalog no. TRCN0000085778, Sigma-Aldrich, St. Louis, MO) were selected using 1.5 µg/mL of puromycin (Sigma-Aldrich, St. Louis, MO). Knockdown of PAX8 was verified by western blotting and mRNA expression levels compared to scrambled control containing a non-mammalian sequence. MOE cells stably expressing CRISPR/Cas9 plasmids were selected using 1.5 µg/mL of puromycin. Indel alterations were confirmed by sequencing of the PAX8 region in question, by western blot for decrease in protein, and by mRNA expression levels. A 2kb region of PAX8 including the CRISPR cut site was amplified by PCR. Forward primer 5’- GCAGGGGAAGAGAAGGGTTG-3’, reverse primer 5’- CTGGGGAGTGAACATCCTGC -3’. PAX8 sequencing primers were 5’- GGGTCTATATGCAGGGTAGC -3’.
MOSE cells stably expressing PAX8 (Transomic, clone ID: pTCN (BC020526)) and empty vector neomycin resistance were selected using 250µg/mL neomycin G418. Stable integration of PAX8 was confirmed via western blot analysis and mRNA expression, and empty vector neomycin was confirmed via mRNA expression using primers for the neomycin resistance mRNA.

MOE cells stably expressing p53R273H (Addgene, plasmid: 16439, donated by Dr. Vogelstein, Johns Hopkins University school of Medicine, Baltimore, MD) were selected using 600µg/mL neomycin G418, and validated by western blotting. MOE cells expressing Pten shRNA, KRAS, myr-Akt, Pten shRNA and p53R273H, Pten shRNA and myr-Akt, p53R273H and KRAS were previously made in the lab by Dr. Sharon Eddie, Dr. Georgette Moyle-Heyrman and Dr. Suzanne Quartuccio (132). MOE Pten shRNA cells stably transfected with KRAS were selected with 200 µg/mL hygromycin, and verified via western blotting.

**Generation of primary PAX8-CRE MOE cells**

Oviducts were isolated from mice heterozygous for PAX8 promoter driving expression of cre-recombinase (PAX8 +/Cre) (164). Two oviducts from the same animal were placed in a 10 cm³ dissection dish in MOE media. The oviducts were minced together using a scalpel. After several days, the epithelial cells begin to crawl out of the oviducts similar to our previous models (83). At this stage, the cells were isolated from the plate, and placed into culture flasks to propagate to confluency. Once confluent, the cells were frozen in regular media supplemented with 10% DMSO. To validate the cell lines, an aliquot of cells were plated in an 8 well chamber slide (Millipore, PEZGS0816, Billerica, MA, USA). Cells were fixed using 4% PFA, washed 3 times with PBS, and permeabilized with 0.2% triton-X 100. Cells were blocked with 10% goat serum and incubated with primary antibodies for one hour: PAX8 (Proteintech group, Chicago,
IL) at 1:200, OVGP-1 (Abcam, Cambridge, MA) at 1:200, Acetylated tubulin (Sigma-Aldrich, St. Louis, MO) at 1:1000, and CK8 (Developmental Studies Hybridoma Bank, Iowa, IA) at 1:200. Following three PBS washes, secondary antibody (goat anti-rabbit Alexafluor 594, goat anti-mouse Alexafluor 488, goat anti-rat Alexafluor 594, and goat anti-rabbit Alexafluor 488, Life Technologies, Carlsbad, CA) was added at 1:200 for one hour. DAPI mounting medium was used to cover slide the slides.

**Luciferase assay:**

“Cells were plated at a density of 25,000 per well into 24-well plates and incubated overnight. Cells were transfected with 0.05 µg/well of an expression construct containing the Smad binding element promoter upstream of the luciferase gene using Mirus TransIT LT1 (Mirus Bio LLC, Madison, WI) according to the manufacturer’s instructions. The Smad responsive element plasmid contains a CAGA sequence repeated twelve times upstream of the luciferase gene (gift from Dr. Aris Moustakas at Ludwig Institute for Cancer Research, Uppsala, Sweden). Plasmids for expression of wild-type p53 or mutant p53 R273H plasmids were transfected into cells at 0.05 µg/mL. Cells were transfected for 24 hr in serum-supplemented media. Cells were then washed with PBS and treated with TGFβ1 at 10 ng/mL (Sigma Aldrich) for 24 hr. SB-431542 (Selleck Chemicals, Houston, TX) was used at a concentration of 5 µM for all luciferase assays. The protocol and SBE-luciferase transfection efficiencies were normalized and run as previously described(165). Normal cell luciferase activity was measured using a Synergy Mx (BioTek, Winooski, VT)” (121).
Proliferation assay:

“Sulforhodamine B (SRB) assays were used to determine cell density. Cells were treated for 48 hr with TGFβ (20 ng/mL) followed by colorimetric assay as previously described(166). Cell survival was calculated by comparing the absorbance values between treated and control wells. Background was subtracted by measuring the absorbance of 0.1 mM of Tris-base alone” (121).

MOE PAX8 shRNA were plated at a density of 500 cells per well in 8 wells of a 96 well plate. MOSE-Neo and MOSE-PAX8 cells were plated at a density of 1000 cells per well. For PAX8 shRNA, a total of 6 plates were prepare, each plate representing one day of growth. Cells were then fixed with 20% TCA solution. For MOSE-Neo and MOSE-PAX8, a total of 5 plates were prepared. One plate was fixed 2 hours after plating, and represented Day 0. For MOE PAX8 shRNA, one plate was fixed every day for 5 days to represent day 1, day 2, day 3, day 4, and day 5. SRB assays were used to determine cell density. For MOSE-Neo and MOSE-PAX8, one plate was fixed every two days for 8 days to represent day 2, day 4, day 6, and day 8. “Cell growth was calculated by comparing the absorbance values between treated and control wells. Background was subtracted by measuring the absorbance of 0.1 mM of Tris-base alone” (121)”

Flow cytometry

“OVCA 420, OVCA 432, and SKOV3 cells were plated into T25 flasks 24 hr before treatment. Medium containing TGFβ (20 ng/mL) or solvent control was added and incubated for 48 hr. Following treatment, cells were trypsinized, washed with PBS, resuspended in 500 µL PBS, then fixed in 4 mL of 70% ethanol, and stored at −20°C overnight. The fixed cells were washed with PBS and stained with 500 µL propidium iodide (PI) solution [50 µg/mL PI, 90 units Rnase A, 0.1% Triton X-100, 4 mmol/L citrate buffer, 10 mM polyethylene glycol (PEG) 4000]. Cells were incubated in the PI solution for 20 min at 37°C before being treated with 500 µL PI salt
solution (1 mg/mL PI, 0.1 mL of 10% Triton X-100, 4 M NaCl solution, 10 mM PEG 4000).

Flow cytometric analysis was done on a Beckman Coulter Elite ESP (Miami, FL) with at least 30,000 individual events per reaction. Data was analyzed with Mod-fit software (Verity Software House, Inc., Topsham, ME)” (121).

MOE cells were plated at a concentration of 100,000 cells per well in a 6 well plate and left to propagate for 24 hours. The next day, cells were treated with either esiRNA for luciferase or esiRNA for PAX8 as previously described. After 72 hours, cells were trysinized, and washed twice with cold 1x PBS. Cells were then suspended in 70% ethanol for at least 1 hour, or stored at -20°C for a maximum of 1 week. Cells were pelleted, and ethanol removed. Resuspension was performed in PBS with 100 µg/mL RNaseA and 40 µg/mL of propidium Iodide and incubated for 30 minutes. Cells were then filtered through a cell strainer of 35 micron pores. Samples were submitted to UIC flow cytometry core for analysis.

**Western blot analysis:**

“Cells were plated at 50,000 cells per well in six-well plates, transfected with appropriate plasmids at 0.05 µg/mL, and treated with TGFβ1 (10 ng/mL) for 24 hr. To induce p53 express, cisplatin (Fisher Scientific, NC9343338) treatment was performed at 125µM for two hours. Protein concentration was determined by BCA assay (Pierce, Rockford, IL). Cell lysate (30 µg) was analyzed by 10% SDS-PAGE and transferred to nitrocellulose. Blots were then blocked with 5% milk in TBS-T and probed overnight with primary antibodies. The antibodies used were human p53 (#9282), p21 (#9247), maspin (#9117), CDC2 (#9112) (Cell Signaling Technology, Inc., Beverly, MA) at a concentration of 1:1000; DKK1 (H-120) and mouse p21 (F-5) (Santa Cruz Technology, Inc, Santa Cruz, CA) was used at a concentration of 1:200; TMEPAI 2A12 (Abnova, Taipei, Tiawan) was used at a concentration of 1:500; and actin (Sigma-Aldrich) at a
concentration of 1:1000. Anti-mouse and rabbit HRP-linked secondary (Cell Signaling Technology, Inc.) was used for all blots at a concentration of 1:1000” (121).

For MOSE-Neo and MOSE-PAX8 cells, cells were plated at a density of 100,00 cells per plate in a 6 well plate and allowed to growth for 24 hours. Protein was harvested as previously described. Antibodies used were N-cadherin (Abcam, Cambridge, MA, ab12221), at 1:300, fibronectin (Sigma-Aldrich, St. Louis, MO, F3648) at 1:1000, and SNAI2 (Abcam, Cambridge, MA, ab106007) at 1:1000. Actin was used as a loading control.

For western blotting of DAQ compunds, “Cells were plated at a density of 50,000 cells in a 6 well plate one day before treatment. Cells were treated with 17.6 µM 4 for 8 h and 24 h. DMSO was used as a solvent control. Western blot gels were run as previously described [16]. An amount of 30 µg of cell lysate was run for each sample. P21 (#9247) and cleaved-PARP (#9541) from Cell Signaling were used to probe protein membranes at concentrations of 1:1000 in 5% milk/TBS-T. Actin (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control at a concentration of 1:1000. Anti-rabbit HRP-linked antibody (cell signaling) was used for all blots at 1:1000. Densitometry was performed using ImageJ software. All samples were performed in triplicate” (167).

**Wound healing assay:**

“Cells were plated at 50,000 cells per well in a 24-well plate and incubated overnight. A uniform wound was created through the cell monolayer using a pipette tip. Cells were washed and treated with TGFβ1 (20 ng/mL) immediately after scratching. Pictures were taken at 0, 24, and 48 hr after scratching, and the area of the scratch was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Percent closure was measured compared to 0 hr and fold change was determined from percent closure of treated compared to untreated” (121).
MOSE PAX8 and MOSE Neo cells were plated at 50,000 cells per well in a 24 well plate until a monolayer had formed. “A uniform wound was created through the cell monolayer using a pipette tip” (121). Pictures were taken at 0, 24, 48, 72, 96 and 120 hours after scratching, and the area of the scratch analyzed using ImageJ. Percent was determined by comparing the area of each time point to 0 hours.

**qPCR:**

For mRNA analysis, cells were collected in 1mL TRIzol (Life Technologies, Carlsbad, CA) per 1x10^5 cells and isolated using chloroform separation, isopropanol precipitation and ethanol washing according to manufactures protocol. RNA concentrations were determined using nanoVue plus spectrophotometer (GE healthcare, product code 28-9569-62). A total of 1µg RNA was made into cDNA using iScript cDNA synthesis kit (Biorad, Hercules, CA). qPCR analysis was performed in a 96 well plate using Life Technologies ABI ViiA7 machine. PCR reaction mixture was as follows: 5µL FastStart SYBR green (2X) (Roche diagnostics, Indianapolis, IN), 0.2µL forward and reverse primers (0.5µM), 2.6 µL DEPC water and 2µL cDNA (2.5ng/µL). PCR run protocol was 10min @ 95°C (hot start polymerase); 10 seconds @ 95°C followed by 30 seconds @ 60°C (40 cycles). All primers were validated for efficiency through serial dilutions and generation of a standard curve. Housekeeping genes included 18s rRNA and GAPDH. Fold change in mRNA expression was determined using the 2^ΔΔCt method. Primer sequences were:

- **PAX8 forward:** 5’- CGGCGATGCCTCACAACCTCG-3’, **PAX8 reverse** 5’-CCGGATGCTGCCAGTCTCGT-3’;
- **WT-1 forward** 5’- CGGGTTTCTCTTCTCCTTTTG-3’, **WT-1 reverse** 5’- CACATGCCCTGGCCTATAAAT-3’;
- **E2F1 forward** 5’- GGTGATACCTTAAGTCCCTGTTC-3’, **E2F1 reverse** 5’- CCCTCTCCTTCTTCCAATAAAT-3’;
- **BRCA1 forward** 5’- CACAGCGTATGCCACAGAAA-3’, **BRCA1 reverse** 5’-
ATCCTGGAGTTTGCATTG -3’; N-Cadherin forward 5’- TTGCTTCAGCGTCTGTGGAG -3’, N-Cadherin reverse 5’- TCGTGACATCCTCAGGTAA-3’; Fibronectin forward 5’- GCCCTTACAGTTCCAAGTTCC -3’, Fibronectin reverse 5’- CAGCGTTGCCACAGTCA-3’, SNAI2 forward 5’-CTGGACACACACACAGTTA-3’, SNAI2 reverse 5’-AGGTGAGGATCTCTGTTT-3’

**Animals, organ culture and immunohistochemistry (IHC):**

“Animals were obtained, treated, and housed as previously described (48). Ovaries and oviducts were dissected and cultured as previously described (168, 169). The growth media consisted of alpha-MEM (Invitrogen), and 1% penicillin/streptomycin (Invitrogen) with 0.1% DMSO, 20 ng/µL TGFβ, 5 µM SB431542 (TGFβ inhibitor), or 20ng/µL TGFβ plus 5 µM SB431542 added as treatment conditions. TGFβ was dissolved in water but 0.1% of DMSO was added to the TGFβ alone condition to control for SB431542 solvent. Bromodeoxyuridine (BrdU, Sigma; 10 µM) was added into the growth media 24 hr prior to tissue fixation. Tissues were prepared for paraffin sectioning and immunohistochemistry was completed as described previously (48)” (121).

**Proliferation imaging:**

“Imaging was performed using a Nikon E600 Microscope with a DS-Ri1 Digital Camera and NIS Elements Software (Nikon Instruments, Melville, NY). ImageJ was used to quantify cell proliferation. Percent proliferation was calculated by dividing the number of epithelial cells staining positive for BrdU by the total number of epithelial cells” (121).
siRNA knockdown

MOE cells were plated in 6 well plates at a volume of 100,000 cells per well 24 hours prior to transfect. A total of 400 ng/mL of PAX8 endoribonuclease small interfering RNA (esiRNA) (Sigma-Aldrich, St. Louis, MO, EMU061581) was transfected into MOE cells using Mirus TransIT X2 (Mirus Bio LLC, Madison, WI) according to manufactures protocol. Cells were incubated for 72 hours in the presence of esiRNA PAX8, and collected for protein analysis. An esiRNA targeting the luciferase gene was used as a negative control (Sigma-Aldrich, St. Louis, MO, EHURLUC)

OVCA5 Cytotoxicity Assay:

OVCA5 cells were cultured in minimum essential media (Life Technologies, 11090-081, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (Life Technologies, 16000-044) 1% L-glutamine (Life Technologies, 25030-081), 1% nonessential amino acids (Life Technologies, 11140-050), 1% sodium pyruvate (Life Technologies, 11360-050) and 1% penicillin/streptomycin (Life Technologies, 15140-122). OVCA5 cells (5000/well) were plated in a 96 well plate one day prior to treatment. The next day, cells were treated with varying doses of the given compound in regular culture media. The doses tested were 10.0 µg/mL, 5.00 µg/mL, 2.50 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.313 µg/mL, 0.156 µg/mL, and 0.0781 µg/mL. Doses higher than 10.0 µg/mL could not be tested due to compound insolubility in DMSO. Test plates were incubated at 37 °C with 5% CO2 for 96 h. After 96 h, media was removed from the cells and washed with cold PBS. Cells were permanently fixed to the culture plate using 5% trichloroactetetic acid (TCA). A sulforhodamine B (SRB) assay was performed as previously reported [15]. Percent survival was calculated by comparing samples treated with 1 or 4, and
samples treated with the relevant volume of DMSO solvent control. Prism 6 GraphPad was used to graph the results and determine the LC50 in µM concentrations. (167)

**Immunofluorescence**

“Cells were plated at a density of 25,000 cells in a chamber slide (Millipore, PEZGS0816, Billerica, MA, USA) one day before treatment. After one day, cells were treated with 17.6 µM of 4 for 8 h and 24 h. After the treatment, cells were washed with 1X cold PBS and fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton-X100 in PBS for 10 min. Cells were then washed twice with 1×PBS and blocked with 10% goat serum in PBS. Phospho-histone H2A.X (Cell Signaling, #9178, Beverly, MA, USA) was incubated on the cells for 1 h at room temperature in 10% goat serum/PBS at a concentration of 1/100. After two PBS washes, cells were incubated with anti-rabbit AlexaFluor 488 (Life Technologies, Carlsbad, CA, USA) for 30 min at room temperature and mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken on a Nikon E600 Microscope with a DS-Ri1 Digital Camera and NIS Elements Software (Nikon instruments, Melville, NY, USA). ImageJ software was used to count cells. The number of DAPI positive cells that were also positive for phosphor-histone H2A.X were expressed as a percentage of total DAPI stained cells. Only cells with defined foci were counted as positive. At least three random fields from three independent experiments were counted” (167).

**Fraction plate library screening:**

Fraction library plates were received at a concentration of 10 mg/mL in 96 well plates. Cells are plated at concentration of 7,500 cells per well of a 96 well plate. Initial dilution plates were prepared at a 1/100 dilution of each fractions, containing 99 µL of regular cell culture media and
1 µL of a given fraction (100 µg/mL). Next, 10 µL from the dilution plate was placed into 90 µL of media on the cells (1/10 dilution), to give a final concentration of 10 µg/mL of each fraction. Each fraction plate had one lane of a solvent control, to which the results of that plate were compared. Once fractions were added to the cells, plates were kept in a 37°C incubator for 96 hours. After 96 hours, each plate was washed once with cold PBS and surviving cells fixed in 20% TCA. A sulforhodamine B was performed according to previous protocols (166). Percent survival was determined by comparing total cell volume of each fraction to solvent control, where solvent control represented 100% survival. Doxorubicin was used as a positive control.

**Ethics statement:**

“All animals were treated in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the established Institutional Animal Use and Care protocol at the University of Illinois at Chicago. The protocol was approved by the Animal Care Committee at the University of Illinois at Chicago (protocol number: A08-250). Animals were housed in a temperature and light controlled environment (12 h light, 12 h dark) and were provided food and water ad libitum. All mice were euthanized by CO₂ inhalation followed by cervical dislocation” (121).

**Statistical analyses:**

“All values are presented as the mean ± the standard error. ANOVA followed by Tukey’s multiple comparison tests were used to assess differences between experimental and control groups. Two-way ANOVA with Sidak posthoc was performed on proliferation, migration and flow cytometry assays with MOSE Neo, MOSE-PAX8, MOE siLuc and MOE siPAX8. For the wound healing assay with TGFβ on ovarian cancer cells, a paired t-test was used to analyze...
control and treated in each cell line, while an unpaired t-test was used when comparing treated groups between two different cell lines. p<0.05 was considered statistically significant” (121).
Chapter III: Mutation or loss of p53 differentially modifies TGFβ action in ovarian cancer.

The following chapter, Chapter III: Mutation or loss of p53 differentially modifies TGFβ action in ovarian cancer, is published in the journal PLOS one, under which I am an first author (121). All sections, including figures and tables, have been reproduced under the Creative commons Attribution 4.0 international license for the purpose of this thesis. This work is licensed under the Creative Commons Attribution 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/ or send a letter to Creative Commons, PO Box 1866, Mountain View, CA 94042, USA. Copyright of Eoghainnín Ó hAinmhire ©

Introduction:

“Ovarian cancer is the fifth leading cause of cancer death and the most lethal gynecologic disease among US women. In 2013, an estimated 22,240 cases of ovarian cancer will be diagnosed, resulting in 14,030 deaths (8). The high mortality rate can be attributed to the fact that over 60% of ovarian cancers will be diagnosed after the disease has spread to distant locations. Once metastasized, the five-year survival rate drops to under 30% (8). Inefficiency of diagnosis is primarily due to a lack of understanding of the initiating events and mechanisms of progression that give rise to ovarian cancer, with few early detection strategies (23). Importantly, if ovarian cancer is diagnosed earlier, survival rates can be as high as 90% (8). These statistics illustrate the fundamental need to better understand early mechanistic events of ovarian cancer that will assist with earlier diagnosis and better prognosis of patients.

The tumor suppressor p53 is the most commonly mutated gene in all human cancers (23). P53 is a transcription factor that controls many cellular functions such as the cell cycle, apoptosis, and response to DNA damage (170). Most TP53 mutations are missense mutations, where a single nucleotide base substitution results in either dysfunction or absence of p53 activity (140). These mutations lead to increased proliferation, invasion, and metastasis in many
The Cancer Genome Atlas Network (CGAN) identified p53 as being mutated in up to 96% of chemotherapy resistant, high-grade serous ovarian cancers, indicating an essential role for p53 mutations in serous ovarian cancer (44). Moreover, the International Agency for Research on Cancer (IARC) TP53 database indicates that the most frequent p53 mutation in serous ovarian cancer is an arginine to histidine conversion at amino acid residue 273 (R273H) within the DNA binding domain, which accounts for 8% of all p53 mutations (131). Mutant p53 R273H has been reported to play a role in promoting breast and lung cancer metastasis (128, 171) by increasing migration and invasion.

Another important signaling pathway that is modified in ovarian cancer is the Transforming Growth Factor Beta pathway (TGFβ) (135). TGFβ is a superfamily of peptide growth factors that regulate growth, differentiation, apoptosis, and migration (135). TGFβ signals by binding to a family of serine/threonine kinase membrane receptors, which phosphorylate downstream signaling molecules, primarily Smads 2 and 3 (136). Once activated, these Smad complexes translocate to the nucleus and interact with various co-activators and repressors to modulate Smad-regulated transcription (136, 172). TGFβ plays an important role in inducing growth arrest in normal ovarian cells (173). In some cancer cells, TGFβ induces apoptosis and cell cycle arrest, while in other cancer cells it loses the ability to induce growth arrest and can instead promote cellular invasion (135). It can play a role in chemoresistance in advanced serous ovarian cancers (138). The core TGFβ pathway components, the TGFβ receptors, and Smad proteins, are rarely mutated or lost in ovarian cancer (21), suggesting that disruption of the TGFβ pathway occurs by other mechanisms.

As p53 and Smads are both transcription factors, p53 is capable of interacting with Smads to modify both the p53 and TGFβ signaling pathways (140). Smads can form a
transcriptional complex with p53 to induce expression of genes that promote cell cycle arrest, such as p21 (140). Smads and p53 bind to their own responsive elements in the promoters of TGFβ-responsive genes to synergistically activate or repress transcription (140). In fact, it has been shown that p53 is required for TGFβ-induced cell cycle arrest (140). Additionally, mutant p53 R273H abrogates TGFβ-induced cell cycle arrest and promotes metastatic behavior by blocking p63 in breast carcinoma cells (128). In these cells, the mutant p53/Smad complex inhibits p63-mediated transcription leading to invasion in the presence of oncogenic Ras (128).

Given the high percentage of p53 mutations in ovarian tumors (44) and the recent evidence that p53 and Smads interact to regulate metastasis in breast carcinoma cells (128), the role of mutant p53 in response to TGFβ signaling in ovarian cancer was investigated. p53 and TGFβ are implicated in many cancers such as breast and lung on their own (174, 175) and in concert (128). In breast and lung cancers, mutant p53 interacts with Smads to alter transcription of genes that regulate metastasis (128), but little is known about how p53 and TGFβ interact in ovarian cancer. Factors necessary for growth and metastasis in breast, lung, and colon cancer may not be necessary in ovarian cancer, leading to tissue specific effects of mutant p53 signaling (176). Two genes (TMEPAI and DKK1) were studied based on their role in metastasis in ovarian cancer. TMEPAI is a TGFβ-induced negative regulator (177) and is involved in TGFβ-induced metastasis in breast carcinoma cell lines (177) but has never been reported to be co-regulated by p53 and Smads. DKK1 is an inhibitor of Wnt signaling, which is often upregulated in metastatic ovarian cancer, and is associated with poor prognosis (178). Maspin, an anti-metastatic protein, was also chosen as it has established regulation by p53 and Smads (179). The current study
evaluated whether expression of one of the most common p53 mutations in ovarian cancer (R273H) alters the cell response to Smad signaling to modulate cell proliferation and migration” (121).
Results:

*TGFβ induces growth arrest in ovarian cancer cells expressing wild-type p53.*

“To better understand the role of p53 in ovarian cancer, six known ovarian cancer cell lines were analyzed for p53 expression (Fig 3-1a). OVCA 420 and 429 cells express low levels of p53 protein, consistent with reports that they have wild-type p53(180). Due to these low levels, cisplatin treatment was used to induce and confirm p53 expression in OVCA 429 (Fig 3-2). SKOV3 and OVCAR5 did not show any p53 protein expression, as is consistent with the previous finding that classified them as p53 null (131). In contrast, OVCA 432, and OVCAR3 exhibited abundant p53 protein expression due to the R277H and R248Q mutations, respectively (Table 3-1) (131).

To evaluate how p53 expression modulates the ability of cells to respond to Smad signaling, a luciferase assay was employed to determine which cells were responsive to TGFβ-induced Smad transcription (Fig 3-1b).

Next, the effect of TGFβ on non-cancerous progenitor cells was investigated. Since ovarian surface epithelium (OSE) and fallopian tube epithelium (TEC) may give rise to ovarian cancer (16), the response of these normal cells to TGFβ was investigated. In order to monitor signaling downstream of TGFβ, SBE-luciferase assays were performed on normal 2D murine OSE (MOSE) and murine TEC (MTEC) cells, as well as human immortalized OSE (IOSE80) and human fallopian tube epithelium (FTSEC). SBE-Luc is a triplet repeat of a SMAD binding element upstream of luciferase. OSE and TEC cells significantly responded to Smad-mediated transcription induced by TGFβ in both mouse and human cell lines (Fig 3-1c). MOSE cells responded with a higher fold activation (21 fold) of the reporter than MTEC cells (7 fold).
expression in MOSE was previous confirmed as being wild-type (48, 181). MTEC cells behaved as wild-type in response to cisplatin (Fig 3-3), while the IOSE80 and FTSEC were functionally null for p53 due to immortalization with SV40” (121).
Figure 3-1: Ovarian cancer cell lines respond to TGF\(\beta\) regardless of p53 status.

*(a)* Western blot analysis of ovarian cancer cell lines demonstrating their p53 status. Actin used as a loading control. *(b)* Six ovarian cancer cell lines (OVCA 420, 429, SKOV3, OVCAR5, OVCA 432, and OVCAR3), along with four primary, non-cancerous cell lines (MOSE, MTEC, IOSE80, and FTSEC) were treated with or without TGF\(\beta\) (10 ng/mL) for 24 hrs using the Smad binding element upstream of luciferase (12xSBE-Luc). ANOVA was performed separately for fold induction (TGF\(\beta\)) and fold repression (inhibitor and TGF\(\beta\) + inhibitor) to analyze significance compared to untreated. Data represented as mean ± SEM, *\(p\leq0.05\)” (121)
### TABLE II: PARENT CELL LINES AND THEIR P53 STATUS

<table>
<thead>
<tr>
<th>Parent cell lines</th>
<th>p53 Wild-type (WT)</th>
<th>p53 absent (null)</th>
<th>p53 mutation (MT)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>X</td>
<td></td>
<td></td>
<td>(158, 159, 180)</td>
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<td></td>
<td></td>
<td>(180)</td>
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<td>(131)</td>
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<td>X</td>
<td></td>
<td>(131)</td>
</tr>
<tr>
<td>OVCA432</td>
<td></td>
<td></td>
<td>X (R277H)</td>
<td>(131, 158, 159)</td>
</tr>
<tr>
<td>OVCAR3</td>
<td></td>
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<td>X (R248W)</td>
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<td>IOSE80</td>
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<td>FTSEC</td>
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Cells were treated with 125µM of cisplatin for 2 hours and cell lysates run on a western blot. Wild-type p53 is stabilized after damage and therefore detectable by western blotting. Actin was used as an internal loading control. (121)
Cells were treated with 125µM of cisplatin for 2 hours and cell lysates run on a western blot. Actin was used as an internal loading control.

Figure 3-3: p53 Status in MTEC cells
“Based upon these results, three cell lines (OVCA 420, OVCA 432, and SKOV3) were chosen to further analyze the impact of TGFβ on proliferation. These cell lines were treated with TGFβ (20 ng/mL) for 48 hr and cell cycle progression was examined using flow cytometry. TGFβ treatment induced G₀/G₁ cell cycle arrest in OVCA 420 (Fig 3-4a). OVCA 432 cells, which express mutant p53, were not growth arrested by TGFβ treatment (Fig 3-4b). Lastly, TGFβ did not induce cell cycle arrest in SKOV3 cells, but rather reduced the number of cells in G₀/G₁ (Fig 3-4c).

To further confirm the mechanism for the p53-Smad cell cycle regulation, expression of p21 and CDC2 were evaluated in OVCA 420, OVCA 432, and SKOV3 cells treated with TGFβ (Fig 3-4d). p21 is a cyclin dependent kinase inhibitor that is regulated by both p53 and Smads, and correlates with TGFβ-mediated cell cycle arrest (140). CDC2 (or CDK1) is a cyclin dependent kinase and its expression is consistent with cell cycle progression (182). TGFβ treatment in OVCA 420 increased p21 protein expression (Fig 3-4d), which was not observed in the OVCA 432 and SKOV3 cells (Fig 3-4d). The OVCA 432 and SKOV3 cells expressed elevated levels of CDC2 as compared to OVCA 420 (Fig 3-4d). Next, immunohistochemistry was performed to monitor proliferation of normal mouse ovarian surface epithelium and oviductal epithelium using a 3D organ culture system (169) treated with TGFβ. After 48 hr, OSE proliferation was significantly decreased with TGFβ treatment compared to DMSO control (Fig 3-4e). Despite being transcriptionally responsive, proliferation was not inhibited by TGFβ treatment in oviductal cells in 3D culture (Fig 3-4f). Immortalization of IOSE80 and FTSEC with SV40T antigen inactivates p53 (94, 161), therefore growth assays with TGFβ were not performed on these cells” (121)
Figure 3-4: *p53* wild-type ovarian cancer cell lines undergo cell cycle arrest in response to TGFβ.

‘(a-c) OVCA 420, OVCA 432, and SKOV3 cell lines were treated with 20 ng/mL TGFβ for 24 hours and subjected to flow cytometry analysis after propidium iodide stain. Distributions of cells in the three phases of the cell cycle are represented by mean percentages +/- SEM. Statistical significance represents a difference between number of cells in each cycle between treated and untreated and represented with * for an increase of treated cells compared to untreated; # represents decrease of treated cells compared to untreated; p ≤ 0.05 [(d)] Western blot analysis of the three ovarian cancer cell lines probed for cell cycle proteins p21 and CDC2. Actin was used as a loading control. (e-f) Proliferation assay performed using BrdU incorporation in 3D organ culture of mouse ovaries and oviducts. One-way ANOVA was performed. Data represented as mean ± SEM *p ≤ 0.05” (121).
**TGFβ-induced cell cycle arrest is abrogated in p53 mutant and null cells**

“Variants of OVCA 420 were created to investigate the role of p53 in TGFβ-induced cell cycle arrest. Using shRNA, endogenous wild-type p53 was effectively knocked down in OVCA 420 (OVCA 420 p53 shRNA) cells as compared to the scrambled shRNA control (OVCA 420 Scr) (Fig 3-5a). Additionally, SKOV3 cells were stably transfected to express mutant p53 R273H (Fig 3-5a). Wild-type p53 could not be stably transfected into SKOV3 cells because the cells underwent senescence and could not be propagated as previously reported (183). Transient transfection of wild-type p53 into SKOV3 cells did not immediately induce senescence, which allowed data collection at shorter time points.

First, the ability of the variant cell lines to respond to TGFβ was investigated. All stable cell lines maintained the ability to induce Smad-mediated transcription of a SBE-luciferase plasmid irrespective of p53 status (Fig 3-5b). Luciferase induction remained the same in the OVCA 420 p53 shRNA and OVCA 420 Scr when compared to the OVCA 420 parent cells (Fig 3-5b). Similarly, the SKOV3 stable mutant p53 R273H cells did not alter TGFβ-induced Smad-mediated transcription of the luciferase gene (Fig 3-5b). Transient expression of wild-type p53 in the SKOV3 cells reduced Smad-mediated transcription in comparison to the SKOV3 mutant p53 R273H cells, but displayed no significant difference compared to the SKOV3 parent cell line (Fig 3-5b).

In order to assess proliferation in response to TGFβ (20 ng/mL), cell growth assays were performed after 48 hr incubation. As expected, OVCA 420 Scr cell growth was repressed in response to TGFβ, which was similar to the parental line (Fig 3-5c). TGFβ induced growth inhibition was lost in the OVCA 420 p53 shRNA. Similarly, TGFβ did not slow proliferation in either the parent SKOV3 or the SKOV3 mutant p53 R273H cell line (Fig 3-5c)” (121).
Figure 3-5: Wild-type p53 cells, but not p53 null or mutant p53 cells, are growth inhibited by TGFβ.

(a) Western blot analysis of stable cell lines demonstrating knockdown of p53 by shRNA plasmid or expression of mutant p53 R273H. C=control, T=TGFβ treated. (b) SB-431542 (5 µM) was used for 24 hrs to inhibit TGFβ signaling. Fold induction measured as transcription initiation from SBE-Luc. For each panel, data represents mean ± SEM p≤0.05 increase over untreated for groups labeled with a, or between treated groups labeled with b. (c) Cell growth. Percentage of TGFβ-treated cell survival compared to untreated. Data represent mean ± SEM, *p≤0.05" (121).
**Mutant p53 R273H expression prevents TGFβ-induced migration of SKOV3 cells**

“In addition to affecting proliferation, TGFβ and p53 have also been shown to influence migration of tumor cells from the breast and lung (128, 171, 184). Previous literature suggests that mutant p53 might function as a molecular trigger allowing TGFβ to induce pro-migratory stimuli (185). Therefore, TGFβ regulation of cell migration in the presence of wild-type, mutant, and null p53 was investigated using a wound healing assay. TGFβ induced migration in both OVCA 420 Scr and OVCA 420 p53 shRNA cells between 0 and 24 hours and 24 and 48 hours (Fig 3-6a). OVCA 420 Scr (p53 wild-type) cells treated with TGFβ migrated significantly more than non-treated control between 0 and 24 hours, but not between 24 hours and 48 hours, whereas OVCA 420 p53 shRNA cells treated with TGFβ migrated significantly more than control between 0 and 24 hours and between 24 and 48 hours (Fig 3-6a). Migratory rates were compared between treated OVCA 420 Scr and OVCA p53 shRNA. Knockdown of p53 allowed for an increased migration compared to wild-type cells (Fig 3-6b).

The ability of SKOV3 null and SKOV3 mutant p53 R273H cells to migrate in response to TGFβ was also analyzed. TGFβ induced migration in p53 null SKOV3 cells compared to untreated cells between both 0 and 24 hours and between 24 and 48 hours (Fig 3-6a). However, expression of mutant p53 R273H in SKOV3 cells inhibited TGFβ-induced migration, with no change between 0 and 24 hours, or 24 and 48 hours when compared to control (Fig 3-6a). SKOV3 cells expressing mutant p53 R273H demonstrated less TGFβ-induced migration than SKOV3 null cells (Figure 3-6b)” (121).
Figure 3-6: TGFβ induces migration in p53 null cells in comparison to p53 wild-type or mutant cells.

(a) Wound healing assays were performed on SKOV3 and OVCA 420 stable cell lines. Cell monolayers were scratched and treated with or without TGFβ at 20 ng/mL for 48 hours. Wound closure was measured as a fold increase or decrease compared to no treatment control. Paired t-test was used with a p < 0.05. (b) Comparison of the fold increase of TGFβ samples from 5(a). Unpaired t-test was used to analyze significance. Significance is represented by * and signifies a statistical difference between cell lines. Data represented as mean ± SEM, *p≤0.05” (121).
Expression of mutant p53 R273H alters TGFβ induced-expression of TMEPAI and DKK1

“In order to elucidate possible mechanisms by which p53 and TGFβ might regulate migration, pro-invasive targets known to be regulated by either p53 or TGFβ in ovarian cancer cells were investigated. Maspin is a serine protease inhibitor that blocks metastasis (179, 186) and is known to be co-regulated by p53 and Smads in mammary epithelial cells(179). Additionally, maspin expression is reportedly lost in ovarian cancers, and this has been associated with poor prognosis and survival rates (187). Maspin was minimally induced with TGFβ treatment in OVCA420 and OVCA432 cells (Fig 3-7a), and was not induced in SKOV3. Surprisingly, maspin did not demonstrate a dependence on TGFβ treatment or p53 expression in OVCA420 p53 shRNA or SKOV3 R273H mutant p53 cell lines compared to parent cells suggesting that additional pathways modify p53 and Smad regulation of maspin in ovarian cancer cells.

TMEPAI is a TGFβ-induced protein that is known to convert TGFβ from a tumor suppressor into a tumor promoter in breast cancer, and is associated with increased migration in prostate and renal carcinomas (177, 188, 189). Overexpression of TMEPAI has been associated with many cancers, including ovarian cancer(190). TGFβ increased expression of TMEPAI in p53 wild-type OVCA 420 cells and null SKOV3 cells (Fig 3-7a). Mutant R277H p53 OVCA 432 cells demonstrated a reduced induction of TMEPAI expression. TGFβ-induced TMEPAI expression in OVCA 420 mutant p53 R273H transient cells was reduced in comparison to wild-type and null p53 cells (Fig 3-7b). Similarly, TMEPAI induction by TGFβ in SKOV3 mutant p53 R273H cells was lower than that of SKOV3 wild-type and null p53 cells (Fig 3-7c).

Lastly DKK1, a Wnt-signaling inhibitor, was selected as it is differentially regulated by wild-type and mutant p53, and is also overexpressed in late stage metastatic ovarian cancers(178). TGFβ induced expression of DKK1 in SKOV3 null p53 cells (Fig 3-7a). This
increase was not seen in wild-type OVCA 420 or mutant R277H p53 OVCA 432 cells. In OVCA 420 cells, overall levels of DKK1 were highest in OVCA 420 p53 shRNA, with a slight induction upon TGFβ treatment (Fig 3-7b). OVCA 420 mutant p53 R273H had the lowest amount of DKK1, with no induction upon TGFβ treatment (Fig 3-7b). In SKOV3 cells, the parent cell line (null p53), displayed higher DKK1 protein after TGFβ treatment as compared to the transiently transfected wild-type and mutant p53 R273H SKOV3 cells (Fig 3-7c)” (121).
Figure 3-7: TGFβ-induced expression of pro-metastatic proteins is upregulated in p53 null cells.

“(a) OVCA 420 (p53 wild-type), OVCA 432 (p53 mutant), and SKOV3 (null p53) cells were treated with 10 ng/mL TGFβ for 24 hours and analyzed by western blotting. Membranes were probed with Maspin, TMEPAI, and DKK1 primary antibodies. Actin was used as an internal loading control. (b) OVCA 420 cell lines were analyzed by western blot and probed for pro-metastatic factors TMEPAI and DKK1. Actin was used as an internal loading control. (c) SKOV3 cell lines were analyzed by western blot and probed for pro-metastatic factors TMEPAI and DKK1. SKOV3 p53 WT was transiently transfected with 100 ng/mL of p53 wild-type plasmid. Actin was used as an internal loading control” (121).
**Discussion:**

“This study investigated the influence of p53 on TGFβ-mediated proliferation and migration in ovarian cancer. Expression of p53 did not alter the ability of the ovarian cancer cells to respond to TGFβ. However, the p53 status did affect proliferation, migration, and expression of pro-invasive genes. Wild-type p53 cells underwent cell cycle arrest and displayed an inhibition of proliferation when treated with TGFβ. Loss or expression of mutant p53 abrogated TGFβ growth arrest. Interestingly, while both the OSE and TEC responded transcriptionally to TGFβ, only mouse OSE were growth inhibited by TGFβ, indicating a unique action in different potential progenitor cells. Stable integration of mutant p53 R273H mitigated TGFβ-induced migration. In correlation with these functional data, TMEPAI and DKK1 were most significantly upregulated by TGFβ in null and wild-type p53 cells, while expression of these proteins was lower in cells expressing mutant p53 R273H. Therefore, although mutant and null p53 ovarian cancer cells are not growth inhibited by TGFβ, the loss of p53 enhances migration and pro-migratory gene expression induced by TGFβ more than mutation of p53.

In breast and lung cancers, mutant p53 interacts with Smads to alter transcription of genes that regulate metastasis, but little is known about how p53 and TGFβ interact in ovarian cancer. Mutant p53 R273H is the most common p53 mutation in ovarian cancer (131), but recent evidence suggests that silencing or null mutations in p53 may be more metastatic in ovarian cancer, and that mutant p53 retains some wild-type activity (191, 192). Additionally, analyses of gene signatures from metastatic serous ovarian cancers highlighted TGFβ’s involvement in the metastatic disease (193). While breast and colon cancers undergo intra- and extravasation in order to metastasize, ovarian cancer metastasizes through direct dissemination into the peritoneal cavity (176). Therefore, signal transduction necessary for metastasis in breast, lung, and colon
cancer may be different in ovarian cancer, leading to tissue specific effects of mutant p53 and TGFβ signaling (176).

The use of TGFβ inhibitors in the treatment of ovarian cancer has been explored and is dependent on many factors (194). Based on the current study, in high-grade serous cancers, if p53 activity is lost, TGFβ inhibitors may provide greater therapeutic value than in mutant p53 R273H tumors. TGFβ inhibitors may also have a differential impact on cancers arising from the OSE or TEC, the two potential cell types of origin. While both the OSE and TEC in mouse and human cell lines respond transcriptionally to TGFβ, the OSE may respond more robustly than the TEC. In addition, MOSE were growth inhibited by TGFβ treatment, while the MTEC were not. Previous data demonstrated ovarian cancer cell metastasis is reduced in response to TGFβ inhibitors; however, the study did not control for the p53 status of the cells grafted and did not identify the cell of origin (194, 195). Several TGFβ inhibitors are currently in pre-clinical and clinical trials as cancer therapeutics (196)

TMEPAI and DKK1 induction by TGFβ was lower in ovarian cancer cells containing mutant p53 R273H, which were also less migratory. TMEPAI is associated with metastatic disease (177) and has been reported to enhance TGFβ-induced migration and an epithelial-to-mesenchymal transition (EMT) in early and late stage tumors of the breast and lung (177, 197). Additionally, the chromosomal region containing TMEPAI has been reported to be duplicated in breast and ovarian cancer(188). DKK1 expression is often upregulated in ovarian cancer and has been associated with poor outcome (178). Although the exact role of DKK1 in the aggressive nature of ovarian cancer is unknown, it has been proposed as a useful marker of disease and may be one of the many factors contributing to high-grade serous ovarian cancer (178). DKK1
induction by TGFβ was higher in cells that lacked p53 when compared to cells with mutant p53
and these cells also displayed the highest level of TGFβ-induced migration.

The early initiation and progression mechanisms of ovarian cancer are not well understood due
to the poor early detection strategies, leading to a deadly, highly aggressive disease. These data
provide some insight into the role of mutant p53 in ovarian cancer and how it intersects with the
TGFβ signaling pathway. In ovarian cancer cell lines with mutated or null p53, growth inhibition
from TGFβ is lost. Mutant p53 R273H did not induce the same pro-migratory function in
response to TGFβ in ovarian cancer as it had in breast and lung cancer cells (184). In addition,
TGFβ inhibited the growth of normal mouse OSE, but not mouse TEC. These findings suggest
that the p53 status of ovarian cancer cells influences their proliferative and migratory behavior
when exposed to TGFβ” (121).
Chapter IV: Mechanistic and functional role of PAX8 in high grade serous cancer, and its potential as a therapeutic target

Introduction

The cell of origin of serous ovarian cancer has recently come under scrutiny (99). For years, serous ovarian cancer was hypothesized to arise from the ovarian surface epithelium (OSE), a single layer of cells surrounding the ovary (16). However, recent evidence suggests that some high grade serous ovarian cancers (HGSC) may arise from the fallopian tube epithelium (FTE) (91). Understanding where ovarian cancer originates may have implications for both research and clinical efforts (16). Many pathways and genes termed dysfunctional or overexpressed in HGSC are determined by comparing tumor profiles to “normal” tissue, with the normal tissue often being OSE cells (16). For HGSC originates from the FTE, these overexpressed proteins and dysfunctional pathways may need to be revised, as they may constitute normal FTE expression. From a clinical point, many early detection methods have focused on detecting tumors while they are still confined to the ovary, before they metastasize. Tumors originating from the FTE that are detected in the ovary would have already metastasized and may not be in fact early stage disease (16). Rarely are HGSC tumors detected when they are confined to the ovary (198). Therefore, understanding both the OSE and FTE molecular profile and comparing them to HGSC tumors profiles will better our research on the disease.

Paired box 8 (PAX8) is often associated with carcinomas of the tissues in which it is expressed and it is expressed and used to classify all high-grade serous tumors. In thyroid carcinomas, PAX8 undergoes translocation with the PPARγ to create a fusion protein (199). This fusion protein can act as an oncogene, and is found in about 35% of follicular thyroid carcinomas (199). The fusion protein consists of exon 1-10 of PAX8 and exons 1-6 of PPARγ, containing
both the DNA binding domain and activation domain of both proteins (199, 200). The exact mechanism of the PAX8/PPARγ fusion protein is unknown to date; however, it may act in a dominant-negative fashion to block wild-type PPARγ tumor suppressor function. In rat thyroid epithelial cells, PAX8 increased cell survival and proliferation through transcriptional inhibition of the p53 positive regulator protein, p53inp1 (201). Knockdown of PAX8 in these epithelial cells induced p53-mediated apoptosis (201). In renal cell carcinomas (RCC), PAX8 promotes tumor growth through regulation of the E2F1-Rb pathway (202). Knockdown of PAX8 in RCC cell lines led to apoptosis through G1/S phase cell cycle arrest. PAX8 directly activated E2F1 transcription by forming a complex with Rb protein on the promoter of E2F1 to drive proliferation (202). These data indicate that PAX8 plays a critical role in cell cycle regulation and tumor survival. Despite its ubiquitous expression and role in other tumor types, almost nothing is known about what PAX8 controls in HGSC.

PAX8 expression is seen in between 80-99% of HGSCs (152, 154, 203), and is often used as a histological marker to distinguish serous ovarian cancers from other types of carcinomas (154, 203). Indeed, PAX8 and Wilms tumor protein-1 (WT1) are used in conjugation to histologically diagnose serous histotype of EOC (152, 155). Interestingly, in ovarian cancer cells molecularly classified as being HGSC, knockdown of PAX8 induced cleaved-PARP, leading to apoptosis of the cells (156). In support of this, Di Palma et al showed that knockdown of PAX8 in SKOV3 ovarian cancer cells led to a decrease in proliferation, migration and invasion (201). Additionally, SKOV3 cells with PAX8 knockdown had reduce anchorage independent growth, and suppression of tumor growth in nude mice (201). In non-transformed IOSE80 cells, expression of PAX8 induced EMT gene and protein expression, with an increase in fibronectin, twist and vimentin (201). While this data gives us an insight in the function of
PAX8 in ovarian cancer, the cellular models used are not ideal. SKOV3 cells have recently been ranked as least likely of all ovarian cancer cells to be high grade serous based on their genomic profile (86). SKOV3 cells also possess a loss of function mutation in p53, instead of the more common GOF mutations of p53 seen in HGSC (86). Similarly, the IOSE80 cells are immortalized with SV40, which precludes p53 from the system (121, 204). In addition, the authors deciphered a potential role of PAX8 in OSE cells, but not in FTE derived cells, which may be the true progenitor cell of HGSC and which normally express PAX8. Since both cell types may give rise to HGSC, understanding the function of PAX8 in non-transformed OSE and FTE cells is imperative in interpreting PAX8 mechanisms of HGSC, and its potential use as a therapeutic target. The aim of this chapter is to further decipher the functional and mechanistic actions of PAX8 in both progenitor cells.

The lack of PAX8 expression in the OSE, coupled with PAX8 expression normal FTE and malignant cells, has lead to increasing speculation that the fallopian tube epithelium is the source of HGSC. However, there are multiple experimental models of HGSC derived from both the FTE (94, 205, 206), and the OSE (84, 157, 207). The most striking thing about OSE derived HGSC models is that after transformation, they begin to express PAX8 (84, 157). Tanwar et al showed that when Pten and Lkb1 tumor suppressor proteins are lost, that PAX8 expression can be seen not only in serous tumors, but also in the OSE (157). McCloskey et al showed that a spontaneously transformed mouse OSE cell line formed tumors in xenografted mice that morphologically resembled HGSC, and had expression of both WT1 and PAX8 (84). These data suggest that PAX8 may play a role in HGSC, regardless of whether it arise from the fallopian tube or OSE. Identifying a common mechanism of tumor progression from both the OSE and FTE would greatly benefit patients by providing a novel and essential therapeutic target that
could be exploited. No mechanism to date has been suggested as to how PAX8 gets expressed in OSE derived tumors, or whether these cells are now dependent on PAX8, similar to HGSC cell lines. Additionally, it is unknown whether fallopian tube epithelium are as reliant on PAX8 expression as HGSC, and if HGSC co-opt endogenous expression of PAX8 to promote tumor progression. Elucidating a common progressive model of HGSC formation from both OSE and FTE would eliminate the need for debate on the cell of origin, and provide a clearer understanding of early stage disease as well as a viable drug target.

The role of PAX8 in both OSE and FTE was investigated in this study. Overexpression of PAX8 in a non-cancerous murine OSE cells (MOSE) increased proliferation and migration, but did not facilitate anchorage independent growth. Knockdown of PAX8 in a murine oviductal epithelial (MOE) cell line did not induce cell cycle arrest and apoptosis, but did decrease proliferation. In contrast, knockdown of PAX8 in Kuramochi cells, a HGSC cell line, induced apoptosis through cleaved-PARP. Alteration in pathways often associated with HGSC can regulate PAX8 expression. Pten$^{shRNA}$ and Kras$^{G12V}$ increased PAX8 expression in MOE, and a combination of Pten$^{shRNA}$ with either oncogenic Ras or p53$^{R273H}$ also increased PAX8 expression. Small molecules that target these pathways are already available and part of their mechanism of action may be to reduce PAX8 expression.
Results:

**PAX8 increased proliferation and migration in MOSE cells**

The OSE does not have endogenous expression of PAX8 (2), yet expression of PAX8 is seen in OSE derived serous ovarian cancer mouse models (84, 157). In order to determine if forced expression of PAX8 in the OSE is a component of tumor formation from that cell type, PAX8 was stably expressed in MOSE cells under control of a constitutently active promoter (MOSE-PAX8) (Fig 4-1a). Expression of PAX8 was confirmed via western blot analysis (Fig 4-1a). Morphology of MOSE-PAX8 cells was altered compared to MOSE-Neo, with MOSE-PAX8 cells having a more mesenchymal morphology (Fig 4-1b). MOSE-PAX8 cells showed an increase in proliferation after 8 days (Fig 4-2). Expression of PAX8 in MOSE cells also increased wound closure, suggesting an increase in migration (Fig 4-2). Five pro-migratory genes were selected for analysis to verify increased migration. Loss of E-Cadherin and increased N-Cadherin are associated with increased migration, and EMT (208). E-cadherin was not tested in this system as OSE cells lack expression of E-cadherin (209). Fibronectin, vimentin and twist are associated with both EMT and migration, and were analyzed by Di Palma et al in their study of PAX8 in IOSE 80 cells (201). Slug (SNAI2 gene) has recent been demonstrated to govern GOF mutant p53 mediated migration in MOE cells (132). N-Cadherin, fibronectin and Slug were all significantly increased in MOSE-PAX8 cells compared to MOSE-Neo control on both a protein and mRNA level. Twist and vimentin did not show any change in mRNA levels in the presence of PAX8 (Fig 4-3). These characteristics are indicative of pro-migratory cells. However, anchorage independent growth was not increased by PAX8 expression, suggesting that the cells were not transformed (Fig 4-2). MOSE-PAX8 show increased proliferation and migration; however, PAX8 is not sufficient to transform cells as evident from a lack of colonies in soft agar.
Figure 4-1: Validation of PAX8 and neomycin expression in MOSE cells
(a) Murine PAX8 cDNA under the control of a constitutently activated promoter was stably integrated into MOSE cells, and validated by western blot analysis. MOSE cells expressing neomycin resistance gene was use as a control (MOSE-Neo). (b) Morphology of MOSE-Neo and MOSE-PAX8
Figure 4-2: MOSE-PAX8 cells show increased proliferation and migration, but not transformation.

(a) MOSE cells expressing PAX8 proliferated more than control cells not expressing PAX8. Unpaired student t-test was used to determine significance. Data represented as mean ± SEM, *p≤0.05. (b) MOSE cells expressing PAX8 demonstrated a significant increase in wound closure, an indication of increased migration. Significance determined by two-way Anova analysis. Data represented as mean ± SEM, *p≤0.05. (c) MOSE-PAX8 cells did not form colonies in soft agar, indicating that they are not transformed. MOSE cells stably expressing SV40-T antigen was used as a positive control and previously characterized by Dr. Shelby King (48). Each experiment was performed in triplicate. Unpaired student t-test was used to determine significance. Data represented as mean ± SEM, *p≤0.05.
Figure 4-3: MOSE-PAX8 cells have increased levels of pro-migratory genes

(a) Western blot analyses of MOSE-Neo and MOSE-PAX8 for pro-migratory proteins. MOSE-PAX8 cells expressed more N-cadherin, fibronectin and slug. Actin was used as a loading control. (b) mRNA expression analysis of MOSE-PAX8 compared to MOSE-Neo. ΔΔCt method between MOSE-Neo and MOSE-PAX8 was used to determine fold change of MOSE-PAX8 mRNA. MOSE-Neo expression is represented as 1, while MOSE-PAX8 is fold change compared to MOSE-Neo. Each experiment was performed in triplicate. 18s rRNA was used as an internal control (housekeeping) gene. Unpaired student t-test was used to determine significance. Data represented as mean ± SEM, *p≤0.05
**Knockdown of PAX8 in MOE cells does not induce apoptosis, but leads to cell cycle arrest**

When PAX8 is overexpressed exogenously in a murine ovarian surface cell line, cells gain additional proliferation and migratory phenotypes. Additionally, previous studies determined that knockdown of PAX8 in HGSC cell lines lead to apoptosis through cleaved-PARP (156). However, no studies have explored the role of PAX8 or the impact of reducing its expression in normal FTE or oviductal cells. In order to test this, the function of PAX8 in cells that have endogenous expression of PAX8 (MOE) was investigated. Three clones of MOE cells stably expressing PAX8 shRNA were created, and validated by western blot and mRNA analysis (Fig 4-4). MOE PAX8 shRNA cells were analyzed, and no significant change in proliferation was detected (Fig 4-4). Next, 3 genes known to be positively regulated by PAX8 in thyroid and kidney cells were analyzed to determine if they were regulated similarly in MOE cells (152, 202, 210). qPCR of WT-1, E2F1, and BRCA1 indicated that they were all significantly decreased in MOE PAX8 shRNA expressing cells (fig 4-4), suggesting that they are regulated in a similar manner in the oviduct, kidney and thyroid.

Upon continuous culturing of MOE PAX8 shRNA cells, PAX8 expression returned to normal levels, and it seemed the cells circumvented the shRNA knockdown (Fig 4-5). Two additional clones of MOE PAX8 shRNA were selected that were stably transfected with PAX8 shRNA, but after several passages, expression of PAX8 returned again. The inability to permanently knockdown PAX8 meant it was not possible to proceed further with this analysis. To circumvent this issue, CRISPR/Cas9 genomic editing was performed to knockout PAX8 expression (211). A gRNA targeting exon 2 of PAX8 was designed and cloned into the pX260 CRISPR/Cas9 nuclease plasmid. CRISPR/Cas9 pX260 plasmid creates a double-stranded break within the gRNA sequence cloned into the plasmid. This double stranded break leads to indels of
various sizes, ranging from 1 base pair to multiple bases. Exon 2 of PAX8 was chosen as it would knockout all known variants of PAX8. Stable transfection of the CRISPR/Cas9 containing a gRNA against PAX8 exon 2 showed a reduction of PAX8 expression in three clones, but not a complete knockout of the protein (Fig 4-5). Sequencing confirmed that there was a heterozygous knockout of PAX8, with one allele possessing an indel of roughly 2-5 bps in clone #3 (Fig 4-6). In order to get a complete knockout, the CRISPR/Cas9 with the PAX8 gRNA was re-transfected in MOE cells, but only heterozygous clones were obtained. Additionally, upon continuous passaging, the wild-type allele of PAX8 compensated for the loss of one allele, and PAX8 expression returned to normal similar as to shRNA.

To try and completely knockout PAX8 in MOE cells, an alternative approach was taken. A new MOE cell line was created from a novel mouse model, where one allele of PAX8 was replaced by Cre-recombinase (CRE) (164). The PAX8 promoter drives expression of CRE, meaning that MOE cells generated from this mouse will only possess one functional allele of PAX8, and thus it might be easier to completely knockout the remaining PAX8 allele. This new MOE PAX8-Cre cell line was validated as being epithelial with expression of CK8, of oviductal origin with expression of OVGP-1, and possessing both ciliated and secretory cells as shown by expression of both PAX8 and acetylated tubulin respectively (Fig 4-7). In addition, two extra gRNA’s targeted at PAX8 (exon 4 and exon 7) were designed and cloned into the pX260 vector. All three gRNA’s (targeting exons 2, 4, and 7) were transfected into MOE PAX8-Cre cells and selected using the same concentration of puromycin as previously used for other MOE stables. Surprisingly, no clones survived selection after transfection of all three gRNA’s. The experiment was repeated, but no clones survived second time around, suggesting that loss of PAX8 in MOE cells may be lethal. This agrees with animal models, as homozygous loss of PAX8 leads to
infertility due to lack Müllerian organ development (150). Additional controls, such as expression of these gRNAs in MOSE cells or MCF7 cells that do not express PAX8 and are not reliant on its expression to survive must be performed to make conclusions regarding this experiment.
Figure 4-4: Knockdown of PAX8 in MOE cells does not alter proliferation
(a) Western blot analysis of MOE-PAX8shRNA demonstrating a knockdown of PAX8 protein. (b) mRNA of PAX8 in MOE-PAX8shRNA demonstrating a reduction in transcript from shRNA. (c) mRNA expression of known PAX8 target genes from studies performed in kidney and thyroid cells. mRNA expression was reduced in shRNA clones with reduced PAX8.
Cas9 is a nuclease enzyme derived from the microbial clustered regulatory interspaced short palindromic repeats (CRISPR) adaptive immune system. CRISPR are short sequences of DNA present in prokaryotic cells, derived from exposure to bacteria, viruses and plasmids infections. These sequences are used as part of the prokaryotic adaptive immune system to identify future infections. Upon recognition of exogenous DNA corresponding to a CRISPR, the Cas9 enzyme is guided to degrade the foreign DNA. In eukaryotic cells, the CRISPR/Cas9 system can be utilized to precisely edit a given gene. A guide RNA (gRNA) is designed corresponding to the gene to be edited. The gRNA associates with the Cas9 enzyme and guides it to the gene in question. Once a match for the gRNA is found, the Cas9 nuclease performs a double-stranded cut at the site. This DNA damage can then be repaired in one of two ways: Non-homologous end-joining (NHEJ) or Homology directed repair (HDR). NHEJ repair can lead to mistakes being introduced into the DNA called indel mutations. Indels are INsertion or DELetion of nucleotides from the original sequence. These indels can range in size from 1 nucleotide to >50 nucleotides in size. Indels can disrupt protein transcription through addition of premature stop codons, or through altering the amino acid frame during translation. Reprinted by permission from Macmillan publishers Ltd: Nature Protocols (217)1010, copyright 2013. 

http://www.nature.com/nature/index.html
Figure 4-5: MOE PAX8<sub>shRNA</sub> cells re-express PAX8.

(a) Western blot analysis of MOE PAX8<sub>shRNA</sub>. MOE PAX8<sub>shRNA</sub> cells continuously passaged for 4 passages until expression of PAX8 returned. (b) Western blot analysis of CRISPR clones shows a decrease in PAX8 protein level after stable transfection with CRISPR/Cas9 plasmid and PAX8 gRNA in clones #3, #4 and #6. Actin was used as a loading control.
Figure 4-6: Confirmation of heterozygous deletion of PAX8.

(a) Chromatogram of MOE PAX8 CRISPR sequence of the area where the gRNA was targeted. Reading output dropped off at the start of the gRNA sequence (5'-CCCGGG-3', with CCG being the PAM sequence) due to multiple sequences because of the heterozygous knockout. (b) PAX8 sequence in wild-type MOE cells, and MOE cells stably transfected with CRISPR/Cas9 plasmid and PAX8 gRNA. Roughly 2-5 bps (designated as 3 bps here) were deleted from one allele of PAX8 of clone A.
Figure 4-7: Characterization of MOE-PAX8 Cre cell line.

MOE-PAX8 Cre recombinase cell line was created from a mouse possessing a knock-in of Cre-recombinase into one of the PAX8 loci. The Cre-recombinase gene is driven by the PAX8 promoter. Every cell in the murine model possesses one functional copy of the PAX8 gene. Staining for CK8, PAX8, OVGP-1 and Acetylated-tubulin (Ac-tub) confirm that the cell line is of oviductal origin.
Knockdown of PAX8 does not induce cell cycle arrest or apoptosis in MOE cells

Based on the data that MOE PAX8\textsuperscript{shRNA} and MOE PAX8\textsuperscript{CRISPR} failed to demonstrate consistent loss of PAX8 expression after creation of a stable cell line, it was hypothesized that PAX8 may be essential for MOE cell survival. A siRNA was used to transiently knockdown PAX8 in MOE cells and lysates were subjected to western blotting and probed for cleaved-PARP. Previously, HGSC cell lines, of unknown origin, showed induction of cleaved PARP when PAX8 was knocked down (156). No increase in cleaved-PARP was seen in MOE-PAX8 siRNA expressing cells, suggesting that knock down of PAX8 in MOE cells did not induce apoptosis (Fig 4-8a). Knockdown of PAX8 in Kuramochi cells, a human HGSC cell line, was next investigated. Increased cleavage of PARP was seen when PAX8 was knocked down, indicating apoptosis similar to previous reports in OVCAR3 (Fig 4-8b). Previous reports have shown that knockdown of PAX8 in thyroid and renal cancer cell lines led to cell cycle arrest in the G1 phase of the cell cycle (202). Therefore, to analyze the effect of PAX8 knockdown on cell cycle in MOE cells, MOE PAX8 siRNA were subjected to flow cytometry after propidium iodide staining (Fig 4-8c). MOE PAX8 siRNA cells as compared to luciferase targeted negative control did not exhibit a change in the percentage of cells at any phase of the cell cycle, suggesting no change in cell cycle. Additionally, no increase was seen in sub G0 peaks, suggesting no induction of apoptosis (Fig 4-8c). Due to the lack of apoptosis and cell cycle arrest and apoptosis seen in MOE cells, the effect of PAX8 on proliferation was analyzed (Fig 4-8d). Knockdown of PAX8 in MOE cells slightly decreased proliferation compared to negative control (Fig 4-8d). These data indicate that knockdown of PAX8 in MOE cells does not induce either apoptosis or cell cycle arrest, but does decrease proliferation.
Figure 4-8: Knockdown of PAX8 does not induce apoptosis or cell cycle arrest
(a) MOE cells transiently transfected with 200 ng/mL of siRNA for luciferase (control) or PAX8 for 72 hours. No change was seen in levels of cleaved-PARP upon downregulation of PAX8 in MOE. Actin was used as a loading control. (b) High-grade serous ovarian cancer cell line Kuramochi was transiently transfected with shRNA for PAX8 for 96 hours. Increased levels of cleaved-PARP indicate that HGSC cells undergo apoptosis when PAX8 is knocked down. Vinculin was used as a loading control. (c) Cell cycle analysis of MOE cells with siRNA for luciferase or PAX8. No change was seen in percent cells at any stage of the cell cycle, suggesting no cell cycle arrest occurred. (d) Proliferation of MOE cells treated with siRNA for PAX8 was decreased compared to control MOE cells treated with siRNA for luciferase.
Pathways associated with HGSC alter PAX8 expression in MOE cells

Given the high expression levels of PAX8 in HGSC, and that PAX8 can be turned on in transformed OSE cells, our aim was to investigate whether pathways associated with HGSC can alter PAX8 expression in MOE cells (Fig 4-9) (157, 203). We hypothesized that pathways associated with HGSC might increase or enhance PAX8 expression and create a reliance on this protein such that in tumor cells its reduction can trigger apoptosis. A panel of MOE cell lines containing pathways known to be altered in human HGSC was generated (212). These alterations include Pten\textsuperscript{shRNA}, p53\textsuperscript{R273H}, constitutively activated Akt (Akt\textsuperscript{MYR}) and oncogenic Ras (Ras\textsuperscript{G12V}) (Fig 4-10) and their various combinations. Levels of PAX8 protein and mRNA were analyzed in all cell lines (Fig 4-10). MOE-Ras\textsuperscript{G12V} and MOE-Pten\textsuperscript{shRNA} had increased levels of PAX8 protein and mRNA compared to the scrambled\textsuperscript{shRNA} and wild-type MOE cells control (Fig 4-10 a and b). Interestingly, both GOF p53\textsuperscript{R273H} and Akt\textsuperscript{MYR} decreased levels of PAX8 as compared to wild-type MOE cells control (Fig 4-10 a and b). MOE-Pten\textsuperscript{shRNA} was combined with MOE-Ras\textsuperscript{G12V}, p53\textsuperscript{R273H}, and MOE-Akt\textsuperscript{MYR}. MOE-p53\textsuperscript{R273H} cells were also combined with Ras\textsuperscript{G12V}. All combinations of altered pathways in MOE cells demonstrated significant increase in PAX8 mRNA (Fig 4-11). However, only MOE-p53\textsuperscript{R273H}/Ras\textsuperscript{G12V} and MOE-Pten\textsuperscript{shRNA}/p53\textsuperscript{R273H} exhibited significantly higher levels of PAX8 protein (Fig 4-11). Interestingly, a combination of Pten\textsuperscript{shRNA}, Ras\textsuperscript{G12V}, and Akt\textsuperscript{MYR} showed no change in mRNA and slightly decreased protein expression (Fig 4-11). Certain pathways associated with HGSC can alter expression of PAX8.

In murine OSE derived HGSC tumors, PAX8 expression can be detected (Fig 4-12). A double knockout of Pten and LKB1 induced serous ovarian cancer derived from the OSE, with strong expression of PAX8 (157). LKB1 knockout also showed expression of PAX8, but at a
lower level (Fig 4-12). Additionally, LKB1 knockout alones developed tumors at a slower rate (157). These data shows that several pathways associated with HGSC can alter PAX8 expression regardless of the cell of origin, indicating that PAX8 may be an essential protein expressed during the formation of HGSC from both the OSE and fallopian tube.
Figure 4-9: Schematic of the Ras/PI3K/Akt oncogenic pathway

Upon activation of Ras by growth factors or activating mutations, it propagates its signal through two mechanisms: PI3K pathway and MAPK/Erk pathway. In the PI3K pathway, Ras activates phosphoinositide 3-kinase (PI3K). PI3K can be deactivated by phosphatase and tensin kinase (PTEN). PTEN is lost in many cancers, including ovarian cancer. PI3K then phosphorylates protein kinase B (Akt), which goes on to promote a pro-survival/proliferative signal. In the MAPK/Erk pathway, Ras activates downstream Raf kinases, which then phosphorylates mitogen activated protein kinase kinase 1 and 2 (MEK1/2). MEK1/2 further phosphorylates mitogen activated protein kinase (Erk), which promotes proliferation/survival and migration.
Figure 4-10: PAX8 expression is altered by pathways associated with HGSC

(a) Western blot analysis of PAX8 expression in MOE stable cell lines. MOE wild-type and scrambled shRNA are used as controls. Actin is used as a loading control. (b) mRNA analysis of PAX8 expression. Relative fold change is represented in log form. 18S rRNA was used as a housekeeping gene. Data represented as mean ± SEM, *p≤0.05
Figure 4-11: PAX8 expression is altered by pathways associated with HGSC

(a) Western blot analysis of PAX8 expression in MOE stable cell lines. MOE wild-type and scrambled shRNA are used as controls. Actin is used as a loading control. (b) mRNA analysis of PAX8 expression. Relative fold change is represented in log form. 18S rRNA was used as a housekeeping gene. Data represented as mean ± SEM, *p ≤ 0.05
Murine OSE cells with knockout of LKB1 and Pten were created from a murine model of serous ovarian cancer derived from the OSE. The cell lines were kindly donated to us by Dr. Teixeira from Michigan State University. Both MOSE LKB1$^{-/-}$ and MOSE Pten$^{-/-}$/LKB1$^{-/-}$ had expression of PAX8, with the double knockout expressing higher levels.
Discussion:

This project evaluated the functional role of PAX8 in MOE and MOSE. Expression of PAX8 in MOSE cells increased pro-cancerous functions including proliferation and migration. However, PAX8 was unable to enhance soft agar colony formation of MOE cells demonstrating a lack of transformation by PAX8 alone. In addition, increased levels of pro-migratory proteins N-cadherin, fibronectin and slug were seen in MOSE-PAX8 cells. MOE-PAX8shRNA cells did not proliferate differently than control, however, expression of PAX8 returned after passaging of the cells, suggesting that they became resistant to the shRNA and have a growth advantage due to retention of PAX8. Attempts to use the CRISPR/Cas9 genomic editing system to knockout PAX8 failed, as all clones were either heterozygous, or died during selection. An additional MOE cell line containing only one functional allele of PAX8, MOE PAX8-Cre, was created to circumvent this issue. All clones of MOE PAX8-Cre stably transfected with CRISPR/Cas9 also died during selection. Transient knockdown of PAX8 using siRNA in MOE cells did not induce apoptosis or cell cycle arrest, but did decrease proliferation. Transient knockdown of PAX8 in Kuramochi cells induced apoptosis through cleaved-PARP. Pathways associated with HGSC in MOE cells modify PAX8 expression. Oncogenic Ras (G12V) and PtenshRNA increased both mRNA and protein levels of PAX8, while p53R273H and Aktmyr decreased levels. Combinations of these pathways also increased both mRNA and protein levels of PAX8. Together, this data shows that PAX8 can modify and control many functional characteristics associated with HGSC, and provides a potential common mechanism of progression of HGSC from both the OSE and FTE.

OSE do not endogenously express PAX8. However, expression of PAX8 is seen in OSE-derived HGSCs (84, 157). PAX8 is known to be involved in cancers derived from cells
endogenously expressing PAX8, however, expression of PAX8 has also been reported in cancers derived from cells that do not endogenously express PAX8 (213). This is similar to OSE-derived tumors, and demonstrates the ability of a tumor to co-opt the expression of PAX8. Interestingly, ovarian inclusion cysts lined with OSE cells lose mesenchymal markers and begin to express epithelial markers such PAX8 (55). MOSE cells expressing PAX8 also under a morphological change, suggesting that stimulation of PAX8 expression in OSE cells could be an important step in progression and possibly transformation. In agreement with this, transgenic mice expressing SMAD2 dominant negative alleles (SMAD2 DN) in the OSE, form epithelial inclusion cysts that do not progress to serous tumor formation (83). These inclusion cysts do not express PAX8 in the epithelial cells lining the cyst (83). PAX8 expression may be required for the SMAD2DN inclusion cysts to progress and form serous ovarian cancer.

Previous work showed that PAX8 expression in human OSE cells had increased levels of certain pro-migratory genes, but they did not investigate migration itself (214). The authors overexpressed PAX8 in a human OSE cell line immortalized with SV40 virus. However, this precludes both p53 and Rb proteins from the experimental system. A GOF mutation in p53, and not loss of p53, is more often seen in HGSC. Our data demonstrates that GOF mutations in p53 repress PAX8 protein expression. Additionally, Rb protein interacts with PAX8 to regulate tumor cell growth, justifying the need to study PAX8 in murine OSE cells (202). Our study uses non-cancerous cells that have not been immortalized with SV40 to study PAX8 activity in OSE cells. Data from MOSE-PAX8 cells concurred with previous studies that pro-migratory genes were increased as well as migration. *Di Palma et al* conclude that PAX8 in OSE cells induces EMT, however OSE cells do not express E-cadherin, which is often lost during EMT. Moreover, expression of N-cadherin, which is upregulated during EMT, was not reported by *Di Palma et al.*
Data from our study found an increase in N-cadherin, while also showing both morphological and functional changes in migration in MOSE-PAX8 cells, demonstrating the power of this experimental model. Additionally, previous studies have demonstrated that PAX8 upregulates Wnt4 in the thyroid (215). Wnt4 is known regulator of cell migration (215), and blocks EMT in squamous cell carcinomas types (216). This delicate balance of PAX8 induced migration, and potential maintenance of epithelial phenotype by Wnt4, could explain the unique characteristics seen in epithelial inclusion cysts. Moreover, Wnt4 is known to play a role in the development of the oviduct and ovarian carcinogenesis in the laying hen model, further indicating a common mechanism of carcinogenesis through PAX8 (217). Further studies need to be performed to understand the function of PAX8 is OSE cells, and its role in tumorigenicity.

Complete knockout of PAX8 in murine oviductal cells (MOE) was not achieved, as it appeared the cells died, although more work must to done to make a firm conclusion. In PAX8 knockout animals (150), Müllerian structures fail to develop properly, leading to infertility of the mice, demonstrating that PAX8 is essential for the development of the oviduct. Non-cancerous MOE cells with knockdown of PAX8, but not knockout, were viable, yet their proliferation was decreased. Additionally, heterozygous loss of PAX8 was also viable, as was evident from PAX8-Cre animals and CRISPR/Cas9 PAX8 heterozygous knockout cells. This is in contrast to HGSC cell lines, where knockdown of PAX8 lead to apoptosis through cleaved-PARP. This is further evidence that HGSC likely becomes more reliant on PAX8 as the cancer progresses, and creates speculation that a common mechanism of progression involving PAX8 may occur from both OSE and MOE. This phenomenon may be unique to HGSC, as Di Palma et al did not report induction of apoptosis when PAX8 was knocked down in SKOV3 cells. SKOV3 cells have been recently designated as not likely to be HGSC, based on their genomic profile (218). Further
studies to understand the co-opting of PAX8 by HGSC from the fallopian tube needs to be done to fully comprehend this mechanism.

Extensive research has been done on the regulation of PAX8 expression in the thyroid by thyroid-specific transcription factors; however, little is known about what regulates PAX8 outside the thyroid (219, 220). Functional analysis of the murine PAX8 identified that PAX8 regulates its own promoter, along with other thyroid specific factors. Further studies demonstrate that PAX8 is regulated by thyroid specific hormone (TSH) and β-human chorionic gonadotrophin (hCG) in thyroid cells (221, 222). Interestingly, hCG is known to upregulate LH receptor in ovarian epithelial cells (223). LH has previously been shown to increase proliferation and soft agar colony formation in murine OSE cells. LH activates oncogenic pathways associated with HGSC in OSE cells, such as Akt and MAPK (73). LH signaling also induces similar pathways, such as p-ERK1/2 signaling, in immortalized baboon oviduct cells (30). This study is the first to identify that many of these oncogenic pathways modify PAX8 expression in oviductal cells. Of particular interest is the fact that PAX8 expression increases in oviductal cells when multiple pathways associated with HGSC, indicating an increased role of PAX8 in the progression of HGSC. In OSE cells, loss of Pten and LKB1 induced expression of PAX8, as did serial passaging of OSE cells (84, 157). However, an exact mechanism for the acquired expression of PAX8 in these experimental systems is unknown. By understanding which pathways modify PAX8 expression in HGSC precursor cells will aid in the development of a HTS to find inhibitors of PAX8 transcription.

PAX8 plays a significant role in HGSC and is expressed consistently in almost 85% of all tumors, and these data suggest that it may contribute to a common mechanism of progression from both the OSE and FTE. This study has helped to decipher the functional role of PAX8 in
non-cancerous cells, and how it may contribute to HGSC. This data suggests that HGSC may progress from a common mechanism regardless of cell of origin, and provides a therapeutic target to treat all HGSCs. Further studies need to be performed to fully understand the oncogenic role of PAX8
Chapter V: Identification of novel therapeutics from aquatic derived *Actinomycetes* through bioassay-guided fractionation

*Introduction:*

Epithelial ovarian cancer is an aggressive disease with a high rate of therapeutic resistance, specifically chemoresistance (46, 47). Ovarian cancer is frequently treated with carboplatin and paclitaxel (46, 47, 194). Unfortunately for most women, ~70% of ovarian cancers reoccur and are chemoresistant (46, 47). Cisplatin-based drugs target many pathways including AKT, death receptor mediated apoptosis, and p53 induced apoptosis (224). Indeed, many cisplatin drugs upregulate p53 in an effort to induce apoptosis (224). Cisplatin-based treatment selects for cancer cells deficient in p53 mediated apoptosis via mutations to *TP53*, thereby circumventing cisplatin induced apoptosis and leading to a more aggressive malignant cancer (225, 226). Therefore, it is not surprising that HGSC has a high level of chemoresistance, given the considerable number of p53 mutations associated with the disease (96-100% of cases) (44). New drugs are desperately needed to allow for effective treatment of relapsed HGSC.

The National Cancer Institute (NCI) 60 anticancer drug screen began in the early 1980’s with the goal of improving clinical activity of anti-cancer compounds (160). Previous screening models utilized murine models, specifically xenografts, to identify compounds (160). However, these methods were expensive and relatively inaccurate. Often times, drugs lacked either specificity or toxicity once they reached human cells (160, 227). The NCI developed a panel of cell lines, representing the majority of solid tumors seen in patients, to screen any potential therapeutic compounds (160). These cell lines included tumors originating from colon, lung, ovarian, CNS, leukemia, breast, prostate, renal and melanoma (160). To date, the majority of FDA approved anticancer drugs have been screened multiple times in the NCI-60 panel (227).
The assay has been hugely successful, for example, it identified drugs such as Bortezomib (multiple myeloma) and Paclitaxel (ovarian, breast and lung) (227, 228). While the NCI-60 is useful in determining toxicity of pure compounds, separation and isolation of active compounds from metabolite extracts needs to be performed prior to screening. Additionally, the NCI-60 panel does not compare toxicity of normal cells to their cancer cell counterparts and therefore may provide rationale for using certain molecules in distinct tumor types but not necessarily based on sparing toxic effects to normal cells. Therefore, bioassay guided fractionation of metabolite extracts needs to be performed, where the active compound is identified from a complex mixture of extracts through screening for activity followed by separation of active fractions.

Actinomycetes are a gram-positive bacteria that contain a high GC nucleotide percentage and their natural habitat is both terrestrial and aquatic (229). Approximately 23,000 bioactive secondary metabolites have been discovered from microorganisms to date, with 10,000 of them being produced by actinomycetes (230). While terrestrial actinomycetes have been mined for their anti-bacterial and anti-cancer activity for generations (231), identification of new scaffolds has slowed due to continuous rediscovery of known agents (232). Recently, the diversity of actinomycetes obtained from aquatic sources has been realized, and these microorganisms are now an intense area of investigation (231). These aquatic actinomycetes are capable of producing new classes of chemical structures due a different set of selection pressures in the oceans and lakes that impact the genetic drift of their genome to produce diversity(231). Because marine and fresh water actinomycetes encountered eukaryotic cells in their microenvironment under these extreme selective pressures, evolution has allowed for the selection of strains producing novel potent chemicals to attack eukaryotic organisms in order to survive (233).
While marine actinomycetes are under-studied for the production of cytotoxic metabolites, many novel and intricate compounds have been isolated from other marine organisms (234). The first marine derived natural compound to receive FDA approval was ω-conotoxin MVIIA, isolated from a fish hunting cone snail (234). The compound ω-conotoxin MVIIA, now known under the trade name Prialt, is indicated for chronic pain as a neuromuscular inhibitor and is more potent than morphine (234, 235). The first marine derived anti-cancer drug approved by the FDA was Ecteinascidin-743, originally isolated from a sea squirt (234). Under the trade name Trabectedin, it is indicated for the treatment of soft tissue sarcoma. Given that some marine actinomycetes species dedicate up to 10% genome to secondary metabolite production, we hypothesize that they may possess a wealth of cytotoxic anticancer compounds (236). Indeed, marine actinomycetes have a wide range of diversity among species (237). In the past several years, novel anticancer compounds have been isolated from marine actinomycetes, but few have reached the clinic. Their activity ranges from proteasome inhibitors to chemopreventative agents (237-239). However, only a small population of diverse marine actinomycetes have been assessed for the secondary metabolite production (237). Therefore, further study of these diverse organisms may find new novel anticancer compounds.

The aim of this study is to identify potential novel therapeutics by performing bioassay guided fractionation of actinomycete secondary metabolite fraction libraries in collaboration with Dr. Brian Murphy’s lab. By performing bioassay-guided fractionation of actinomycete fractions, novel compounds can be identified based on activity instead of chemical class or structure. Multiple different cell lines were used to determine cytotoxicity, including genomically validated HGSC cell lines (OVCAR4 and Kuramochi), standard NCI-60 ovarian cancer cells (SKOV3 and OVCAR5), and non-cancerous p53 wild-type cells (MOSE and MOE). OVCAR5 and SKOV3
cells have been in use by the NCI-60 since its foundation in the 1980s, and much work has been
done on anticancer drug mechanisms in these cell lines. By screening compounds in these cells,
mechanistic analysis can be compared to the vast NCI-60 database available. OVCAR4 and
Kuramochi cells have recently been validated as highly similar to HGSC tumor profiles (218),
and provides an accurate tumor model of HGSC. OVCAR5 and SKOV3 cells are null for p53,
mimicking loss of p53 pathway seen in many chemoresistance cancers, while OVCAR4 and
Kuramochi cells posses GOF mutations in p53, mimicking the mutations most often seen in
HGSC. In addition to these cell lines, compounds were screened in non-cancerous primary
murine cell lines (MOSE and MOE). These cell lines represent the “normal” control to screen for
selective cytotoxicity, and posses no known mutations in p53. All fraction library fractions
collected and processed by Dr. Murphy’s lab were screened for cytotoxicity, and positive hits
were further analyzed to isolate and identify the active molecule.
Results:

Screening of marine actinomycete fraction libraries

The goal of this study is to identify novel molecules that may be used as a potential therapeutic in ovarian cancer. Aquatic actinomycetes are collected from around the world, and secondary metabolites isolated and screened for cytotoxicity (Fig 5-1). Dr. Murphy and his lab collected aquatic actinomycete bacteria from around the world and made fraction libraries of the secondary metabolites. A total of 1536 fractions, from 16 fraction plates, each containing 96 individual fractions, were screened in OVCAR5, SKOV3, MOE and MOSE lines. Cytotoxicity was determined by less than 50% percent survival at 10 µg/mL (Table S5-1). OVCAR5 cells showed cytotoxicity in 24% of fractions, with SKOV3 showing cytotoxicity in 12% of fractions. Several fractions showed differential or selective cytotoxicity against OVCAR5 and SKOV3 compared to MOE and MOSE cells (under 50% in cancer cells, over 50% in non-cancerous cells) (Table S5-1). Fractions that were selected for further analysis were then screened in the HGSC tumor model cell lines, Kuramochi and OVCAR4.
Figure 5-1: Collection, isolation, fractionation and testing of aquatic actinomycete secondary metabolites  
(a) Samples are collected from around the world by SCUBA diving or by PONAR and brought back to the lab.  
(b) Collections are grown up and actinomycete bacteria are isolated from the samples.  
(c) Once actinomycetes are isolated, they are grown up individually in large cultures, filtered and dried in order to collect secreted molecules (secondary metabolites).  
(d) The secondary metabolites are re-suspended and fractioned into four initial fractions for each strain and placed in a 96 well plate.  
(e) Fraction libraries are screened against ovarian cancer cell lines for cytotoxic activity. Cytotoxic fractions undergo further isolation to yield the active molecule
Bioassay guided fractionation of anticancer compounds:

Disclaimer: All fractions that showed selective cytotoxicity were projects performed in collaboration with Dr. Brian Murphy and Vanessa Nepomuceno in Dr. Murphy’s lab. All fractionation, fractionation trees and structure elucidation were performed by Vanessa Nepomuceno.

From our initial screening of the fraction library, we chose fractions that showed selective cytotoxicity to perform bioassay-guided fractionations on (Table S5-1). Selective cytotoxicity is defined by having cytotoxic activity (percent survival of under 50%) in the EOC cell lines at a given concentration (10 µg/mL in initial fraction plate screening), while showing lower levels of cytotoxicity in our non-cancerous cell lines (percent survival of over 50%). Fractions displaying selective cytotoxicity in ovarian cancer cell lines were given preference. Dereplication of fractions identified potential novel molecules, and these samples were then ranked based on both cytotoxicity and novelty (Table S5-2). After dereplication (performed by Dr. Murphy’s lab), these strains were regrown in larger quantities and re-fractionated to identify the active compound.

Strain K011 was isolated from a strain collected in Iceland. From the fraction library screening, fraction 3 of K011 showed selective cytotoxicity and was subject to bioassay-guided fractionation. A large scale re-grow was performed of strain K011 in 30 liters, in order to obtain large quantities of the fraction for isolation and structure elucidation. The media containing the secondary metabolite compounds was fractionated into 13 fractions (Fig 5-2). All fractionations were performed by Vanessa Nepomuceno in Dr. Murphy’s lab. The 13 fractions were screened for cytotoxic activity in our cancer cell lines, and non-cancerous cell lines (Fig 5-3a). For K011, toxicity in OVCAR4 and Kuramochi cell lines was a focus due to their genetic similarities to HGSC (218). During the initial screening, fractions were tested in dose response starting at 100 µg/mL, and decreasing 2 fold each well to 0.78 µg/mL. Fractions 1 through 8 did not show any
cytotoxicity. However, fractions 9 through 13 showed cytotoxicity of under 0.78 µg/mL (Fig 5-3a). Specifically, fractions 10-13 showed differential cytotoxicity (Fig 5-3a). After analysis of the fractions, it was determined that the majority of the active compound was in fraction 9. Therefore, a dose response of fraction 9 was performed at doses lower than 0.78 µg/mL (Fig 5-3b). As expected, K011 F9 showed selective cytotoxicity against the ovarian cancer cell lines (Fig 5-3b). Further fractionation of K001 F9 was performed by Dr. Murphy’s lab, to produce 4 additional fractions (K011 fraction 9-F1 through F4). After dose response, K011 fraction 9-F3 showed activity. LC$_{50}$ activity was determined for all 4 cell lines: OVCAR4 - 0.55µg/mL, Kuramochi - 0.22µg/mL, MOSE – 1.18µg/mL, and MOE – 1.17µg/mL. Upon dereplication of K011 fraction-F3, the active compound was identified as mithramycin, an already known and classified anti-cancer drug (240). These data proves that successful bioassay guided fractionation can be performed to identify anticancer compounds from aquatic actinomycetes.
Figure 5-2: Detailed fraction tree of strain K011

Fractionation tree: All fractionations and dereplications were carried out by Vanessa Nepomuceno. K011 was grown up in 30L and initially fractionated into 13 separate fractions. From this initial fractionation, F9 showed differential cytotoxicity against our cancer cells. F9 was further fractionated into 4 fractions, with 9-3 showing activity.
Figure 5-3: K011 F9-13 show cytotoxicity in EOC cell lines.

(a) LC\textsubscript{50} values of fractions 9 through 13 of K011 in all four cell lines. Dose response was performed at a concentration 100 \(\mu\)g/mL, 50 \(\mu\)g/mL, 25 \(\mu\)g/mL, 12.5 \(\mu\)g/mL, 6.25 \(\mu\)g/mL, 3.125 \(\mu\)g/mL, 1.56 \(\mu\)g/mL, and 0.78 \(\mu\)g/mL. Prism GraphPad was used to determine LC\textsubscript{50} values (b) Dose response curve of fraction 9, which was chosen for further isolation and analysis.
**Diazaquinomycin, isolated through bioassay guided fractionation, shows cytotoxicity in OVCAR5 cells**

“The following section, Diazaquinomycin shows cytotoxicity in OVCAR5 cells, is published in the journal Marine drugs, under which I am an author (167). All data, including figures and tables, have been reproduced under the Creative commons Attribution 4.0 international license for the purpose of this thesis. This work is licensed under the Creative Commons Attribution 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/ or send a letter to Creative Commons, PO Box 1866, Mountain View, CA 94042, USA.”

Strain F001 was obtained from sediment collected from a protected reef off the coast of Kri, Raja Ampat, Birds Head, Papua, Indonesia (S 033’54.72 E 130 40’35.04) using SCUBA at a depth of 9 meters. Based on the fraction plate screening, and successful bioassay-guided fractionation of mithramycin from K011, strain F001-F1 to F3 was chosen for bioassay guided fractionation of the active molecule. “A screening of our aquatic-derived actinomycete fraction library against a cisplatin-resistant ovarian cancer cell line (OVCAR5) led to the isolation of novel diaza-anthracene antibiotic diazaquinomycin E (DAQE; 1), the isomeric mixture of diazaquinomycin F (DAQF; 2) and diazaquinomycin G (DAQG; 3), and known analog diazaquinomycin A (DAQA; 4)” (Michael Mullowney performed isolation and structure elucidation, Dr. Brian Murphy’s lab) (Fig 5-4). Compounds 1 and 4 were tested for in vitro cytotoxicity against the ovarian cancer cell line OVCAR5. Dose response analysis of the isolated compounds revealed an LC50 of 9.0 µM for 1 and 8.8 µM for 4 after treatment of cells for 96 h” (Fig 5-5a) (167). After 72h treatment of additional cell lines, compound 1 had LC50 values of 4.3µM and 9.4µM in OVCAR4 and Kuramochi respectively, and 22µM and >28µM in MOSE and MOE (Fig 5-5b) “Further cell-based experiments were performed on 4 due to its high yield. Western blot analysis of OVCAR5 cells treated with 17.6 µM (LC100) of 4 showed increased levels of p21 (a cell cycle inhibitor) after 8 h (Fig 5-6). Interestingly, levels of p21 decreased after 24 h when compared to solvent control (Fig 5-6).
Reduction of p21 protein after 24 h correlated with an increase in cleaved-PARP, an indication of apoptosis (Fig 5-6). The induction of cell cycle arrest, leading to apoptosis suggests significant DNA damage. To address this possibility, immunofluorescence for phospho-histone H2A.X was performed on OVCAR5 cells treated with 4 at 17.6 µM. Enhanced DNA damage, as monitored by increased phospho-histone H2A.X staining, was seen after 8 h and 24 h treatment with 17.6 µM of 4 when compared to solvent control” (Fig 5-7) (167).
Figure 5-4: Structure of DAQA and DAQE.

Chemical structures of DAQA (4) and DAQE (1). Chemical structures courtesy of Michael Mullowney in Dr. Murphy’s lab, as previously published (167).
Figure 5-5: Dose response analysis of 1 and 4 in OVCAR5 cells, and dose response of compound 1 (DAQA) in HGSC and non-cancerous cell lines.

“(a) Concentrations represented as log of nM. The LC50 value was determined using a non-linear curve fit on prism 6 GraphPad. These data represents average ±SEM from three replicates” (167). (b) LC50 values of DAQA in HGSC cell lines and non-cancerous MOSE and MOE cell lines (241)
Figure 5-6: Compound 4 induces cell cycle arrest followed by apoptosis.

“OVCA5 cells treated with 17.6 µM of 4 showed induction of cell cycle arrest through increased p21 after 8h, and induction of apoptosis through increased cleaved-PARP after 24 h. Densitometry of westerns was performed from three replicates. Statistical significance is donated by * for increased expression, and # for decreased expression. Student t-test was performed for all statistics and are represented as SEM +/-, *, and # p ≤ 0.05” (167).
Figure 5-7: Compound 4 induces DNA damage in OVCAR5 cells.

“(a) H2AX foci images taken after treatment of OVCAR5 cells with DAQA at 17.6 µM 8 h and 24 h; (b) Quantification of phospho-histone H2AX foci as a fold increase over DMSO solvent control. Statistical significance is donated by * using student t-test. * p ≤ 0.05” (167).
Discussion:

In this study, actinomycete fraction libraries were screened in a variety of ovarian cancer cell lines, and normal non-cancerous to identify novel therapeutic hits through bioassay-guided fractionation. Successful bioassay-guided isolation of an active anticancer compound, mithramycin, was performed on strain K011 isolated off the coast of Iceland. Mithramycin is a known anticancer compound, and demonstrates the ability to identify unknown compounds through bioassay-guided fractionation of aquatic actinomycetes (242). Strain F001 was isolated from a protected reef coast in Indonesia, and produced the compounds Diazaquinomycin E through G (167). DAQA, an already known compound, showed similar structural features along with cytotoxicity to DAQE. DAQA induced DNA damage in OVCAR5 cells, leading to cell cycle arrest and apoptosis. DAQA has since been screened in several other cell lines and demonstrated similar cytotoxicity activity (241).

Activity from secondary metabolites isolated from strain K011 showed considerable cytotoxicity, with LC$_{50}$ values in the 500nM range. The active compound from fraction 9/F3 was an already a characterized molecule (240). Mithramycin is a member of the chromomycin class of compounds (240). Mithramycin binds to GC rich regions of DNA, and blocks transcription from promoters with high GC content (240). Mithramycin was able to block transcription of many genes, thus preventing aberrant transcription seen in cancer (240). Specifically, in ovarian cancer cell lines, mithramycin blocked expression of genes such as Sp-1, VGEF and hTERT (242). In xenograft models, mithramycin blocked Sp-1 transcription and slowed tumor formation (242). Similar LC$_{50}$ values were seen between our K011 fractions and mithramycin in ovarian cancer cells as previous reports (240). The authors of these studies state that mithramycin does not work through p53 dependent mechanisms, which could be the reason it shows considerable toxicity in ovarian cancer cells (240). Ovarian cancer harbors mutations of p53 in nearly 100% of
cases (44), and thus any potential chemotherapeutic drug will not be able to take advantage of the p53 pathway.

Many secondary metabolites secreted by actinomycete bacteria often contain various analogues of the same compound. In this study, 4 novel diazaquinomycins (243), and an additional 2 diazaquinomycin molecules were isolated between strains B006 and B026 (241). While the focus of our study was F9 from the initial K011 fractionation due to the high yield of metabolites, fractions 9-13 showed activity in our cell lines. Further analysis of these will be performed to potentially find novel analogues of mithramycin that show either increased cytotoxicity, or increased differential activity among our cancer and non-cancerous cell lines. As a proof of principle, this data shows that our model of screening actinomycete libraries has the potential to produce active therapeutics lead compounds.

Diazaquinomycin is a relatively understudied, but known class of compound. In our studies, we have identified a novel analogue of the diazaquinomycin, DAQE, and showed that it has similar toxicity to DAQA (167). DAQA has been well-studied for its antibiotic activity, specifically as a thymidylate synthase inhibitor (243). Studies showed that cytotoxicity could be circumvented by the addition of folate or thymidylate (243). In the same study, DAQA was tested against two mammalian cell lines, Vero and Raji cells, two common cell lines used in natural product screening (243). DAQA showed anti-growth activity against both mammalian cell lines. However, these cell lines are not part of the NCI60 panel of cell lines, and do not represent human epithelial carcinomas. Vero cells are an epithelial cell type isolated from the African green monkey, and do are not a carcinoma (244). While Raji cells are human, they are a Burkitts lymphoma cell line and do not represent epithelial carcinomas (245). Therefore, anticancer activity has not been investigated in carcinomas, the most common form of cancer.
Another study further demonstrated the potential anticancer activity of diazaquinomycin related compounds (246). In this study, they showed that DAQ-like compounds inhibited ERK5 activation through inhibition of the JNK pathway and p38 (246). While these studies were performed in a variety of different cell lines, ranging from lymphomas to breast carcinomas, they did not show apoptosis was due to DNA damage, suggesting that DAQA and DAQE may have a different mechanism in EOC cell lines (167, 246). Our study is the first to report cytotoxic activity in epithelial ovarian cancer.

DAQA has shown activity against thymidylate synthase (TS) in previous studies (243). While our study demonstrated that DAQA induces apoptosis through DNA damage, TS inhibition was not assessed in ovarian cancer cells, and thus cannot be ruled out as a possible mechanism proceeding DNA damage. DAQA may inhibit TS, leading to an increase level of deoxyuridine monophosphate and DNA damage, similar to that seen with 5-fluorouracil (5-FU) drugs (247). Indeed, one mechanism of 5-FU cytotoxicity is through DNA damage leading to upregulation of p21, and induction to cell cycle arrest and apoptosis (248). Recent published data demonstrated that DAQA does not inhibit the activity of human TS enzyme in a cell free assay. Further studies of DAQA in ovarian cancer cells need to be performed to verify that DAQA does not inhibit TS in a cellular model. Another potential mechanism leading to DNA damage may be through DNA intercalation. DAQA is a planar compound that suggests it may bind into DNA and block replication of the cells. Preliminary competitive DNA intercalating assays showed that DAQA may not work through this mechanism, but further evaluation of the mechanism needs to be performed.
Chapter VI: Supplemental data on fractions screened:

**TABLE III: CYTOTOXICITY OF MARINE ACTINOMYCETE FRACTIONS IN OVARIAN CANCER NCI-60 CELL PANEL AND NON-CANCEROUS CELL LINES**

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### Table III: Cytotoxicity of marine actinomycete fractions in cancer and non-cancerous cell lines.

Values represent the % survival of the respective cell lines against the fraction in question. Each bacteria is designated a name based on where it was isolated from. The letter F denotes the fraction number from the initial separation. All fractions are suspended in DMSO, and % survival is determined by comparison with DMSO solvent control. Fractions that were cytotoxic against OVCAR5 and SKOV3 are listed. Fractions are firstly ranked in order of cytotoxicity to OVCAR5 cells, and secondly in order of cytotoxicity to SKOV3 cells. Corresponding cytotoxicity in non-cancerous MOE and MOSE are also listed. MOE and MOSE percent survivals labeled in green are fractions that showed differential or selective cytotoxicity in the ovarian cancer cell lines percent survival of under 50%. All strains were collected by Dr. Murphy and his lab.

<table>
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<tr>
<th>Name</th>
<th>Fraction</th>
<th>% Survival OVCAR5</th>
<th>% Survival SKOV3</th>
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### Table IV: Percent Survival of All Four Cell Lines Against Selectively Cytotoxic Fractions

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<th>SKOV3</th>
<th>MOE</th>
<th>MOSE</th>
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<td>73%</td>
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<td>133%</td>
</tr>
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<td>24%</td>
<td>137%</td>
<td>100%</td>
</tr>
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<td>16%</td>
<td>107%</td>
<td>138%</td>
</tr>
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<tr>
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<td>94%</td>
</tr>
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<td>114%</td>
<td>66%</td>
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<td>D063-F3</td>
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<td>92%</td>
<td>123%</td>
</tr>
<tr>
<td>7</td>
<td>J131-F3</td>
<td>&gt;5%</td>
<td>32%</td>
<td>74%</td>
<td>89%</td>
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*Table IV: Percent survival of all four cell lines against selectively cytotoxic fractions. All cytotoxic activity is represented as percent survival compared to solvent control. Selectively cytotoxic fractions were ranked based on both cytotoxicity and potential novelty of chemical structures (after dereplication was performed).*
**Selectively cytotoxic compounds:**

**K025 –F3 and F4:** K025 is a strain collected off the coast of Iceland during the same collection as K011, which yielded mithramycin. K025 also should differential cytotoxicity in our screening assay (Table S5-2). Upon regrow, the activity of the fractions only showed moderate cytotoxicity and was not differential towards the cancer cells (Fig 5-8). Fraction 4 from the regrow showed the most activity, and was further separated into 8 fractions (K025 4-x) (Fig 5-8). Bioassay screening identified fraction 4-F8 as having the most cytotoxic activity, and this was further fractionated and rescreened to follow cytotoxicity (K025 4-8-x) (Fig 5-8). K025 4-8-x were never screened for cytotoxicity because dereplication of the fractions showed that the compound present was nonactin, a macrotetralide antibiotic. The structure of nonactin is already known (249) and well characterized. Due to the low cytotoxicity of the fractions, and the fact that the structure was previously elucidated, this project was discontinued.

**E003-F2:** E003-F2 is from a strain of actinomycete collected in the queue that showed differential cytotoxicity in our screening (Table 5-2). Upon regrow of this strain, all cytotoxic activity was lost in all cell lines and the project was discontinued.

**D063, H032, and J002:** All three strains showed excellent cytotoxicity in our screening assay, with preference for ovarian cancer cells (Table 5-2). Upon regrow of all strains, activity was lost, and thus the active molecule could be further characterized. All three strains are currently in re-grow to try and retrieve activity.
**Figure 5-8: Fractionation tree and cytotoxicity of strain K025**

(a) Fractionation tree of strain K025 from regrow.  (b) LC\textsubscript{50} values of the active fractions from K025
Additional marine actinomycete fractions:

A046: Strain A046 was collected at Urrel beach, MA. This strain showed cytotoxicity towards both OVCAR5 and SKOV3 cells from our fraction library screening (Table 5-1). This strain was re-grown by Anam Shaikh from Dr. Murphy’s lab, and bioassay guided fractionation was performed to identify the active compound. After several rounds of fractionation, it was determined that several active molecules were present in this strain, the most abundant being Antimycin. Antimycin is a known anticancer agent, which works through inhibiting β-catenin and blocking mitochondrial electron transport. In some cancers, analogs of antimycin A also block Bcl-XL, offering a novel treatment of chemoresistant cancers (250). Other molecules isolated from the active fractions include Kitamycin A and N-formyl antimycic acid methyl ester. Due to the relative low abundance of these molecules in the fractions, they have not been tested as isolated compounds for cytotoxicity to date.

G039: sediment sample collected by PONAR at a depth of 22 m, from ca. 3.3 miles off the coast southeast of Cát Bà Peninsula in Vietnam. Fractions from G039 showed under 50% survival at 10 µg/mL in the initial fraction plate screen. Fraction of G039 fractions led to the discovery of a novel compound. The structure of this compound is currently being deciphered by Dr. Murphy’s lab. Upon dose response screening of a near pure compound, LC50 values in our cell lines were: OVCAR4 – 1.15 µg/mL, Kuramochi – 3.62 µg/mL, MOSE – 4.78 µg/mL and MOE – 2.8 µg/mL. Further analysis of this compound will be performed in the future to identify the mechanism of action.

B006 and B026: Both B006 and B026 strains were both isolated from Lake Michigan, and produce very similar compounds. Strain B026 showed activity against both OVCAR5 cells and SKOV3 cells, while B006 only showed activity against OVCAR5 cells (Table 5-1). Strain
B006 was regrown in a 30L volumes to try and isolate large quantities of the active compound. B008-30L bioassay guided fractionation followed activity until a near pure compound was isolated. Upon structure elucidation by Dr. Murphy’s lab, the active compound was determined to be two novel analogs of diazaquinomycin, termed DAQH and DAQJ. The LC$_{50}$ value of the active fraction containing both analogs was 0.47 µg/mL, significantly more cytotoxic than DAQE, which was previously published (167). The individual novel DAQ analogs have not been tested to date. Strain B026 contained identical DAQ analogs as B006. Comparison of secondary metabolites of B006 and B026 suggest that both strains may actually be the same strain. This data was recently published (241)

**G003:** Strain G003 was isolated from a sediment sample collected by PONAR at a depth of 22 m, from ca. 2.0 miles off the coast southeast of Cát Bà Peninsula in Vietnam. From the fraction library screening, fractions from this strain showed cytotoxicity of under 50% survival at 10 µg/mL in OVCAR5 cells (*Table 5-1*). This strain was re-grown in larger volumes and subject to further bioassay guided fraction. However, during the fractionation process, the product either degraded or was in too low abundance to isolate and characterize. The project was terminated at this stage.

**B007:** Strain B007 was collected from lake Michigan by diving at a depth of <10m. Initial screening from the fraction plate library showed high levels of cytotoxicity amongst all four fractions of B007 (*Table 5-1*). B007-F4 was regrown and further fractionated, and bioassays performed for cytotoxicity. B007 F4/1 had an LC$_{50}$ <1.25 µg/mL in OVCAR5 cells, while B007-F4/3 had an LC$_{50}$ <0.78 µg/mL. Dereplication was performed on both B007 F4/1 and B007 F4/3. Dereplication identified staurospprine like molecules present. Staurosporine is a
well characterized molecule known to have anti-cancer activity. It works through inhibition of PKCα and survivin, and through cleavage of caspase-3(251, 252). Due to the research already performed on staurosporine and its derivatives, this project was terminated.
Conclusions and future directions

Mutation or loss of p53 differentially modifies TGFβ action in ovarian cancer

The purpose of this thesis was to identify therapeutic targets in ovarian cancer through characterization of mechanisms involved in the disease, and to potentially discover natural product derived anticancer drugs that can be used specifically in ovarian cancer. The need for new and novel therapeutics in ovarian cancer cannot be overstated, with 5 year survival rates barely improving over the past 30 years (8). By understanding some of the key mechanisms of progression, metastasis and chemoresistance of the disease, new drug targets can be identified and exploited to better treat patients, and create a more directed therapeutic strategy specific to ovarian cancer. Additionally, screening of secondary metabolites from actinomycetes bacteria in an array of ovarian cancer cell lines with varying p53 status, and in non-cancerous epithelial cells representing the two potential cells of origin of ovarian cancer, aided in discovery of novel drug leads that can be used to treat the disease by refining standard screens with more mechanism and comparison to normal cells.

Both GOF mutations in p53 and TGFβ are known to increase migration and metastases in various different cancers, independently and through crosstalk with each other (124, 128, 253). However, this study demonstrated that in ovarian cancer, crosstalk between GOF mutations in p53 and TGFβ does not increase migration, and that the TGFβ induction of cell cycle arrest and migration is dependent on p53 status. In the presence of wild-type p53 (121), TGFβ induced cell cycle arrest and a decrease in proliferation of ovarian cancer cells that was abrogated upon mutation of p53 (both loss and gain of function mutations). Furthermore, TGFβ increased migration of ovarian cancer cells possessing wild-type and no p53, but did not induce migration of cells possessing a GOF mutation in p53. Concurrently, TGFβ-induced expression of two pro-
migratory genes associated with ovarian cancer, was diminished in GOF p53 ovarian cancer cells, suggesting that GOF mutations may suppress TGFβ pro-migratory genes. This study demonstrated the importance of p53 in ovarian cancer for patients that might be receiving TGFβ inhibitors as a therapeutic. Patients with loss of p53 function would benefit most from the use of TGFβ inhibitors, with little effect seen in patients possess a GOF mutation in p53. In fact, for ovarian tumors that still maintain wild-type p53, the use of TGFβ inhibitors may prove detrimental, as a functional TGFβ pathway would slow proliferation through p21 induction and cell cycle arrest.

For HGSC, the cell of origin may also have an impact on the potential use of TGFβ inhibitors as a therapeutic. Our study showed that while both the OSE and FTE respond to TGFβ signaling, only the OSE was growth inhibited in both 2D and 3D assays. For HGSCs derived from the FTE, inhibiting TGFβ may not have a detrimental effect, as the normal cells are not growth inhibited by TGFβ.

Adorno et al identified that GOF mutations in p53 cooperate with TGFβ-induced SMAD signaling to co-opt p63, and block transcription of p63 regulated anti-migratory and anti-invasive genes in lung and breast cancer (128). The expression levels of p63 were not measured in this study, and may be required to mediate TGFβ-induced invasion and migration in ovarian cancer. A future study would co-express p63 with GOF mutation in p53 in ovarian cancer cells to monitor TGFβ induced phenotype. However, the TCGA did not identify the p63 pathway as being dysfunctional in HGSC, while mutations in p53 are seen in nearly 100% of cases, suggesting that this particular mechanism may not be relevant to HGSC (44).

Endogenous expression of TMEPAI and DKK1 are associated with a pro-migratory phenotype, and have shown to be drivers of migration in cancer cells (177). Indeed, knockdown
of TMEPAI in MDA-MB-231 breast cancer cell lines decreased TGFβ-induced migration (177). MDA-MB-231 cells possess a GOF mutation in p53 at R280K, similar to the R273H tested in this study (177). DKK1 is known to be both p53 regulated and TGFβ regulated, but previously no study has analyzed the role of crosstalk of these pathways on DKK1 expression (254, 255). DKK1 is also associated with increased migration in hepatocellular carcinomas (256). To validate the specific role of both genes in TGFβ-induced migration of ovarian cancer cells, TMEPAI and DKK1 should be knocked down in SKOV3 cells and TGFβ induced migration analyzed. Based on the data in this study, TGFβ induced migration would be reduced in the absence of both genes, mimicking the down regulation seen in GOF mutant p53 ovarian cancer cells.

Finally, to verify the translational aspect of this study, cells should be xenografted into athymic nude mice and allowed to form tumors with stable alterations in p53. Once tumors had been formed, the mice would be treated with a TGFβ inhibitor currently in pre-clinical trials and tumor size, and metastasis analyzed (194). Considering the data presented in this study, the hypothesis would be that TGFβ inhibitors would decrease metastasis of p53 null cells, while having limited to no effect of GOF mutant p53 cells. Cells possessing wild-type p53 may have a larger tumor burden, but metastasis would also be reduced.

Mechanistic and functional role of PAX8 in high-grade serous cancer, and its potential as a therapeutic target

MOSE-PAX8 cells did not exhibit anchorage independent growth, which is a hallmark of transformation. Anchorage independent growth allows for a population of cells to grow in the absence of an extracellular matrix or contact with neighboring cells (257). However, while anchorage independent growth is indicative of cellular transformation, it does not definitively
designate tumorigenicity. In order to determine if MOSE-PAX8 cells are tumorigenic, the cells have been xenografted into athymic nude mice. MOSE-PAX8 and MOSE-Neo have been xenograft both subcutaneously (s.c) and intraperitoneally (i.p). Cells injected s.c will determine whether MOSE-PAX8 cells are tumorigenic. Cells injected i.p will demonstrate the ability of MOSE-PAX8 cells to metastasize within the peritoneal environment.

Little research has been done on PAX8’s biological function HGSC, including many of the pathways it may regulate. Primarily it is used as a marker for histotyping. With so many different pathways being dysfunctional in later stage HGSC, when the majority of HGSCs are diagnosed, identifying the function of any one protein in the progression of the disease can be difficult. Importantly, PAX8 seems to be expressed at high levels in almost all HGSC. This study used a unique system with non-cancerous cells from both the OSE and oviduct, to investigate the function of PAX8. MOSE-Neo control and MOSE-PAX8 cells have already been submitted for global RNA sequencing to identify transcripts altered by PAX8. Understanding the downstream targets of PAX8 may aid in elucidating the mechanisms whereby PAX8 regulates migration and proliferation once acquired in OSE cells. Additionally, PAX8 cDNA is currently being design to possess a FLAG-tag sequence at the 5’-end of the DNA. This will produce a functional PAX8 protein with a unique sequence on the N-terminal. Antibodies directed at this FLAG sequence can be used to perform chromatin immunoprecipitation (ChIP) assays, which is important as currently available commercial antibodies are not good for ChIP of murine PAX8. A future goal would be to perform genome wide ChIP-sequencing using the FLAG-tag antibody directed towards the PAX8-FLAG to identify DNA elements that PAX8 directly binds. By comparing the data obtained from MOSE-PAX8 cells RNA sequencing and genome-wide ChIP-sequencing of
the PAX8 protein, both direct and indirect targets of PAX8 induced transcription will be determined and a better understanding of signaling properties of PAX8 will be uncovered.

A pitfall of this study would be that PAX8 induced tumor characteristics are not directly caused by PAX8 induced transcription. Recent evidence from renal development suggests that PAX2 may act as an epigenetic modifier, more than a transcription factor (258). PAX2 shares a high level of homology with PAX8, and is often co-expressed in similar tissues (259). To address this, a future goal of this project could be to make use of the PAX8-FLAG to perform a co-immunoprecipitation (Co-IP). Co-IP of PAX8 would be performed, along with mass spectrometry analysis to identify the transcriptional complexes associated with PAX8. Specific genes that demonstrated altered RNA expression from the PAX8 RNA seq experiment, but that did not possess a PAX8 binding site according to the Chip-seq experiment, could be analyzed for their epigenetic modifications. While RNA-sequencing and ChIP-sequencing are powerful tools to identify genes that are directly bound and regulated by PAX8 DNA-binding, the role of PAX8 as a co-repressor or co-activator of transcription through epigenetic modifications would be missed.

PAX8 is essential during development of the thyroid and Müllnerian duct, and knockdown of PAX8 induces apoptosis in HGSC cell lines (150, 156). Also, PAX8 is known to control epithelial cell survival (201). In agreement, homozygous knock out of PAX8 in MOE cells could not be achieved using the CRISPR/Cas9 system, which may be due to the importance of PAX8 in epithelial cell survival. However, knockdown of PAX8 did not lead to cell cycle arrest or apoptosis in MOE cells, suggesting that knockdown of PAX8 can be tolerated by MOE cells, but not homozygous knockout. The major difference between this study and previous studies, is that MOE cells are derived from mouse, while other models are derived from human cells. In order to
determine whether this is a species difference, immortalized fallopian tube epithelium cells (FTSEC) previous published, will be treated with siRNA for PAX8 (94). Furthermore, attempts will be made to create a stable PAX8 homozygous knockout using CRISPR/Cas9 in FTSEC, MOSE or other non-expressing cells, such as MCF7. These data will determine whether the species differences are a factor in this study. If species difference in the role of PAX8 is not a factor, HGSC cell lines transiently treated with PAX8<sup>shRNA</sup> will undergo RNA sequencing. The results of the sequencing of HGSC cell lines with PAX8 knocked down, will be compared with the results of MOSE-PAX8 RNA sequencing. The mechanistic role of PAX8 in HGSC can be identified by analyzing commonly altered pathways between cancerous and non-cancerous cells.

Pathways associated with HGSC increased levels of PAX8 protein in both MOE and MOSE cells, suggesting that PAX8 may become more important for during tumor progression. This could be very important if ultimately a therapy is developed that reduces PAX8 expression in the oviductal cells, because this would have very little toxic effect on the normal cells. One of the initial hypotheses of this study was that HGSC becomes “addicted” to PAX8. Since MOE cells did not undergo cell cycle arrest or apoptosis when PAX8 was knocked down, a future direction would be to test whether any of the MOE cells containing multiple altered pathways associated with HGSC are addicted to PAX8. PAX8 siRNA will be used to knockdown PAX8 in all MOE cell lines that showed an increase in PAX8 levels. As before, cell cycle analysis and apoptosis will be examined, along with proliferation. In addition, PAX8 will be knocked down in MOSE Pten<sup>−/−</sup>/LKB1<sup>−/−</sup> cells, to determine whether OSE cells expressing PAX8 are now reliant on it. Determining which pathways lead to reliance on PAX8 for survival will aid in understanding the transformative mechanisms of HGSC. Determining which pathways lead to reliance would
also allow for combination therapies that reduce PAX8 and reduce signals that contribute to PAX8 enhanced expression.

**Identification of novel therapeutics from marine derived Actinomycetes**

Strain F001 showed cytotoxicity in OVCAR5 cells in the initial fraction library screening, and subsequently underwent fraction to isolate the active molecule. The active molecule was a diazoquinomycin (DAQ) E, an analog of diazoquinomycin A. Further structure elucidation performed by Dr. Brian Murphy’s lab determined that up to 3 separate DAQ analogs were present in the fractions, DAQE through G (167). Due to limited quantity, synthetic DAQA was tested for cytotoxicity and mechanism of action. DAQA showed an LC$_{50}$ value of 8.8µM in OVCAR5 cells. Similarly, DAQA exhibited an LC$_{50}$ value of 4.3µM in OVCAR4 cells and 9.4µM in Kuramochi cells. Of particular interest was the LC$_{50}$ values in non-cancerous MOE and MOE cells had LC$_{50}$ values of 22 and >28µM respectively, significantly higher than in the ovarian cancer cell lines (241). DAQA induced cell cycle arrest in OVCAR5 cells, leading to apoptosis through cleavage of PARP. Cell cycle arrest was induced by DNA damage (167). Additional studies performed by Dr. Murphy’s lab and his collaborators determined that DAQA was not inhibiting TS synthase, as previously report (241, 243). Future studies will aim to identify the mechanism leading to DNA damage, and why DAQA shows a significantly higher LC$_{50}$ value in non-cancerous MOSE and MOE cells, compared to ovarian cancer cells. This difference in LC$_{50}$ values is interesting, with LC$_{50}$ values being 2-3 fold higher in the non-cancerous cells (241). Understanding why MOSE and MOE cells are more resistant to the DNA damaging affects of DAQA could give insight into some of the pathways gained by ovarian cancer during transformation, regardless of cell of origin.
DAQA displayed decent cytotoxicity levels in ovarian cancer cells. Additionally, DAQA displayed lower LC$_{50}$ values in breast, colon and melanoma cells lines, suggesting that the mechanism of cytotoxicity may not be unique to ovarian cancer. Future work would involve submitting DAQA to be screened in the NCI-60 cell panel. OVCAR5 and SKOV3 cells are part of the NCI-60, and could be used as positive control cell lines. By comparing molecular pathways of DAQA sensitive cancer cells, additional mechanisms of anticancer activity can be identified. This is based on the observation that different molecules targeting the same anti-growth pathway will show similar levels of cytotoxicity in the cancer cells. By identifying sensitivity and resistance of the NCI-60 panel of cancer cells to DAQA, and comparing and aligning this profile to anticancer drugs of a known mechanism, the cytotoxic pathway leading to DNA damage can be identified (160).

Many issues were experienced through this study, most notably was reproducibility of fraction library toxicity. Upon re-grow of various strains that showed cytotoxicity in the initial fraction library, cytotoxicity was often lost. Prospective work would include optimization of the re-grow procedure, included potential co-culturing of the strain with eukaryotic organisms to stimulate production of the active molecule. An additional issue experienced was identification of already characterized molecules. This is an issue experienced throughout the natural product discovery field, and all possible measures have been taken through dereplication to address this issue. Genotyping of active strains, coupled with dereplication procedures may provide a more powerful technique to circumvent re-isolation of known molecules. Strain identity and dereplication UV profiles could be aligned with known compounds produced by similar strain to estimate if an active compound is already known or not.
A major issue in the ovarian cancer field is chemoresistance of the disease. HGSC is often diagnosed in later stages (stage III or IV). Although patients often respond well to initial treatment, most will have recurrence of the disease, with a median survival of 15 months. Second line therapy only shows response in between 10-25% of patients, highlighting the need for new therapeutics. One active area of research is to find new drugs that can confer chemo-sensitivity on chemoresistant ovarian cancers (260). Cancers treated with platinum based chemotherapies often alter specific pathways to circumvent the cytotoxic activity of chemotherapy (224, 261). The hypothesis is that targeting agents to block these specific pathways can confer chemo-sensitivity again to the disease. A future study would screen strain fractions from the actinomycete library in chemoresistant ovarian cancer cell lines, both with and without cisplatin. The goal would be to identify molecules that circumvent the chemoresistant pathways associated with ovarian cancer.

With so many active fractions identified from the initial fraction library screening, not all active fractions have been re-tested or re-evaluated for this study. The same fraction libraries have been screened for many other diseases, such as tuberculosis and HIV. These studies have identified novel molecules from the fractions that may have shown cytotoxicity in ovarian cancer, but not yet re-tested. These newly identified compounds will be screened for cytotoxic activity in the ovarian cancer cell lines to determine whether they were the active compound seen in the initial screen of these cell lines.

**Concluding remarks:**

This dissertation aimed to identify new therapeutic targets for ovarian cancer by studying common mechanisms of progression and metastasis of the disease, and by screening novel compounds isolated from aquatic actinomycetes. Targeting transcription factors, or the signaling
pathways that activate them, can provide a more specific therapeutic strategy for treating disease. Carcinomas, specifically HGSC, are diseases of altered pathways. Identification of common transcription factors or signaling molecules that drive carcinogenesis of the altered pathways can provide a multi-pathway inhibitor to treat the disease. Targeting transcription factors can be achieved through blocking their transcription, targeting their downstream effects or through epigenetic modifications altering either their transcription or activity.

Therapeutics for ovarian cancer is in dire need, with current treatments failing in many recurrent diseases. By using data presented here, patients can be screened for their p53 status prior to treatment with TGFβ inhibitors to improve translation of the drug into the clinic. Only patients with loss of p53 would benefit from treatment with TGFβ inhibitors, and thus clinical trials should only be performed with patients possessing a loss of p53 in their tumors. For patients possessing other types of p53 mutations, inhibitors of PAX8 transcription could provide an alternative therapy. These inhibitors would provide a novel therapeutic for inhibiting the progression of ovarian cancer, regardless of whether it comes from the OSE or fallopian tube, and may be useful as a chemopreventative treatment. Finally, unique compounds isolated from natural sources provide a foundation for the development of therapeutics. Identifying selectively cytotoxic fractions can help decipher the pathways co-opted in HGSC progression that are not essential for normal cell survival. Moreover, many of the natural product drugs can be used to treat chemoresistant HGSCs and provide new hope for patients that have exhausted all available options.
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DOB: 9/30/1987
Nationality: Irish

Education

• 2010 – Present: P.hD candidate in Pharmaceutical biotechnology, University of Illinois at Chicago. Current GPA 3.9

• 2009: CCIP Diploma in Scientific and Technological French

• 2006-2010 – Bachelors of Science (B.Sc) in biotechnology, National University of Ireland, Galway (NUIG). GPA: 3.8 (equivalent)

• 2005: Diploma in Irish language from Aras Mhairtín Uí Chadhain (NUIG), An Cheathrú Rua, Co. Galway

Experience:

2010 – Present: University of Illinois at Chicago: P.hD candidate in Pharmaceutical biotechnology (current GPA: 3.9)

I am currently working on understanding the crosstalk and relationship between TGFβ and p53 in ovarian cancer metastasis. We are trying to determine if the early missense mutations of p53 are acting as a molecular switch. Making TGFβ switch from being anti-proliferative to pro-metastatic in later stages. Additionally, I perform bioassay screening of extracts from collaborating labs. We are looking for any compound that might either make chemotherapy resistant ovarian cancer cells chemo-sensitive again, or that can target cancer cells specifically.

2013 – Takeda Pharmaceuticals – Translational Pharmacology (Intern, 3 months)

At Takeda, I worked on biomarker profiling of varies CNS diseases to aid translation of candidate drugs from pre-clinical trials to clinical trials. Our goal was to increase drug efficacy and improve transition from pre-clinical disease models to humans. We validated several of our biomarkers in pre-clinical studies and will proceed to include them in several IND application
2010 – Regenerative medicine institute (REMEDI) - Student research assistant (4 months)

Worked on the transdifferentiation of mesenchymal stem cells from human bone marrow into insulin producing β-cells of the pancreas. We successfully created a 10 day transdifferentiation protocol and tested our findings via insulin ELISA tests, qPCR, dithizone staining, western blotting and morphological changes in the cell structures. For this research, I won the Alpha technology gold medal award.

2009 - University of Heidelberg – Student research assistant (4 months)

Worked on creating constructs to tag the ciliary margin zone (CMZ) stem cells in the eye’s of zebrafish in order to study the migration and differentiation of them during development. This involved creating constructs of GFP under the control of a CMZ specific promoter via gateway cloning and injecting the constructs in zebrafish fertilized embryos at the one cell stage. The embryos would then be grown up until hatching and monitored via fluorescent microscopy over this period. The CMZ cells were then tracked along their migration through the developing eye.

Awards:
2014 ISRS 2nd place platform presentation award
2012 Graduate student council travel award
2012 Larry Ewing Memorial Trainee Travel Fund (LEMTTF)
2011 Alpha technology gold medal award for my work on mesenchymal stem cells
2003 Presidents gold medal award for community service (Ireland)

Fellowships and scholarships:
2014 CCTS Pre-doctoral Education for Clinical and Translational Scientists (PECTS) scholarship
2014 Paul Sang scholarship
2013 Chancellors graduate research fellowship
2012 Myron Goldsmith scholarship

Presentations:
2014- AACR/Marsha Rivkin Ovarian Cancer Symposium (poster)
2014- ISRS platform talk (presentation)
2014- College of Pharmacy research day (4 posters)
2013- ISRS at southern Illinois University (2 posters)
2013- Takeda research symposium (presentation)
2013- College of Pharmacy (COP) research day (poster)
2012- ISRS at Northwestern (poster)
2012- Society for the study of reproduction (SSR) conference at Penn state university (poster)
2012- IIEOCC conference at Notre Dame (poster)
2012- Cancer center forum at UIC (poster)
2012- College of Pharmacy (COP) research day (poster)
2011- Illinois symposium on reproductive sciences (ISRS) at UIUC (poster)
2010- Alpha technology award ceremony (presentation)
Honors:
• Member of the Illinois division of Phi Kappa Phi (2013)
• Chair of the Webster-Sibilsky lectureship committee (2012 and 2014).
• Chosen to participate on the Illinois symposium on reproductive science (ISRS) conference committee (2013)
• Community outreach program to educate high school students on a career in science
• Awarded a competitive summer internship at Takeda Pharmaceuticals

Techniques:
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