Oncolytic Virus Therapy in Combination with Chemotherapy

for Ovarian Cancer

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2013

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Abstract

Purpose. Novel therapeutic regimens are needed to improve dismal outcomes associated with late-stage ovarian cancer. Oncolytic viruses have shown efficacy against ovarian cancer. We studied the application of an oncolytic herpes simplex virus expressing two anti-tumor genes: ICP34.5 under the stem cell-specific nestin promoter, and anti-angiogenic molecule Brain-specific Angiogenesis Inhibitor-1 (BAI1) under the strong viral promoter IE4/5 for use against ovarian cancer. We also studied the use of this viral vector in combination with a second-line standard of care chemotherapeutic drug, doxorubicin.

Experimental Design. The 34.5 Expressing Nestin-driven Vasculostatin-120 Expressing (34.5ENVE) virus was tested for treatment of ovarian cancer in vitro and in vivo. Efficacy of the virus, and its antiangiogenic effects on endothelial cells were assessed in vitro in cancer cell lines and in primary patient ascites samples. Scope of cytotoxic interactions between 34.5ENVE and chemotherapeutic agent doxorubicin were evaluated using Chou-Talalay synergy analysis. Efficacy of oncolytic viral therapy in combination with doxorubicin was evaluated in vivo in the murine xenograft model of progressive human ovarian cancer.
Results. 34.5ENVE showed robust efficacy against ovarian cancer cell lines, and even greater efficacy against ex vivo mouse and patient ascites. When combined with doxorubicin, 34.5ENVE killed synergistically with a robust increase in caspase-3/7 cleavage. The combination of doxorubicin and 34.5ENVE significantly prolonged survival in nude mice bearing intraperitoneal ovarian cancer tumors.

Conclusions. This study establishes the potential for use of oncolytic HSV in combination with doxorubicin for the treatment of late-stage ovarian cancer.
Acknowledgements

I dedicate this document to my support team throughout many years, with very special thanks to my adviser, Dr. Balveen Kaur, and the members of my committee, Drs. McCarty, Munson and Kaumaya.
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Introduction

Ovarian cancer (OvCa) is the 5th most deadly cancer in women, and two-thirds of women present with disease that has spread to abdominal organs or distant sites. While many women show initial regression of disease with standard therapy, nearly 70% recur\(^1\). Recurrent tumors are often resistant to first line therapeutics, cisplatin and paclitaxel\(^1\). Second line therapies include chemotherapeutic agents such as doxorubicin or topotecan, and antiangiogenic agents such as VEGF monoclonal antibody, bevucizumab\(^1\). However, less than 30% of patients presenting in late stages have a 5-year survival\(^1\). Thus novel therapies are needed as many patients present with late-stage disease or recur with chemotherapy-resistant disease.

Furthermore, while standard therapies may be quite effective against the differentiated bulk of tumor, it has been shown that tumors are composed of a small subpopulation of cancer stem-like cells that maintain or rapidly develop resistance to chemotherapy. This population is able to survive treatment and repopulate the tumor\(^2\). As the primary therapy for ovarian cancer, platinum-based compounds show good efficacy initially, but nearly 70% of women relapse\(^1\). This may be due to the reservoir of resistant stem-like cells that survive therapy, and reinitiate aggressive tumor growth\(^3,4\). Because of this, it is of interest to identify and develop therapies that are able to target these cancer-initiating cells.
Nestin is a type II intermediate filament originally described as a neuronal stem cell marker for neural progenitor cells during CNS development. After differentiation, neuronal cells lose nestin expression and instead express tissue-specific intermediate filaments. Thus, nestin is a marker for undifferentiated cell populations. Since its discovery, nestin has been shown to be upregulated in stem cell-like subpopulations of many cancers, including glioblastoma, gastric, prostate, breast, melanoma, and pancreatic cancer. Elevated expression in these tumors is associated with tumor aggression (migration, invasion), metastatic potential, and decreased survival. Nestin expression has also been shown to be elevated in the proliferating tumor-associated neovasculature in gastric cancer, colorectal cancer, prostate cancer, glioblastoma, and malignant melanoma.

Recently, OvCa subpopulations termed stem-like cells or OvCa initiating cells (OCICs) have been shown to display increased nestin expression compared to adherent cells, differentiated cells, and bulk OvCa tumor. These stem-like/initiating cells have been shown to be critical for re-initiation of tumor growth after primary treatment, and are capable of serial propagation of the original tumor phenotype in animals. Chronic chemotherapy treatment with low doses of cisplatin and paclitaxel induces stem cell-like phenotype in culture from OvCa cell lines. Qin et al reported that nestin expression in advanced serous ovarian carcinoma was correlated with more aggressive disease, and with poor prognosis and chemotherapy resistance. However, a study performed by Hetland et. al. did not see any correlation between nestin expression and disease prognosis or
chemotherapy resistance, although nestin expression was seen to be elevated in tumor samples\textsuperscript{27}. Given the aggressive properties of stem-like ovarian cancer cells, we hypothesize that a treatment regimen designed to preferentially target nestin-expressing cancer cells will have improved therapeutic efficacy and prolong survival.

The process of angiogenesis contributes greatly to the progression of disease in ovarian cancer. Angiogenesis has been shown to be important in oncogenesis, and expansion and viability of ovarian cancer\textsuperscript{28}. Angiogenic stimulation results in malignant ascites, a major burden of disease\textsuperscript{29,30}. Peritoneal ascites accumulate due to an increase in vasculature leakiness and aberrant function of lymphatic vessels caused by angiogenic stimulation\textsuperscript{31-33}. Ascites fluid acts to facilitate tumor spread throughout the peritoneal cavity, providing nutrient-rich fluid in which tumor cell spheroids (which detach from the primary tumor) travel, and depositing cells onto the omentum and other peritoneal surfaces\textsuperscript{34}. Angiogenic stimulation also affects the biology of tumor cells and tumor stroma, regulating proliferation, differentiation and survival\textsuperscript{29}. Targeting angiogenic pathways regulated by vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietins, and fibroblast growth factor (FGF) is currently being evaluated for use in the clinic either as single agent treatments or in combination with standard chemotherapy regimens. Therapies targeting VEGF pathway, such as anti-VEGF monoclonal antibody Bevacizumab (Avastin ®)\textsuperscript{35} and fusion protein VEGF-trap with picomolar affinity for VEGF-A (Aflibercept/ZALTRAP ®)\textsuperscript{36}, and PDGF pathway inhibitor Imatinib mesylate (Gleevec)\textsuperscript{37} have shown clinical efficacy in patients, highlighting the importance of angiogenic pathways. Furthermore, although angiogenic pathways are presumably
independently regulated, there is evidence that there are strong connections between different pathways (e.g. correlation between levels of VEGF and PDGF-BB in malignant ascites), and altering one pathway may affect others\textsuperscript{38}.

Herein, we examine the use of an antiangiogenic agent to both prolong survival and reduce tumor-associated ascites burden in a murine xenograft model of human ovarian cancer. Brain-specific angiogenesis inhibitor-1 (BAI1) is an anti-angiogenic protein that contains five thrombospondin type I repeats, and was originally described to be a p53-transactivated protein\textsuperscript{39}. BAI1 has been shown to reduce neovascularization in vitro\textsuperscript{39}, and reduce tumor angiogenesis and delay tumor growth in vivo\textsuperscript{40-42}. It was originally described to be down-regulated or absent in glioblastoma patient samples, while continuing to be expressed in normal brain\textsuperscript{39,41}. Since its discovery, BAI1 has been shown to be down-regulated in several different tumor types regardless of p53 expression (colorectal\textsuperscript{43,44}; pulmonary adenocarcinoma\textsuperscript{45}; non-small cell lung\textsuperscript{46}; gastric\textsuperscript{47}; renal\textsuperscript{48,49}), with reduced expression being associated with increased angiogenesis and elevated intratumoral microvessel density, increased tumor aggression, and decreased survival in patients.

BAI1 is a transmembrane protein, and its extracellular fragment, called Vasculostatin-120 (VStat120), can be cleaved at its G protein coupled receptor proteolysis site (GPS) via autoproteolysis, and released extracellularly. VStat120 contains the five thrombospondin type I repeats, and modulates the potent antiangiogenic activity of BAI1 by binding cell surface glycoprotein CD36, which is
present on microvascular endothelial cells\textsuperscript{50,51}. Binding of CD36 induces downstream signaling through src kinases fyn and lyn, activating p38 MAP kinase which leads to caspase activation and endothelial cell apoptosis\textsuperscript{51}. Two thrombospondin-1 mimetic proteins, ABT-510 and ABT-898, have been tested in the orthotopic, syngenic model of epithelial ovarian cancer with promising results. Treatment with ABT-510 or ABT-898 induced tumor regression, reduced ascites fluid volume and intraperitoneal dissemination, and prolonged survival. Treatment also reduced aberrant tumor-associated vasculature, decreased VEGF expression, and reduced tumor hypoxia. ABT-510 showed effects against OvCa in vitro, indicating that its effects were not solely mediated via tumor vasculature\textsuperscript{52}. Combination therapy of ABT-510 with paclitaxel or cisplatin resulted in increased apoptosis of both the tumor cells and the tumor vasculature, mediated via vasculature normalization\textsuperscript{53}. Thus, we hypothesize that an anti-angiogenic therapy that utilizes the fragment of BAI1 that contains the thrombospondin type I repeats (VStat120) will be a potent inhibitor of angiogenesis and tumor progression in our ovarian cancer model.

Oncolytic viral (OV) therapy has been examined for treatment of many types of cancer, including ovarian cancer. Oncolytic viruses target lytic replication to cancer cells while sparing normal cells. Many different OVs have been examined for use as treatment for ovarian cancer, including oncolytic herpes simplex virus (HSV)-1\textsuperscript{54-58}, HSV-2\textsuperscript{59}, oncolytic or conditionally-replicating adenovirus\textsuperscript{60-63}, oncolytic measles virus\textsuperscript{64-67}, vaccinia virus\textsuperscript{68,69}, vesicular stomatitis virus\textsuperscript{70-72}, Sindbis virus\textsuperscript{73,74}, and oncolytic myxoma virus\textsuperscript{75}. Many OV are being evaluated clinically, for several types
of cancer. Currently, patients with late-stage or recurrent ovarian cancer are being recruited for clinical trials to evaluate the use of oncolytic measles virus, or Reovirus in conjunction with paclitaxel (NCT00408590, NCT01199263).

Oncolytic HSV has shown similar activity in OvCa, regardless of p53 status or chemotherapy-resistance\(^{56}\). Furthermore, combination strategies of OV with chemotherapy have been examined for use in ovarian cancer. Oncolytic adenovirus has been studied in conjunction with paclitaxel, cisplatin, epirubicin and gemcitabine\(^{62,76-78}\). Sindbis virus has increased efficacy when used in conjunction with irinotecan due to non-overlapping natural killer (NK)-dependent and NK-independent anti-cancer mechanisms\(^{79}\). Administration of cyclooxygenase-2 inhibitors permit repeated administration of vaccinia virus by limiting production of neutralizing antibodies against the virus. Furthermore, oncolytic HSV has been shown to synergize with DNA-damaging chemotherapy in other tumor models, such as glioblastoma and thyroid cancer\(^{80-83}\). We hypothesize that combining our oncolytic HSV with standard chemotherapy may yield a more potent therapeutic regimen against ovarian cancer.

In the study herein, we utilize two engineered oncolytic HSV, both of which selectively replicate in tumor cells due to a truncation of UL39, which encodes ICP6. This truncation ablates the viral ribonucleotide reductase (RR). RR catalyzes the reduction of ribonucleotides into deoxyribonucleotides, precursors for \textit{de novo} synthesis of DNA. Host RR is highly expressed in mammalian cells during S phase and under DNA damage and repair conditions\(^{84-87}\). By encoding its own RR, HSV is
capable of replication independent of host cell cycle. Deletion of ICP6 restricts viral replication to cycling cells which complement the RR function\(^{88}\). RR subunit 1, encoded by ICP6, has other known functions including the capability to interfere with apoptotic host defense mechanisms by interacting with caspase-8 receptor-interacting protein 1 (RIP1)\(^{89,90}\). It is also reported that ICP6 deletion ‘molecularly targets’ viral replication to tumor cells with oncogenic mutations, such as tumor suppressor p16 mutant cells, even if these cells are maintained in a quiescent state\(^{91}\).

The vector backbone utilized herein is also doubly deleted at both Infected Cell Protein (ICP) 34.5 loci, which results in an attenuated virus with enhanced safety due to the marked reduction in cytotoxicity\(^{92}\). ICP34.5 acts in modulating the host-cell innate immune response to viral infection\(^{93}\). ICP34.5 facilitates escape from host antiviral defense mechanisms. The infected host cell induces an antiviral signaling cascade which attempts to restrict viral protein synthesis by shutting off host protein synthesis. HSV evades this response, and prevents cellular shutoff of protein synthesis via dephosphorylation of host translation Initiation Factor 2a, eIF2a\(^{94,95}\). Furthermore, ICP34.5 is known to bind Beclin-1, an autophagy protein, and inhibit its incorporation into the PI3 kinase complex that catalyzes autophagosome formation\(^{96}\). Autophagy is another host mechanism of defense against pathogens. Host DNA damage protein GADD34/MyD116 functionally complements the carboxyl-terminal domain of ICP34.5, and is expressed in many cancer cells as a response to DNA damage\(^{95,97}\). ICP34.5 deletion restricts the replication of HSV in normal cells, but aberrant host cell expression of GADD34 can complement this activity, and
permit viral replication. However, host complementation does not fully recapitulate the potency of viral ICP34.5 function. In normal cells, oncolytic HSV does not yield a productive replication cycle and is eliminated by the host anti-viral immune response.

To model clinical manifestations of late-stage ovarian cancer, we utilize a murine xenograft model of ovarian cancer which mimics progressive disease, including abdominal dissemination of cancer cells, tumor nodular formation through the intraperitoneal cavity and tumor-associated ascites. 65 With this study, we examine the use of oncolytic HSV-1(oHSV) in conjunction with the conventional chemotherapeutic drug doxorubicin for treatment of the murine xenograft model of ovarian cancer. Oncolytic vector 34.5ENVE (γ34.5-Expressed by Nestin promoter and Vasculostatin-120 Expressing) is engineered to re-expresses viral ICP34.5 under the nestin promoter, restricting its expression to tumor cells expressing stem cell marker nestin and thereby enabling virus killing of the cell population. 34.5ENVE also expresses antiangiogenic molecule Vasculostatin-120 under viral Immediate Early 4/5 promoter. 98 The second virus we examine, called revertant ENVE, is a virus that was generated by removing the expression cassette containing the ICP34.5 and VStat120 genes from 34.5ENVE, and serves as a control for the effect of the specific anti-tumor genes. We also examine the effect of combining chemotherapeutic agent doxorubicin with our viral vector. Doxorubicin has not been studied in conjunction with oHSV for the treatment of ovarian cancer.
We found that OV induced cell killing of ovarian cancer cell lines, ex vivo ascites isolated from mice, and primary human ovarian cancer ascites. The 34.5ENVE virus was more potent than revertant ENVE. Furthermore, 34.5ENVE produced a functional antiangiogenic agent that reduced endothelial cell migration in vitro and reduced the presence of ascites in the murine model in vivo. Finally, when combined with doxorubicin, 34.5ENVE killed ovarian cancer cells synergistically (as analyzed by Chou-Talalay analysis), and significantly prolonged survival in vivo.
Chapter 1: Introduction to Ovarian Cancer

Ovarian cancer is the most deadly gynecologic malignancy, and the fifth most deadly cancer in women. It is a heterogeneous disease that originates in the ovaries, but is capable of spreading throughout the body. In fact, the majority of women (~70%) present with ovarian cancer in advanced stages of disease in which the cancer has disseminated throughout the abdominal cavity or to distant sites, such as the lung or bone. Late stages of disease are markedly more difficult to treat, and 5-year survival rates are less than 30\(^1\). Current standard of care includes aggressive surgery to remove all visible tumors, followed by chemotherapy. However, despite initial response, 70-80\% of women will have recurrent tumors\(^1\). In fact, 69\% of all ovarian cancer patients will succumb to their disease, as compared to 19\% of breast cancer patients.

Incidence, Risk Factors, and Morbidity & Mortality

In 2013, the American Cancer Society estimates that 22,240 new cases of ovarian cancer will be diagnosed, and 15,500 women will die of ovarian cancer in the United States. These estimates indicate that approximately 1 in 100 women will die from ovarian cancer. The vagueness of symptoms associated with disease is often attributed to belated diagnosis. Symptoms include: bloating (persistent/unusual); pelvic or abdominal pain (persistent/unusual); trouble eating or feeling full quickly; and urinary symptoms, such as urgency\(^1\).
Clinically, ovarian cancer is classified by stages which are based on tumor spread, lymph node involvement and metastasis. Pathologists grade tumor biopsies based on these contributing factors\(^1\).

- **Stage I.** Cancer is confined to one or both ovaries. Five-year survival rate: 89%.

- **Stage II.** Cancer is present in one or both ovaries, and has spread to nearby pelvic organs (i.e. fallopian tubes, uterus, bladder, rectum). Cancer has not spread to the peritoneum, lymph nodes or distant sites. Five-year survival rate: 66%.

- **Stage III.** Cancer has spread to organs within the pelvis and to nearby abdominal organs (i.e. small intestine, surface of peritoneum) and/or the lymph nodes. Five-year survival rate: 34%.

- **Stage IV.** Cancer has spread beyond the peritoneal cavity to organs such as the lungs, liver and/or bone. Cancer cells present in pleural fluid are the most common indicator of Stage IV ovarian cancer. Five-year survival rate: 18%.

The causes of ovarian cancer remain unknown, but certain factors and lifestyles are known to impact a woman’s risk of developing ovarian cancer. Risk factors include\(^1,99\):

- **Personal history.** This includes a personal history of breast cancer, or familial history of breast, ovarian or colorectal cancer.

- **Age.** Older women are at highest risk for developing ovarian cancer. Most deaths from ovarian cancer occur in women age 55 and older.
• **Weight.** Obese women (BMI of 30 or greater) have a higher risk, and higher death rate from disease. In a study by the American Cancer Society, the most obese women had a 50% increase in risk of developing ovarian cancer\(^\text{100}\).

• **Hormone therapy.** Long-term use of estrogen replacement may increase risk of developing ovarian cancer. However, risk associated with the use of estrogen and progesterone is less certain, and requires more research and longitudinal clinical studies\(^\text{101}\).

• **Specific genetic mutations.** BRCA1 and BRCA2 mutations are responsible for only a small number of cases.

Some factors and lifestyles also reduce a woman’s chance of developing ovarian cancer. These include\(^1\):

• **Reproductive History.** Having multiple children early in life lowers risk, with each pregnancy further lowering one’s risk. Breast feeding further lowers risk of developing ovarian cancer.

• **Use of hormonal birth control.** Women who take oral contraceptives for more than 5 years have a reduced risk.

• **Diet.** Women who consume a low fat diet have a slightly lower risk of developing ovarian cancer.

**Ovary Structure & Function**

The ovary is the female reproductive organ responsible for producing and releasing oocytes in the process of ovulation, and acts as an endocrine glad which releases hormones, including estrogen and progesterone. A normal female has two ovaries located in pelvic region of her body. Ovaries interact directly with the fallopian tubes,
which are two tubes lined with ciliated epithelial cells that shuttle the oocyte released from the ovary to the uterus for potential insemination and implantation. Each ovary alternates ovulating, producing one egg per month, unless one ovary is absent or dysfunctional in which case the remaining healthy ovary produces an egg every month.

The ovary has an outermost layer called the ovarian surface epithelium (OSE), which is composed of squamous-to-cuboidal epithelial cells. This outer layer acts analogously to the mesothelium of the peritoneal cavity, and covers the ovary. The innermost area is called the medulla of ovary. This inner area of the tissue does not contain egg-producing follicles, and is highly vascularized. It provides the structure to which the ovarian ligament attaches to the peritoneal wall. Between the OSE and medulla is the ovarian cortex. The ovarian follicle, the primary unit responsible for producing oocytes, and stromal support tissue compose the ovarian cortex.

The OSE is responsible for transport of materials to and from the peritoneal cavity, and participates in ovulation, including the processes of ovulation and repair\textsuperscript{102}. The OSE undergoes regulated, localized apoptosis near the time of ovulation to permit the release of the oocyte to the fallopian tube. This process is induced by prostoglandins and perhaps by Fas-mediated signaling\textsuperscript{103-105}. The OSE is also responsible for repairing the voids left by ovulation, and the proliferative capacity of the OSE is highest directly after ovulation\textsuperscript{106}. The OSE also gives rise to both the epithelial cells, and connective tissue-like components of the extracellular matrix of the ovarian cortex\textsuperscript{107-109}. These processes, including increased replication, are
regulated by hormones, including human chorionic gonadotropin, luteinizing hormone, and follicle-stimulating hormone\textsuperscript{106}. The OSE is implicated as the source of approximately 90\% of ovarian cancers\textsuperscript{110}.

The ovarian follicle is the site where the primary oocyte develops into the mature oocytes, and is the fundamental functional unit of the ovary. Throughout the menstrual cycle, multiple follicles are stimulated to grow and develop, but eventually one of these follicles becomes the dominant follicle, and results in the release of its egg. There is usually a release of only a single functional oocyte per month; the non-dominant follicles atrophy and are reabsorbed by the ovarian tissue. This process of ovulation is the second phase of the menstrual cycle.

The menstrual cycle is generally divided into three phases: the follicular phase, ovulation, and the luteal phase. This cycle is controlled by hormones produced by the endocrine system, and the cyclical rise and fall of Follicular Stimulating Hormone (FSH), Luteinizing Hormone (LH), estrogen and progesterone regulate this ovulation cycle. Increasing amounts of estrogen stimulate the production of follicles and maturation of oocytes. Ovarian follicles are present from birth and may remain dormant until stimulated for up to 50 years. LH (predominantly) and FSH stimulate the release of the oocyte. After release of the oocyte, progesterone secretion increases in order to thicken the uterine lining in preparation for implantation of an egg. If an egg does not implant during that time, the uterine lining is shed in the process of menstruation.
Pathology & Site of Origin

Tumors in the ovary exhibit a wide variety of histological features. Ovarian cancer is classified by its histology, and this classification indicates course of treatment and correlates with survival. There are four major histological classifications of ovarian cancer:

- **Surface epithelial carcinoma**, also known as epithelial ovarian carcinoma. This is the most common form of ovarian cancer, and accounts for most ovarian cancer. It originates from the ovarian surface epithelium of the ovaries.

- **Sex cord-stromal (mesenchymal) carcinoma**, which originates from either the endocrine cells located in the ovary or the structural stromal tissue. Such tumors are generally low-grade and are diagnosed as Stage I disease (confined to the ovary).

- **Germ cell carcinoma**, which originate from primordial oocytes. Most germ cell carcinomas are benign teratomas; these tumors tend to present in younger women (late teens to early twenties).

- **Mixed carcinomas**, which present with more than one of the above tumor types.

The predominant type of ovarian cancer is epithelial ovarian carcinoma (EOC), which accounts for approximately 84% of ovarian cancer cases. These cancers most likely arise from the ovarian surface epithelium. Several different histological subtypes occur, which tend to resemble epithelial cells from nearby normal tissue of the fallopian tube, endometrium or intestine. EOC is pathologically sub-characterized by the predominant epithelial cell type present within the tumor, and morphology,
immunophenotype, and genotype can help classify tumors into subtypes.

Classification is imperative for disease treatment. There are four major categories:

- **Serous or serous-papillary carcinoma.** Tumor has glandular or papillary architecture; it resembles surface epithelium of the fallopian tube. Found predominantly in stage II or IV. ~43% of cases.

- **Mucinous carcinoma.** Tumor resembles endocervical glands or normal colonic mucosa. ~5% of cases.

- **Endometrioid carcinoma.** Endometriod-like glands from the endometrium compose tumor bulk. Disease is associated with endometriosis, and resembles endometrioid cancer of the uterus. Tend to remain confined to ovary. Highly associated with endometriosis. ~10% of cases.

- **Clear cell carcinoma.** This tumor shares characteristics of both serous and endometriod EOC. Tend to remain confined to ovary. Highly associated with endometriosis. ~5% of cases. (Population statistics are summarized from the NCI Surveillance Epidemiology and End Results (SEER).)

**Theories of Disease Origination**

How and from where ovarian cancer arises is still somewhat unclear. The anatomical site of origin for high-grade serous carcinomas is generally accepted as the single layer of ovarian surface epithelium\textsuperscript{111,112}. Cause of oncogenesis is also an area of continued study, and several theories exist, including:

- Incessant Ovulation Theory\textsuperscript{113-115}
- Theory of Gonadotropin Stimulation\textsuperscript{116-119}
- Theory of Inflammation\textsuperscript{120-122}
Incessant Ovulation Theory of Disease Origination

The Incessant Ovulation Theory, first proposed by Dr. Fathalla in 1971 in a letter to The Lancet indicates that there is epidemiological evidence that there may be a relationship between the frequent process of ovulation, and cancers that arise from the epithelial surface that is involved in this process\textsuperscript{113}. Dr. Fathalla notes, “compared with other mammals, the human female appears to be very extravagant with her ova.” Whereas other mammals only ovulate during mating season or after copulation, humans ovulate monthly for decades. He hypothesized that, although continuous ovulation may have been evolutionarily favorable, most ovulation cycles are now unfruitful, and the regular cycle acts as a contributor to ovarian neoplasms\textsuperscript{113}. In his letter, Dr. Fathalla notes that women who are “denied the ovarian physiological rest periods afforded by pregnancies—nuns and unmarried and infertile women” have a higher incidence of ovarian cancers. He also notes that in countries where twins are more likely (e.g. Scandinavian countries), there is a higher incidence in ovarian cancers, whereas the opposite is true in Japan. This evidence suggests that an increase in ovulation events may contribute to an increase in ovarian cancer\textsuperscript{113}.

These epidemiological observations are also supported by scientific evidence. Ovulation involves follicular rupture and subsequently creates a void in the ovarian
surface epithelium, which is repaired via migration of nearby epithelial cells into the empty space\textsuperscript{102}. The process of proliferation, migration and surface repair results in inclusion cysts that generally repair the wound site, and are reabsorbed into the surrounding tissue. This process bears striking similarity to the wound repair process\textsuperscript{126}. Ovulation is a process which repeatedly exposes the surface epithelium to estrogen-rich follicular fluid, and follicles are repeatedly developed, ruptured and reabsorbed. Frequent ovulation and subsequent surface repair allows for the potential of ovarian epithelium to become trapped in inclusion cysts\textsuperscript{127,128}. These cysts may not resolve themselves, and thus provide an environment that contributes to malignant transformation due to loss of basement membranes, changes in hormone receptor expression and sensitivity, and changes in oxidation response\textsuperscript{127,129}.

Proper repair of the epithelial surface post-ovulation requires growth factors and steroids which may contribute to ovarian oncogenesis. Hormones and growth factors are key modulators of cell proliferation, differentiation and apoptosis. Estrogen has been shown to be mitogenic to epithelial cells\textsuperscript{130}, and estrogen stimulates the release of growth factors, such as epidermal grown factor (EGF). EGF is secreted by ovarian cancer cells, and these cells express EGF receptors which may allow autocrine growth loops to be established\textsuperscript{131,132}. Secretion of steroids such as progesterone, androstedione, testosterone and estradiol varies depending on tissue type and tumor location, but normal and tumor ovarian epithelium both express steroidal receptors\textsuperscript{133-135}. The exact involvement of hormones and steroids and their receptors in ovarian cancer is still unclear. Similarly, cytokines are known
to regulate both normal and cancerous ovarian epithelial cells, but exact mechanisms of involvement remain to be elucidated\textsuperscript{136,137}.

Repeated cell damage and repair associated with ovulation would increase the likelihood of aberrant DNA repair causing inactivation of tumor-suppressor genes and/or activation of oncogenes. The OSE cells undergo repeated cycles of replication to permit migration and repair of the tissue void left by the ovarian follicle after ovulation. Mutations or inactivation of tumor suppressor protein p53 is reported in 46\% of invasive ovarian tumors, but only 8\% of low malignant potential tumors. Mutations are nearly non-existent in benign tumor and normal ovarian tissue\textsuperscript{123}. Schildkraut reported that women with a greater mean number of ovulation cycles had an increased likelihood of developing p53-positive ovarian cancer, but did not have an increased likelihood of developing p53-negative cancer\textsuperscript{138}. p53-positive cancers over-express a mutant form of the p53 tumor suppressor protein that accumulates in the tumor cells, and does not function properly; p53 dysfunction has been seen in precursor ovarian cancer lesions, such as inclusion cysts\textsuperscript{127}. Mutant p53 is indicative of DNA damage. In this mutant form, p53 no longer acts as a tumor suppressor, and facilitates transformation. Normal p53 repairs damaged DNA, however loss of the normal protein may permit aberrant replication of DNA-damaged cells leading to tumor development, and compounded genetic mutation.

\textit{Gonadotropin Theory of Disease Origination}

The Gonadotropin Hypothesis suggests that gonadotropins secreted by the pituitary gland (e.g. luteinizing hormone [LH] and follicle-stimulating hormone[FSH]) stimulate
the ovarian surface epithelium, and promote the development of cancer\textsuperscript{116-119}. Inclusion cysts that occur when surface epithelium becomes trapped after ovulation are known to be stimulated by estrogen. However, it is understood that high levels of estrogen cannot be enough to stimulate transformation and oncogenesis as pregnancy, a known protective factor against ovarian cancer, is a reproductive event during which women’s estrogen levels are exceptionally high\textsuperscript{139}. Thus, high and persistent levels of gonadotropics (LH and FSH) are perhaps required for adequate stimulation and transformation of the epithelial cells to develop cancer. LH and FSH are known to stimulate pathways important in cell division and apoptosis, and to stimulation the production of hormones such as estrogen. The epidemiology behind the gonadotropin theory of disease was based on women in early post-menopausal years whose higher incidence of ovarian cancer correlates with an increase in gonadotropin release\textsuperscript{139}. The relationship between gonadotropin and ovarian cancer is not totally clear. Ovarian cancer cells can be stimulated in vitro with gonadotropins\textsuperscript{140,141}, and both benign and malignant tumors express gonadotropin receptors\textsuperscript{142}. Furthermore, in a study of women’s levels of gonadotropins in serum over the years, higher levels of LH or FSH do not seem to correlate with development of ovarian cancer\textsuperscript{143}. Thus, it is unlikely that gonadotropins are the sole or primary cause of disease, although it is likely they contribute to oncogenesis.

\textit{Inflammation Theory of Disease Origination}

Ness & Cottreau review epidemiological data and propose the possible role of inflammation in ovarian epithelial cancer\textsuperscript{120,144}. Firstly, many of the normal processes
and conditions that occur or are present in the ovary (e.g. high levels of hormones and gonadotropins, ovulation) increase inflammation. The authors propose that this inflammation may be the underlying modulator of carcinogenesis in the ovary. Inflammation induces DNA damage (and subsequent repair), oxidative stress, and an increase in cytokine production and prostaglandins\textsuperscript{120,143}. All of these processes may be mutagenic. Furthermore, additional risk factors, such as asbestos and talc exposure, endometriosis, and pelvic inflammatory disease are not related to ovulation, but do induce the inflammatory cascade, and are associated with an increased risk of developing cancer. Also, they note that epidemiological studies of women who use fertility drugs that cause hyper-ovulation do not seem to correlate strongly with an increase in ovarian cancer. Ness and Cottreau state that if ovulation were the causative process of disease, women who chemically induce hyper-ovulation should have an increased risk of developing ovarian cancer.

Furthermore, epidemiological data regarding the effect of hysterectomies and bilateral tubal ligations on a woman’s risk of developing ovarian cancer supports the Theory of Inflammation. Both of these operations are associated with a reduced risk of disease development\textsuperscript{145-148}. Greene et. al. proposed that this reduction in cancer is due to the physical consequences of hysterectomy/ligation in which the canal which connects the lower and upper parts of the genital tract is severed. This severing prevents the ovarian epithelium from being exposed to pro-inflammatory stimulants from the lower genital tract, such as talc, or the causative agents of pelvic inflammatory disease (e.g. sexually transmitted agents), and this reduced inflammation may account for the protective effect\textsuperscript{148}. 
Molecular Genetics of Disease

Concordant with the pathological subtyping of ovarian cancers, a study by Marquez et. al. found that gene expression profiles of varying EOC subtypes correlated to the normal cells that the cancer resembles. This study confirmed that the cell types that EOC resembles pathologically, it also resembles on the molecular level. Thus, for example, serous EOC that histologically resembles the epithelial surface of the fallopian tube also shares a similar genetic profile to the cells of the epithelial surface of the fallopian tube.

Several signaling pathways are associated with the development of ovarian cancer, including NF-kB pathway, Jak-STAT3 pathway, MAPK pathway, tyrosine-protein kinase Src pathway, ErbB activation pathway, lysophosphatidic (LPA) pathway, Mullerian inhibitory substance receptor pathway, EGF and VEGF pathways, and ER-beta pathway.

Drs. Ie-Ming Shih and Robert Kurman of Johns Hopkins Medical Institute have developed a subtyping schematic that delineated ovarian cancers by their tumorigenic course, and then matched histological subtypes with genetic changes. They delineate between two tumor types, called Type I and Type II. Type I tumors are low-grade neoplasms that arise via step-wise, identifiable transformations from a well-defined precursor lesion. Type I tumors include low grade serous-papillary carcinoma, endometrioid carcinoma, and borderline tumors with low malignant potential. Type II tumors are high-grade neoplasms with poorly
understood genetic alterations, which arise from a unknown and disputed precursor. High-grade serous carcinoma is the prototypic type II tumor $^{151,152}$.

Type I cancers are generally well-differentiated, with an indolent disease course that can last over 20 years, and overall prolonged survival (on average, 82 months). Patients generally present at a young age, with tumors confined to the ovary. Type I tumors arise from identifiable tumorigenic pre-cancerous lesions, such as cystadenomas, and tend to be chemoresistant. Tumors are characterized by accumulating genetic changes over time, with mutations in BRAF and KRAS seen in serous tumors, KRAS mutations seen in mucinous tumors, and β-catenin and PTEN mutations seen in endometrioid tumors $^{153}$. Tumor suppressor protein p53 mutations are rare in these cancers; these cells have a low proliferation index $^{154}$.

Type II cancers are induced by poorly understood genetic events. Patients with type II tumors are generally post-menopausal, and have a shortened survival of only 30 months. An in situ precursor lesion not yet never been identified by pathologists, so it is believed that type II cancers arise directly from the surface epithelium or inclusion cysts. This continues to be disputed, however $^{152}$. Although these tumors tend to be chemosensitive (at least during primary treatment), they grow quickly and disseminate rapidly; they are nearly always diagnosed in late-stage of disease. Widespread DNA copy number gains and losses involving all chromosomes indicate genetic instability $^{155,156}$. Furthermore, up to 80% of patients have p53 mutations, and this is the most frequent genetic abnormality seen in Type II EOC $^{154,157}$. Mutations in cyclin E1 are also common in these patients. These mutations are seen in tumors of
all stages, which indicate that the mutation may develop as a primary event in early stages of disease progression. Type II tumors have a high proliferation index.

**Conclusion**

Ovarian cancer is an incredibly heterogeneous disease. It is also incredibly lethal. Although the pathway to disease is not yet completely understood (and likely varies between disease subtypes and even patients), effective therapy will hinge on the ability of doctors, pathologists and scientists to determine the best course of treatment for each of the varying types and stages of disease.
Chapter 2. Ovarian Cancer Stem Cells

The cancer stem cell hypothesis is based on the concept that even within the disordered microenvironment of a tumor, a hierarchy of organization exists. Some cells within the tumor microenvironment have limited capacity of self-renewal, and are restricted to a particular cell lineage. Other cells have a less limited or unlimited capacity of self-renewal, and are capable of differentiating into a number of cell lineages. This is similar to normal tissues, wherein there are certain subpopulations called stem cells that are capable of self-renewal and multi-lineage differentiation. These populations have proliferative properties that are different from the rest of the tissue. In a tumor, this sub-population is believed to be ultimately responsible for the formation and growth of the tumor, and only through eradicating this subpopulation are we able to completely ablate the tumor’s ability to regrow. Stem-like cells, also called tumor-initiating cells, have been discovered in types of tumors, including prostate, colorectal, pancreatic, brain and ovarian cancer. Cancer stem cells compose typically 0.01-1.0% of the tumor bulk, with the capacity to undergo symmetric or asymmetric division to yield a tumor identical to that from which the stem cells were isolated.
Cancer stem-like cells have several key properties, including:

- Self-renewal and the ability to renew indefinitely while in undifferentiated state;
- Unlimited proliferation potential;
- High DNA repair capacity;
- Resistance to chemotherapy; and
- The ability to drive the expansion of malignant cells.

While tumor initiating cells present similar properties to normal stem cells, one important difference is the capacity and management of self-renewal. Normal stem cells self-renew and differentiate according to tightly regulated needs presented by the organ in which they reside. However, cancer stem cells self-renew and differentiate in a deregulated fashion, producing cells of various differentiation capacities\textsuperscript{169,170}. Thus, while cancer stem-like cells are capable of producing cells of different lineages or differentiation statuses, they are not under the strict regulatory controls which direct normal stem cells during organogenesis.

Furthermore, while standard therapies may be quite effective against the differentiated bulk of tumor, it has been shown that cancer stem cells have or rapidly develop resistance, and are able to survive and repopulate the tumor\textsuperscript{171}. As the primary therapy for ovarian cancer, platinum-based compounds show good efficacy initially, but nearly 70% of women relapse. This may be due to the reservoir of resistant stem cells that survive therapy, and reinitiate aggressive tumor growth. Due to the complexity of ovarian cancer, its different histological subtypes and still-unclear understanding of where more aggressive EOC originates, stem cell study of
ovarian cancer has been controversial. Furthermore, with its ease of metastasis and EMT (and reverse) transitions, it is also difficult to study the stem cells as they can assume quiescent or proliferative states depending on cellular microenvironment and cell stressors such as chemotherapy\textsuperscript{23,172}. Furthermore, EMT yields cells that have more stem-cell-like qualities, including increased resistance to conventional chemotherapies and surface molecule expression.

Stem-like cells are an identifiable population within the tumor, and maintain a distinct pattern of surface markers (e.g. CD133, CD44, Thy1 or SCA1), and non-surface markers (e.g. aldehyde dehydrogenase activity). These markers allow for cell isolation, and when implanted into immunodeficient mice, these tumor-initiating cells are capable of completely reproducing a tumor identical to that from which they were originally isolated. CSCs can only proliferate in certain environments, and the cross-talk between cancer cells and microenvironment is critical for the development or re-establishment of tumors. Bapat et al were the first to report the isolation and characterization of stem-like cells from OvCa patient samples. This subpopulation was grew in spheroids in anchorage-independent stem cell media\textsuperscript{(Bapat 2005)}. These cells expressed CD44, EGFR, e-cadherin, and stem cell factor CD117. Since then, many other reports of OvCa stem cells have been published. OvCa stem cells have been reported to have constitutive NFkB activity, and secrete cytokines such as IL6, IL8 and GRO\textalpha\textsuperscript{(Silasi J Biol Med 2006, Chen Oncogene 2008)}. Fibroblasts, endothelial cells and immune cells all contribute to the establishment of tumors through the secretion of soluble and insoluble factors, and ECM proteins. Immune cells have also been shown to play a critical role in tumor promotion\textsuperscript{173}. 

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Ovarian Cancer Stem Cell Markers

CD133 & Aldehyde Dehydrogenase (ALDH)

Stem cell marker CD133 is one of the best-described ovarian cancer stem cell markers. It was first described on hematopoietic stem cells, and has since been shown to be a marker for many normal and cancer stem cells. CD133 is a transmembrane protein that promotes stem-like properties by suppressing differentiation. Expression has been correlated with resistance to doxorubicin, camptothecin and radiotherapy. Aldehyde dehydrogenases are a group of highly reactive electrophilic enzymes that catalyze the oxidation of aldehydes. Endogenous aldehydes are generated by multiple metabolic processes, including lipid peroxidation, amino acid catabolism, and vitamin and steroid metabolism. Exogenous aldehydes can be generated from compounds such as chemotherapeutic drugs\textsuperscript{174}. ALDH play roles in biosynthesis and metabolism, as well as detoxification pathway. Importantly, ALDH metabolizes retinal into retinoic acid, which acts in "stem cell self-protection" \textsuperscript{175}. ALDH expression has been shown to be related to cyclophosphamide resistance. Observations regarding CD133 and ALDH in ovarian cancer are summarized in Table 1.
Table 1. CD133 and Aldehyde Dehydrogenase expression in ovarian cancer stem cells.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Ref.</th>
<th>Cell System</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD133</td>
<td>176,177</td>
<td>primary tumor samples (41 ovarian, 8 normal, 5 benign)</td>
<td>* Normal and benign tumors have significantly lower expression of CD133 than malignant.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>* CD133+CK7+ primary cells have a greater colony forming potential.</td>
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<tr>
<td>CD133</td>
<td>167</td>
<td>Subpopulations of xenograft tumors established from primary ovarian cancer (serous and clear cell) patients</td>
<td>* Studied the ability of subpopulations of cells to be isolated, implanted and subsequently recapitulate the primary tumor.</td>
</tr>
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<td></td>
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<td></td>
<td>* CD133-high expressing cell fraction had greater tumor-initiating capacity compared to CD133-low.</td>
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<td></td>
<td></td>
<td></td>
<td>* CD133+ cells injected into immunocompromised mice resulted in tumors with CD133+ and CD133- cells, which looked similar to the primary tumor.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>* None of the primary tumor xenograft tumors expressed CD117.</td>
</tr>
<tr>
<td>ALDH</td>
<td>178</td>
<td>Established cell lines and primary human tumors</td>
<td>* ALDH+ cells were able to initiate tumors identical to primary tumor, with both ALDH+ and ALDH- cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>* ALDH+ cells were 50 times more tumorigenic, and chemoresistant.</td>
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<td></td>
<td></td>
<td></td>
<td>* ALDH+ correlated with worsened prognosis in patients.</td>
</tr>
<tr>
<td>CD133 and ALDH</td>
<td>179</td>
<td>Established cell lines and primary human tumors</td>
<td>* CD133- cells that were ALDH+ were far more tumorigenic when implanted into immunocompromised mice.</td>
</tr>
<tr>
<td></td>
<td>172,180</td>
<td></td>
<td>* CD133+/ALDH+ had an even greater tumor-initiating capacity, with decreased latency period.</td>
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<td></td>
<td></td>
<td></td>
<td>* CD133+/ALDH- were unable to initiate tumor growth, although this could be attributed to the sorting process.</td>
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<td></td>
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<td></td>
<td>* CD133-/ALDH+ and CD133+/ALDH+ cells, when implanted in mice, were highly angiogenic, and robustly recruited vasculature.</td>
</tr>
</tbody>
</table>
CD44 & CD117

CD44 is also a well-established marker for cancer stem cells, having been identified as a marker in many cancer types, such as breast, prostate, colorectal, pancreatic, head and neck squamous cell carcinomas. It is a cell-surface glycoprotein that acts as a receptor for hyaluronic acid. It is involved in cell-cell interactions, cell adhesion and migration. CD44 is also involved in cell functions including lymphocyte activation, hematopoiesis, and tumor metastasis. CD44 expression in ovarian cancer tumors has shown to correlate with disease-free survival. Patients who have low or no CD44 expression display significantly longer disease-free progression than patients with CD44-positive tumors. CD44 expression also has been shown to correlate with chemoresistance to paclitaxel and carboplatin, and monoclonal antibody tratuzumab. CD117, also known as c-kit, is a cytokine receptor, and binds to stem cell factor, also called c-kit ligand. CD117 is a receptor tyrosine kinase whose signaling is important in cell survival, proliferation and differentiation. Observations regarding CD44 & CD117 in ovarian cancer are summarized in Table 2.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Ref.</th>
<th>Cell System</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>186</td>
<td>Murine ovarian cancer cell line and human ovarian cancer ascites</td>
<td>• Use Hoechst dye exclusion to identify side population (SP) of cells.</td>
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<td></td>
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<td>• SP cells were enriched for CD117 expression.</td>
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<td></td>
<td>• Human ascites SP did not display elevated CD117 expression</td>
</tr>
<tr>
<td>CD44 &amp; CD117</td>
<td>187</td>
<td>Spontaneously immortalized cell lines (19 total) from a patient with serous ovarian cancer</td>
<td>• Clones had up-regulated CD44, CD117 and scatter factor (ligand for CD117).</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>• Only one clone had tumor-initiating capacity when injected into immunocompromised mice.</td>
</tr>
<tr>
<td>CD44 &amp; CD117</td>
<td>24</td>
<td>tumor spheroids generated from serous ovarian cancer patient</td>
<td>• After ~10 passages in stem cell media, the remaining spheroids were highly enriched in CD44 and CD117.</td>
</tr>
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<td></td>
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<td>• Spheroids were compared to parental cancer cells and spheroids grown in differentiating media.</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>• RT-PCR indicated up-regulation of stem cell markers: Bmi-1, stem cell factor, Notch-1, Nanog, nestin, ABCG2, and Oct-4.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• CD44+/CD117+ cells were resistant to chemotherapy.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• When implanted into mice, these cells produced tumors that recapitulated the primary tumors from which they were isolated.</td>
</tr>
<tr>
<td>CD117</td>
<td>188</td>
<td>Subpopulations of xenograft tumors established from primary ovarian cancer tumors.</td>
<td>• Three of the 17 primary tumor xenografts produced isolatable CD117-high subpopulation of cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• CD117+ cells were capable of serial transplantation into mice and asymmetric division that yielded a tumor with both CD117+ and CD117- cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Presence of CD117+ cells in tumor correlated with chemoresistance.</td>
</tr>
</tbody>
</table>
**CD24**

CD24 is a mucin-like cell surface glycoprotein that has been identified as a stem-cell marker in pancreatic\textsuperscript{161} and liver cancer\textsuperscript{189}. It functions in cell adhesion, Wnt receptor signaling, cell activation, and apoptotic signaling. It has been implicated in tumor formation and metastasis in experimental models. Variation in CD24 expression in different cancers may be related to different tissues of origin, and its effects may be modulated via Nanog regulation\textsuperscript{189}. Expression of CD24 is correlated with resistance to cyclophosphamide, gemcitabine, and radiotherapy. Observations regarding CD24 in ovarian cancer are summarized in Table 3.
Table 3. CD24 expression in ovarian cancer stem cells.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Ref.</th>
<th>Cell System</th>
<th>Observations</th>
</tr>
</thead>
</table>
| CD24, CD133 & CD117    | 23   | Primary cell lines from human patients with serous and mucinous ovarian cancer | • CD24 is putative marker for ovarian cancer stem cells.  
  • CD24+ cells were quiescent, chemoresistant, and had the capacity to initiate tumors when implanted in immunocompromised mice.  
  • RT-PCR indicated that CD24+ cells had up-regulated stem cell factors, such as Nestin, β-catenin, Bmi-1, Oct4, Oct3/4, Notch1 and Notch 4, as compared to CD24- populations.  
  • Approximately 1% of CD24+ cells co-expressed CD133, and ~1% of CD24+ cells expressed CD117.                                                                                                                                  |
| CD24 & CD117           | 190  | Cell lines generated from cells at the periphery of tumor and at the center of tumor. | • Hypothesis: Leading edge of tumor (e.g. periphery) would be enriched for tumor-initiating cells.  
  • Clones from periphery were enriched in side population (SP) cells.  
  • SP cells were enriched for CD24 and CD117. Approximately 1% of SP co-expressed CD24 and CD117.  
  • Tumor-initiating capacity was not reported.                                                                                                                                                                                                                                                   |
| CD24, CD44 and EpCam   | 191  | Established cell lines treated with chemotherapy                            | • Chemotherapy treatment increased the percentage of SP cells that are triple-positive for CD24, CD44 and Epithelial Cell Adhesion Molecule (EpCam).  
  • “Triple Positive” cells, as compared to “triple negative” cells displayed greater invasion into Matrigel and more rapid growth when implanted into mice.  
  • Set of three markers were not assessed in human tumors.                                                                                                                                                                                                                                       |
**Nestin**

Nestin is a type VI intermediate filament, predominantly expressed in normal nerve cells where it acts in axon development\(^5\). Although it was originally found to be a marker for neural stem cells\(^5\), it is now utilized as a marker for stem/progenitor cells in many adult tissues. Nestin expression correlates with poor prognosis in some cancers, and plays a role in several processes, including growth, migration and invasion, and metastasis. However, in ovarian cancer, the research is still fairly limited.

Qin et al. studied CD133, a more common stem cell marker, and Nestin positivity, and found that dual staining was seen in >30% of serous ovarian cancer patient tissues\(^26\). In this study, nestin expression was shown to be associated with cisplatin-resistance, whereas CD133 expression was not. Furthermore, this group determined that nestin expression was an independent prognostic factor for patient survival. Microvessel density of tumors, in addition to VEGF and EGFR expression were elevated in patients with higher nestin expression. The results of this study indicate that increased nestin levels correlate with more aggressive and chemoresistant disease in this patient sample set\(^26\).

Nestin and class III beta-tubulin were examined in clinical samples of advanced serous ovarian cancer to determine role in clinical outcome by Hetland et al\(^27\). This group examined correlation between expression of these two proteins with clinical parameters, such as chemotherapy resistance and survival. Both nestin and beta-tubulin were expressed in tumor cells in >95% of samples. Level of expression did
not change pre- or post-chemotherapeutic treatment. Also, expression levels did not correlate with age, tumor grade, FIGO stage, surgical intervention, or residual disease volume. Class III beta-tubulin was found to be a prognostic indicator for poor overall patient survival, whereas nestin expression was not seen to correlate with chemoresistance or overall survival\textsuperscript{27}.

Ma et. al. studied how to propagate a subpopulation of chemotherapy-resistant cancer stem cells or initiating cells from the ovarian cancer cell line SKOV3.ip1, with the desire to make a system for studying such cell populations for treatment efficacy\textsuperscript{25}. SKOV3.ip1 cells were grown under selective pressure of levels of cisplatin and paclitaxel in serum-free stem cell culture system. The spheroids that resulted displayed elevated levels of several known stem cell markers, including nestin, Nanog, Oct4, sox2, ABCG2, CD133 and CD117. These spheroids were also more tumorigenic. DNA microarray analysis indicated that pathways involved in tumorigenesis (e.g. angiogenesis, extracellular matrix, integrin-mediated signaling, cell adhesion and cell proliferation) were altered compared to parental adherent cell lines\textsuperscript{25}.

Gao et. al. also examined stem-cell like populations \textit{ex vivo}, but these cells were isolated from patient tumor samples\textsuperscript{23}. CD24-positive subpopulation of cells isolated from patient samples showed a stem cell phenotype. Compared to CD24-negative subpopulation, CD24-positive cells were more tumorigenic, and chemoresistant. Furthermore, they possessed the capacity of self-renewal and subsequent differentiation. CD24-positive cells expressed higher mRNA levels of nestin, Bmi-1,
Oct4, Oct3/4 Notch1 and Notch4, and lower levels of E-cadherin than the CD24-negative population\textsuperscript{23}.

Zhang et. al. published with the objective to take patient samples, disaggregate, and isolate, identify and characterize a sub-population of cells that were capable of serial propagation of the original tumor phenotype in animals\textsuperscript{24}. Patient samples were disaggregated and grown in stem cell tissue culture conditions. A subpopulation of cells (called ovarian cancer initiating cells, OCICs) that was CD44-positive and CD117-positive were discovered to have the ability to initiate tumor recapitulation in mice with the injection of as few as 100 spheroid cells. This subpopulation was found to be chemoresistant to cisplatin and paclitaxel, and displayed increased expression of many stem cell markers (e.g. nestin, Bmi-1, stem cell factor\textsuperscript{5}, Notch-1, Nanog, ABCG2, Oct4) when compared to either primary tumor bulk or to OCICs grown in differentiating conditions (e.g. with serum)\textsuperscript{24}.

**Inflammation and Cancer Stem Cells**

In normal tissues, injury to the tissue results in an expansion of cells to repair the damage. This tissue remodeling is carried out by adult stem cells, which divide and differentiate to facilitate normal wound healing. Once the damage is repaired, the adult stem cells return to their quiescent state. Although many of the capabilities of normal stem cells have also been observed in cancer stem cells, cancer stem cells do not maintain the same tight levels of control\textsuperscript{192}. In tumor tissue, cancer stem cells are critical in sustaining tumor regrowth after chemotherapy or surgery. Surgery or chemotherapy acts as an “injury,” and may stimulate the stem cells to respond,
secrete inflammatory cytokines, undergo proliferation and differentiate to recapitulate the original tumor.

Clinical and epidemiological studies suggest a correlation between chronic inflammation and cancer in many different types of cancer. Development of ovarian cancer has been linked to chronic inflammation; and inflammatory pathways having been shown to initiate, sustain and enhance the growth of ovarian cancer. The NFkB pathway is a major source of pro-inflammatory cytokines, which may contribute to resistance to chemotherapy seen in cancer. Constitutive activity of the NFkB pathway has been shown to be a characteristic of CD44+ OvCa stem cells, and activation of this pathway has been shown to regulate cell proliferation, angiogenesis, chemoresistance, metastatic potential, and apoptotic suppression.

Immune inflammatory cells also modulate cancer stem cells, and in fact can facilitate their survival. The immune response includes both tumor-promoting and tumor-killing subclasses of immune cells. Paradoxically, the tumor-associated immune response can in fact induce tumor cells to maintain or acquire stem cell-like properties. This occurs due to the presence of certain molecules in the tumor microenvironment that are produced by immune cells. The NFkB pathway and MAP kinase pathway both stimulate an inflammatory response, characterized by the release of cytokines and chemokines. These pathways can be signaled by Toll-like receptor (TLR) signaling through MyD88; cellular debris resultant from released during surgery of chemotherapy treatment can be recognized by TLRs present on
immune cells. Proteins such as EGF, FGF and IL6 reduce cell death and enable stem cell onset and maintenance \(^{197-199}\). Hypoxia has also been shown to help maintain stem cell populations; glycolitic metabolism is associated with activated oncogenes and mutated tumor suppressor genes\(^{200}\).

**Conclusion**

Ovarian cancer stem cells are an important subpopulation of cells that modulate many tumor properties, including aggression, chemotherapy resistance, and recurrence. Although they compose a small portion of the bulk tumor, they are the most difficult to target and eradicate. Their eradication is of key importance for long-term patient survival. Development of novel therapies that preferentially target this subpopulation is critical in order to improve disease management and patient survival.
Chapter 3: Ovarian Cancer and Metastasis

Metastasis, the spread of tumor cells from their primary site of development to distant organs, is considered a hallmark of cancer, and is a major indicator of prognosis in patients. Invasion and metastasis is regulated by a complex set of intracellular and environmental signals that stimulate the cell to modify its cytoskeleton and metabolism, which enable the cell to survive the metastatic journey, and recolonize the secondary site\textsuperscript{201}. While metastasis via the vasculature is a hallmark of most other cancers, EOC frequently spread via the direct extension of tumor cells from the primary tumor site to nearby organs, such as the peritoneal lining or the large bowel. The primary step in which tumor cells or clumps of cells detach from primary tumor site is followed by a secondary passive step in which these cells float to their site of deposition within the peritoneal cavity. This movement is facilitated by the normal movement of fluid within the peritoneal cavity. Extensive seeding of the intraperitoneal cavity is often associated with ascites in patients.

High grade serous carcinomas grow very efficiently within the peritoneal cavity, but rarely metastasize to outside organs. In fact, even when cancer cells are infused into the venous system as a byproduct of a shunt implanted to relieve pain and discomfort associated with ascites, most patients with a shunt did not develop secondary metastasis due to hematological metastasis\textsuperscript{202}. This evidence supports Paget’s “Seed and Soil” theory, which outlines that certain tumors have a micro-
environmental preference which results in predilection for metastasis to specific organs. For ovarian cancers, the “soil” is the mesothelium, a lining that covers all the organs within the peritoneal cavity\textsuperscript{203}. Histologically, the mesothelium in a monolayer of cells attached to a basement membrane that is rich in fibronectin, integrins and collagen types I and IV\textsuperscript{204,205}. The tumor cells implant into the mesothelium, but rarely invade deeper\textsuperscript{206,207}. However, the obstruction and disease caused by dissemination of cancer cells to these sites is frequently the cause of death in patients.

Prior to detaching from the primary tumor site within the ovary, tumor cells must first undergo an endothelial-mesenchymal transition (EMT) to prepare for survival while floating through in the peritoneal fluid to their primary metastatic site. Interestingly, not all areas of the mesothelium are colonized equally in patients with peritoneal involvement. The omentum and peritoneum are the most common sites for tumor metastasis. Furthermore, although the peritoneum is ~9sq ft in the average person, only the small area on the right side of the diaphragm called the omentum, and the small bowel mesentery have preference for metastatic tumor colonization\textsuperscript{203}. Because the tumor-stromal interaction is a strong mediator of the “soil’s” affinity, these areas presumably provide a more hospitable environment for tumor growth. It is unknown whether this environment is “prepped” by the primary tumor. However it has been shown that some tumors are capable of secreting factors that activate bone marrow cells which act to prime the metastatic niche for tumor cell colonization\textsuperscript{208}.
In another report by Sugarbaker, surgeons note that the metastatic sites differ with the primary site of malignancy\textsuperscript{209}. Furthermore, metastatic nodules seem to congregate along the sites of preferential flow, and especially at site of fluid stasis. Tumors also tend colonize where peritoneal fluid is absorbed, as the tumor cells may be pulled towards these locations via natural peritoneal fluid movement\textsuperscript{210}. Surgery itself may cause increase in tumor cell seeding of the IP cavity, as fibrin deposition, inflammatory cells and growth factors that facilitate normal wound healing, are also capable of facilitating tumor cell implant\textsuperscript{211}.

**Epithelial-Mesenchymal Transition**

Upon undergoing EMT, epithelial tumor cells reduce attachments to their basement membrane and loosen intracellular adhesions. This is mediated by E-cadherin down-regulation, and N- and P-cadherin up-regulation\textsuperscript{212,213}. This is a well-known process to transition into an invasive phenotype, and is referred to as the “cadherin switch.” Both the detached tumor cells and the metastatic nodules have lower E-cadherin expression than the primary tumor\textsuperscript{214}. N-cadherin expression promotes interaction with endothelial and stromal cells. Furthermore, reduced E-cadherin expression is correlated with increased invasion, and with poor patient prognosis/survival\textsuperscript{214-216}.

Several signaling molecules promote the EMT in ovarian cancer cells. Transforming Growth Factor-beta (TGF\textbeta) is a cytokine first identified for its ability to induce changes in cytoskeleton arrangement and induce anchorage-independent growth\textsuperscript{217}. It has since been understood to mediate processes such as migration, proliferation and apoptosis. Expression and overexpression of TGF\textbeta have been associated with
malignant disease in ovarian cancer, and other cancers\textsuperscript{218}. TGF\(\beta\) signals via its receptors, and increases migration and invasive capacity by activating cytoskeletal proteins such as b-actin and filamin-a and -b\textsuperscript{219}. Epidermal Growth Factor (EGF) induces EMT via binding of EGF receptor (EGFR). EMT stimulated by EGF is associated with reduced keratin expression, increased motility, expression of pro-MMP-2 and -9, and activation of the ERK and integrin-linked kinase (ILK) pathways\textsuperscript{220}. Hepatocyte Growth Factor (HGF), endothelin-1 and bone morphogenic protein-4 (BMP4) are also important inducers of EMT in ovarian cancer cells.

After undergoing EMT, tumor cells detach from the primary site either as single cells or as clumps of cells. They look more like fibroblasts, and are better able to survive hypoxic conditions with this phenotype. Cells proliferate into spheres as they float through the peritoneal cavity. These spheres are responsive to mesenchymal growth signaling\textsuperscript{221}. Receptor and ligand expression on the tumor cells and the surrounding cells in the microenvironment are critical for facilitating metastasis. Adhesion molecules such as \(\alpha5\beta1\)-integrin and its ligand, fibronectin, are upregulated on the surface of spheroid cells\textsuperscript{222}. Fibronectin has an important role in growth and attachment of the spheroid; there are several different isoforms of fibronectin that are present within the ascites. Other integrins, such as those that bind laminin and type IV collagen are also important. All of these ligands are abundant in the peritoneal ascites, and their presence seems to indicate the reason for the restrictive environment in which ovarian cancer cells grow. Furthermore, fibronectin, laminin and collagen are the most abundant molecules present in the peritoneum.
Proteolytic molecules, such as matrix metalloproteinases (MMPs), are also important in metastasis, especially the initial detachment of tumor cell from ovary. Activated membrane-type 1 MMP (MMP-14) is critical in cleavage of α3-integrin, which allows tumor cells to detach from primary tumor\textsuperscript{223}. MMP-2 expression is up-regulated in floating spheroids, possibly to allow quick disaggregation of spheroid upon attaching to mesothelium\textsuperscript{224,225}.

Interestingly, studies of mammary epithelial cells linked the induction of EMT with the induction of stem cell phenotype, with an elevation in CD44 expression and a decrease in CD24 expression and the ability to form spheroids\textsuperscript{226}. These stem cell-like properties may be acquired following the activation of the Ras-MAPK pathway that follows EMT stimulation.

The tumor-stromal interaction is a strong mediator of the “soil’s” affinity. The primary interaction between the mesothelium and cancer cell/spheroid is mediated by integrins, especially β1 integrins (which heterodimerize with a number of different α subunits). In fact, anti-β1 antibodies inhibit adhesion of tumor cells to stromal cells\textsuperscript{227}. Mesothelial cells express receptors such as vascular cell adhesion molecule-1 (VCAM-1), which binds α4β1-integrin, and this interaction facilitates binding of the spheroid to the metastatic site\textsuperscript{228}. Antibodies against adhesion molecules inhibit migration and metastasis in a xenograft model\textsuperscript{227}. CD44 also facilitates binding of ovarian cancer cells to the mesothelium via hyaluronic acid; it is also a known stem cell marker\textsuperscript{229}. Stromal cell derived factor 1-α (SDF-1 α) has also
been shown to enhance metastasis by mediating attachment and adherence of ovarian cancer cells to the mesothelial cells\textsuperscript{230}.

As an indicator of the importance of proteolytic molecule expression, it has been demonstrated that a type IV collagenase MMP-9 is critical for tumorigensis. This molecule is critical, but is expressed by the host cells. As demonstrated \textit{in vivo}, MMP9-knockout mice have impaired macrophage infiltration, angiogenesis and tumor growth\textsuperscript{231}. MMP9 is secreted by host cells, but is critical in tumor development. Furthermore, tumor cells upregulate MMP2 upon binding mesothelium, which allows cell to bind more tightly\textsuperscript{225,232}.

When these spheres deposit onto the peritoneum, cells undergo a mesenchymal-to-epithelial transition. Once cells regain an epithelial phenotype, they become capable of responding to growth factors and sustain rapid growth. Interestingly, intact mesothelium provides a barrier and it is capable of preventing the invasion of tumor cells. The invasion into the mesothelium is an important step in metastasis. Only after the mesothelium has been disrupted, via apoptosis of the mesothelial cells for instance, can the tumor cells invade through to the extracellular matrix beneath. Although this mechanism is not totally understood, a mechanism has been proposed using colon cancer cells as a model. In this model, tumor cells secrete the Fas ligand, which binds to its receptor, CD94 on the mesothelial cells and induces apoptosis\textsuperscript{233}. Furthermore, there is data supporting the hypothesis that CD95 could support and enhance tumor growth through its non-apoptotic activities, so this secretion of Fas ligand could have both autocrine and paracrine activities\textsuperscript{234}. 

44
Upon apoptosis induction of the mesothelium, the cancer cells can invade into the ECM which promotes tumor growth\textsuperscript{207}. The ovarian cancer cells adhere to preferentially to the abundant type I collagen\textsuperscript{235}. The tumors that develop at the secondary sites of metastasis have been shown to be clonal to the primary tumor, according to a 1992 gene expression study\textsuperscript{236,237}. This is contradictory to metastases seen in other types of tumors, and may be due to a less-involved process of spreading throughout the peritoneal cavity. Furthermore, it indicates that the underlying reason for rapid metastasis may be that the primary tumor cells do not need to undergo further mutations in order to be capable of colonizing distant sites in the peritoneal cavity. After depositing at the site of metastasis, the tumor cells must also attract a new blood supply by secreting pro-angiogenic factors such as VEGF, and placental growth factor (PGF).

**Conclusion**

Because many ovarian cancer patients present with advance stage disease involving tumor spread to abdominal organs, therapeutics should target yet another tumor cell subpopulation: cancer cells which have undergone EMT but have not yet deposited onto their secondary site of metastasis. Treating this population of malignant spheroids that are in transit from the primary tumor to their metastatic niche may be an important part of managing disease and promoting patient survival.
Chapter 4: Ovarian Cancer and Angiogenesis

The process of angiogenesis contributes greatly to the progression of disease in ovarian cancer. As tumors grow in size, they require their own dedicated system of vasculature in order to receive enough nutrients from blood, and to remove waste materials that accumulate. Angiogenesis, the essential process by which tumors recruit and develop blood vessels, is critical for progression of ovarian cancer as it provides the tumor with nutrients and facilitates waste removal. The tumor secretes pro-angiogenic molecules and the tumor microenvironment itself (which is hypoxic, acidic, under mechanical stress from the tumor growth, and inflamed) also acts to stimulate the recruitment of neovasculature. This immature vasculature system is often dysfunctional and disregulated, with tortuous and aberrant vascular beds. The endothelial cells that form the new blood vessels are also abnormal, as the tumor microenvironment undergoes an “angiogenic switch” which encourages rapid and unrestrained growth\(^{238-240}\).

Angiogenesis has been shown to be important in oncogenesis, expansion and viability of ovarian cancer. Angiogenic stimulation results in malignant ascites, a major burden of disease. Peritoneal ascites accumulate due to an increase in vasculature leakiness and aberrant function of lymphatic vessels caused by angiogenic stimulation\(^{241-245}\). Angiogenic stimulation also affects the biology of tumor cells and tumor stroma, regulating proliferation, differentiation, and survival.
Therapies that target the process of tumor progression via angiogenic stimulation are of interest because many ovarian cancer patients succumb to chemoresistant disease, and thus targeting that tumor’s blood supply may be a good alternative treatment option. In fact, reduced blood perfusion and hypoxia can enhance chemoresistance of tumors. The signaling pathways that promote angiogenesis not only promote endothelial cell proliferation, migration and survival, but also affect the tumor microenvironment. Angiogenic signaling stimulates cancer growth and metastasis into the peritoneal cavity, in addition to the development of ascites.

**Ascites**

Ascites are caused by metastatic disease that has spread throughout the peritoneal cavity. "Malignant ascites" contain malignant tumor cells, an influx of immune cells and high lactate dehydrogenase levels\(^{246,247}\). Ascites fluid acts to facilitate tumor spread throughout the peritoneal cavity, providing nutrient-rich fluid in which tumor cell spheroids travel, and depositing cells onto the omentum and other peritoneal surfaces. There are many contributing factors to the formation of ascites. Firstly, obstruction of subperitoneal lymphatic channels prevents reabsorption of the nearly 1 liter of fluid per day that is naturally produced by the cavity. Furthermore, secretion of VEGF-increased vascular permeability and promote ascites formation. It is unclear if ascites are present prior to or as a result of metastatic disease (although the latter would be the likely clinical suggestion).
VEGF ligand & VEGF receptor

One major rate-limiting event of tumorigenesis is the development of tumor-associated vasculature which provides nutrients and removes waste products from the tumor microenvironment\(^{248-250}\). Tumors are incapable of growing beyond 100-200µm without obtaining a vasculature system of some sort. During this pro-angiogenic phase, tumor cells secrete pro-angiogenic soluble proteins that stimulate mature, quiescent vasculature to begin to replicate, migrate and develop new vasculature sprouts that direct blood flow to the tumor. One major modulator of angiogenesis is VEGF family and its receptors. This family of growth factors, with seven ligands (along with multiple isoforms of some ligands) and three receptors, mediate their activity via cell surface tyrosine kinase receptors which are activated upon binding to ligand\(^{251,252}\). Ligand binding induces receptor dimerization, and subsequent down-stream signaling. Soluble growth factors, such as placenta growth factor (PIGF), and cell surface proteins such as Neuropillins have been shown to enhance angiogenic signal when combined with VEGF signaling\(^{253,254}\). VEGF ligands and receptors are regulated by hypoxia-inducible factor (HIF), the ETS family of transcription factors, and reactive oxygen species, and the tumor microenvironment induces increased expression.

VEGF expression is at least partially responsible for the presence of ascites in ovarian cancer patients. One of its effects is that it increases leakiness of capillaries and increased capillary surface area. VEGF is up-regulated in ovarian cancer, as it is in many tumors, where it modulates both tumor angiogenesis and tumor growth. Stressors common to the tumor microenvironment, such as hypoxia, acidosis and
mechanical stress induce the expression of VEGF. VEGF in turn induces endothelial cell recruitment, proliferation, survival and subsequent differentiation. This stimulation yields tortuous and leaky blood vessels that are characteristic of tumor vasculature. Lymphangiogenesis and lymphatic remodeling is another hallmark of angiogenesis in OvCa. It also contributes to the accumulation of malignant ascites. VEGF secreted from CD11b+ macrophages has been shown to be responsible for producing the dysfunctional lymphangiogenesis observed in OvCa\textsuperscript{255}.

VEGF also contributes to metastasis in ovarian cancer. VEGF signaling results in leaky vasculature which causes fluid accumulation in the peritoneal cavity and the development of ascites\textsuperscript{32,241}. Tumor cells utilize the peristaltic movement of this fluid to seed other locations through the cavity. Furthermore, this increase in intraperitoneal fluid results in restructuring of the extracellular matrix, making it less rigid and thus increasing the ability of tumor cells, endothelial cells and fibroblasts to migrate through it. Furthermore, neovasculature have fenestrated basement membranes, making them good avenues for dissemination\textsuperscript{248}.

**VEGF Signaling Pathway**

VEGF intracellular signaling is mediated through one of three receptors, all of which consist of seven extracellular domains, a single transmembrane-spanning region, and intracellular kinase signaling domains. The intracellular signaling domains consist of an ATP binding domain (called TKD1), a kinase insert domain (KID), and a phosphotransferase domain (TKD2). VEGFr1 is primarily expressed on monocytes and macrophages, VEGFR2 is primarily expressed in vascular endothelial cells, and
VEGFr3 is primarily expressed on lymphatic endothelial cells. VEGFR2 is believed to be the main modulator of pathogenic angiogenesis stimulated by VEGFA during cancer progression.

Upon binding the VEGF ligand, the receptors homo- or hetero-dimerize and induce the signaling cascade. Co-receptors such as neuropilins and integrins can associate with the ligand-receptor complex and modulate signaling. The binding of the ligand and receptor dimerization induces a conformational change that exposes the ATP-binding site in the intracellular kinase domain of the receptor complex. Exposure of the ATP binding site permits trans or auto-phosphorylation of the intracellular tyrosine kinase residues. All three VEGF receptors have multiple tyrosine kinase residues, and phosphorylation of each can modulate different processes including endothelial cell proliferation, migration, or organization into a three-dimensional structure (e.g. vascular tube). VEGFR2 can stimulate a plethora of pathways, including MEK/ERK (via RAS/RAF) to induce proliferation; PLCb3 and p38 MAPk to induce migration; and blockade of caspase-9 and caspase-3/7 activation to prevent apoptosis256.

**Thrombospondins as anti-angiogenic agents**

Thrombospondins TSP-1 and TSP-2 are potent endogenous inhibitors of angiogenesis. They modulate direct effects on endothelial cells, and their signaling can inhibit migration and proliferation, suppress nitric oxide signaling, and reduce endothelial cells survival by inducing apoptosis. Furthermore, thrombospondins antagonize the pro-angiogenic activities of VEGF via inhibition of its release into the
extracellular matrix, inhibition of VEGF interaction with its receptor via direct binding, and inhibition of signal transduction\textsuperscript{257-259}.

Given the extensive effects that thrombospondins have on the angiogenic pathway, they have become molecules of interest for use as a clinical therapy. One TSP1 mimic, ABT-510, is currently in clinical trials for several tumor types, including soft tissue sarcomas, metastatic melanoma, and glioblastoma\textsuperscript{260-263}. While these clinical trials have established the safety profile of using thrombospondin mimic peptides, the clinical outcomes are still unclear. It is possible that such peptides will be more efficacious when combined with chemotherapy or other forms of cancer treatment.

ABT-510 and ABT-898, two thrombospondin-1 mimic proteins, have been tested in the orthotopic, syngenic model of epithelial ovarian cancer with promising results. Treatment with ABT-510 or ABT-898 induced tumor regression, reduced ascites fluid volume and intraperitoneal dissemination, and prolonged survival. Treatment also reduced aberrant tumor-associated vasculature, decreased VEGF expression, and reduced tumor hypoxia. ABT-510 showed effects against OvCa in vitro, indicating that its effects were not solely mediated via tumor vasculature\textsuperscript{52}. Combination therapy of ABT-510 with paclitaxel or cisplatin resulted in increased apoptosis of both the tumor cells and the tumor vasculature, mediated via vasculature normalization\textsuperscript{53}. Thus, we hypothesize that an anti-angiogenic therapy that utilizes the fragment of BAI1 that contains the thrombospondin type I repeats (VStat120) will be a potent inhibitor of angiogenesis and tumor progression in our ovarian cancer model.
Brain Angiogenesis Inhibitor-1 (BAI1)

Brain-specific angiogenesis inhibitor-1 (BAI1) is an anti-angiogenic protein that contains five thrombospondin type I repeats, and was originally described to be a p53-transactivated protein\(^ {39}\). BAI1 has been shown to reduce neovascularization \textit{in vitro}\(^ {39}\), and reduce tumor angiogenesis and delay tumor growth \textit{in vivo}\(^ {40-42}\). It was originally described to be down-regulated or absent in glioblastoma patient samples, while continuing to be expressed in normal brain\(^ {39,41}\). Since its discovery, BAI1 has been shown to be down-regulated in several different tumor types regardless of p53 expression (colorectal\(^ {43,44}\); pulmonary adenocarcinoma\(^ {45}\); non-small cell lung\(^ {46}\); gastric\(^ {47}\); renal\(^ {48,49}\), with reduced expression being associated with increased angiogenesis, elevated intratumoral microvessel density, increased tumor aggression, and decreased survival in patients.

The extracellular fragment of BAI1, called Vasculostatin-120 (VStat120), can be cleaved at its G protein coupled receptor proteolysis site (GPS) via autoproteolysis, and released in a soluble form. VStat120 contains the five thrombospondin type I repeats, and modulates the potent antiangiogenic activity of BAI1 binding cell surface glycoprotein CD36, which is expressed by microvascular endothelial cells\(^ {50,51}\). Binding of CD36 induces downstream signaling through src kinases fyn and lyn, activating p38 MAP kinase which leads to caspase activation and endothelial cell apoptosis\(^ {51}\). The TSR of BAI1 have been shown to be sufficient to prevent endothelial cell growth\(^ {264}\). The anti-proliferative activity of this domain has also been shown to be mediated by binding $\alpha_v\beta_5$ integrins on endothelial cells.
In patient glioblastoma samples that were positive for BAI1 staining, there was a loss of expression in tumor cells surrounding pseudopalisading foci. These foci are known to be hypoxic, and the down-regulation may be related to the lower local oxygen levels\(^{41}\). (Thrombospondin-1 has been shown to be down-regulated in hypoxic environments\(^{265}\).) In a study by Nam et.al, BAI1 expression was found to be associated with a better response to radiation therapy in human glioblastoma samples\(^{266}\). Furthermore, several individual factors were associated with favorable outcomes in a univariate survival analysis (e.g. low VEGF, high p53), but were not determined to be independent factors in the multivariate analysis. However, when patient samples were separated into groups by mRNA levels of VEGF and BAI1, a difference in survival rate was seen. Patients with high VEGF and no BAI1 were more often associated with a poor clinical outcome (on average, 6 months survival), whereas patients with tumors expressing BAI1 and lower VEGF survived nearly 14 months\(^{266}\). These clinical outcomes suggest that BAI1 expression is one factor that affects outcome and treatment susceptibility. The balance of angiogenic and anti-angiogenic factors is important in a tumor's ability to resist clinical therapy.

Histidine-rich glycoprotein (HRGP) has also been described as a functional modulator of BAI1. HRGP is a CD36 decoy protein present in the circulation that blocks that anti-angiogenic activity of thrombospondin-1. HRGP was shown by Klenotic et. al. to bind Vasculostatin120\(^{51}\). This interaction is mediated by the CLESH domain present in HRGP. In a subcutaneous model of glioblastoma cells which stably express VStat120, co-expression of HRGP increased tumor growth and vascularity. Furthermore, 85% of human GBM tissue stained positive for HRGP. This
data indicated that when HRGP is present in the tumor microenvironment, it binds Vasculotstatin120, and prevent its interaction with CD36 and subsequent antiangiogenic activity.

**Antiangiogenic Agents being used to treat Ovarian Cancer.**

Targeting angiogenic pathways regulated by vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietins, and fibroblast growth factor (FGF) are currently being evaluated for use either as single agent treatments or in combination with standard chemotherapy regimens. Therapies targeting the VEGF pathway, such as anti-VEGF monoclonal antibody Bevacizumab (Avastin®)267,268, fusion protein VEGF trap with picomolar affinity for VEGF-A (Aflibercept/ZALTRAP ®)269, or tyrosine kinase inhibitor Imatinib mesylate (Gleevec)270-272 have shown clinical efficacy in patients, highlighting the importance of angiogenic pathways. Furthermore, although angiogenic pathways are presumably independently regulated, there is evidence that there are strong connections between different pathways (e.g. correlation between levels of VEGF and PDGF-BB in malignant ascites), and altering one pathway may affect others 38. In the context of ovarian cancer, the VEGF-EGFR signaling pathways have been shown to have a good deal of relevance in disease progression. This is most clearly displayed by the clinical efficacy of anti-VEGF therapies, notably a phase II trial of bevacizumab for treatment in patients with persistent or recurrent epithelial ovarian cancer273. In clinical samples, VEGF expression is seen in tumor and malignant ascites samples, with elevated levels found in malignant tumor types, as compared
to intermediate or benign tumors\textsuperscript{274}. Furthermore, increased VEGF expression correlates with progression in tumor staging, with higher levels of expression seen in late-stage disease. VEGF has been described as an independent poor prognosis indicator\textsuperscript{275}. Table 4 outlines recent studies which examine the use of different antiangiogenic agents, with different mechanisms of action, for patients with ovarian cancer.
Table 4. Anti-angiogenic agents in Phase I clinical trials for use against ovarian cancer.

<table>
<thead>
<tr>
<th>Target</th>
<th>Treatment</th>
<th>Mechanism of Action</th>
<th>Patient Population</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>Bevacizumab (Avastin)</td>
<td>mAb against VEGF-A</td>
<td>recurrent; platinum-resistant &amp; -sensitive</td>
<td>35,268</td>
</tr>
<tr>
<td>VEGF-A, VEGF-B, PIGF-1, PIGF-2</td>
<td>Aflibercept (VEGF Trap)</td>
<td>extracellular domain of VEGFR1 and VEGFR2 fused to the Fc of human IgG1</td>
<td>recurrent platinum-resistant</td>
<td>276</td>
</tr>
<tr>
<td>ABL, c-kit, PDGFR tyrosine kinase receptors</td>
<td>Imatinib mesylate (Gleevec)</td>
<td>tyrosine kinase inhibitor (binds intracellular pocket of TK, preventing ATP binding and inhibiting subsequent activation of TK signaling)</td>
<td>recurrent; platinum-resistant &amp; -sensitive</td>
<td>270,277</td>
</tr>
<tr>
<td>ABL, Src, PDGFR, c-kit</td>
<td>Dasatinib</td>
<td>dual SRC/ABL kinase inhibitor</td>
<td>recurrent</td>
<td>NCT 0067 1788</td>
</tr>
<tr>
<td>Raf, PDGFR, VEGFR</td>
<td>Sorafenib</td>
<td>synthetic compound that blocks RAF kinase and VEGFR/PDGFR signaling</td>
<td>recurrent; platinum- resistant &amp; -sensitive</td>
<td>278</td>
</tr>
<tr>
<td>VEGFR, PDGFR, c-kit, Ret, FLT-3</td>
<td>Sunitinib</td>
<td>Tyrosine kinase inhibitor</td>
<td>recurrent; platinum- resistant &amp; -sensitive</td>
<td>279</td>
</tr>
<tr>
<td>VEGFR, PDGFR, c-kit</td>
<td>Cediranib</td>
<td>Tyrosine kinase inhibitor</td>
<td>recurrent; platinum- resistant &amp; -sensitive</td>
<td>280,281</td>
</tr>
<tr>
<td>VEGFR, PDGFR, c-kit</td>
<td>Pazopanib</td>
<td>Tyrosine kinase inhibitor</td>
<td>recurrent; platinum- resistant &amp; -sensitive</td>
<td>282</td>
</tr>
</tbody>
</table>
Furthermore, combination therapy utilizing standard of care chemotherapy in combination with anti-angiogenic agents may produce a more favorable outcome due to the targeting of multiple mechanisms. Clinically, combination therapies are easier to implement in clinical trials because they augment the standard of care chemotherapy with a secondary therapeutic. Table 5 summarizes recent Phase II-III trials combining antiangiogenic agents with various types of chemotherapy.
Table 5. Phase II-III clinical trials combining antiangiogenic agents with chemotherapy for use against ovarian cancer.

<table>
<thead>
<tr>
<th>Chemotherapy</th>
<th>Antiangiogenic Agent</th>
<th>Trial Phase</th>
<th>N</th>
<th>Response Rate</th>
<th>Patient Population</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>carboplatin + paclitaxel</td>
<td>bevacizumab</td>
<td>III (ICON 7)</td>
<td>1528</td>
<td>RR 78.5% (control arm, 57.4%)</td>
<td>first line therapy</td>
<td>283</td>
</tr>
<tr>
<td>carboplatin + paclitaxel</td>
<td>bevacizumab</td>
<td>III (GOG 218)</td>
<td>Increase in PFS by 3.5 months</td>
<td>recurrent, platinum-res &amp; -sens</td>
<td>284</td>
<td></td>
</tr>
<tr>
<td>carboplatin + gemcitabine</td>
<td>bevacizumab</td>
<td>III (OCEANS)</td>
<td></td>
<td></td>
<td>recurrent, platinum-res &amp; -sens</td>
<td>285</td>
</tr>
<tr>
<td>cyclophosphamide</td>
<td>bevacizumab</td>
<td>II</td>
<td>70</td>
<td>partial RR 24%; stable disease 63%</td>
<td>recurrent, platinum-res &amp; -sens</td>
<td>286</td>
</tr>
<tr>
<td>topotecan</td>
<td>bevacizumab</td>
<td>II</td>
<td>18</td>
<td>partial response 25%; stable disease 35%</td>
<td>recurrent, platinum-res</td>
<td>287</td>
</tr>
<tr>
<td>gemcitabine</td>
<td>sorafenib</td>
<td>II</td>
<td>26</td>
<td></td>
<td>recurrent, platinum-res</td>
<td>288</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>bevacizumab</td>
<td></td>
<td></td>
<td>response rate 60%; stable disease 26%</td>
<td></td>
<td>289</td>
</tr>
<tr>
<td>pegylated liposomal doxorubicin (PLD)</td>
<td>bevacizumab</td>
<td>II</td>
<td></td>
<td>RR 33%; 73% CR/PR/SD</td>
<td>recurrent or progressive</td>
<td>290</td>
</tr>
<tr>
<td>PLD + carboplatin</td>
<td>bevacizumab</td>
<td>II</td>
<td></td>
<td>RR 72.2%</td>
<td>recurrent, platinum-sens</td>
<td>291</td>
</tr>
</tbody>
</table>
Conclusion

The angiogenic pathway is an important modulator of tumor progression. Many anti-angiogenic agents have shown some clinical efficacy via reducing tumor progression or via disease stabilization in ovarian cancer patients. Developing novel strategies that incorporate anti-angiogenic capabilities with other forms of therapy may yield a more potent reduction in tumor burden and prolong patient survival. Targeting multiple pathways may be able to overcome single-mechanism resistance that often develops when single therapies are used to treat ovarian cancer patients.
Chapter 5: Oncolytic Viral Therapy

Oncolytic viruses (OV) are derived from natural viruses that have the capability to replicate and lyse cells as a part of their normal life cycle. The concept behind use of OV as cancer therapeutics adheres to the following paradigm (from Russell, et.al. 292): Delivery of OV to tumor yields killing of the infected cells during the first round of infection, and nearby cells (“bystander effect”) that are infected after subsequent rounds of infection. This initial “viral debulking” of the tumor is further enhanced by a host immune response to the viral-infected /viral-lysed cells, which in turn induces an immune response to cancer antigens. Clearance of residual un-infected cells is mediated by the anti-tumor immune response, which was induced during the anti-viral immune response.

Viruses have natural tropism for cells which act as the natural reservoir (for instance, HSV for neurons, HIV for T helper lymphocytes, and adenovirus for epithelial cells). However, many viruses have been found to have preferential tropism for tumor cells. This is mostly due to alteration in biology of the cancer cells, as compared to normal cells. Tumor cells often have reduced regulation of their host anti-viral immune response, such as the interferon pathway which reduces protein transcription upon infection of virus 292. They also are more resistant to apoptosis. Thus, the aberrant signaling of the cancer cells provides a more ideal environment for virus replication, and this allows viruses to preferentially replicate in cancer. Some oncolytic viruses
are used in an unmodified context because they simply appropriate natural tropism and capacity to act as OV therapy for some cancers. Examples of such “oncolytic wild viruses” include reovirus, myxoma virus, New Castle Disease virus, and vesicular stomatitis virus (VSV). Most OV, however, are modified to alter tropism, or modulate/enhance their natural cytolytic activity. Viruses can be modified to target cancer cells in several ways: translational targeting via deletion of wild-type viral genes; transcriptional targeting; and/or cellular or transductional targeting\textsuperscript{292}.

Transcriptional targeting involves engineering a virus to express essential or toxic genes under the direction of tumor or cell type-specific promoters and enhancers. This restricts gene expression to cell populations that express certain types of genes. For example, nestin has been shown to be upregulated in cancer stem-like cells. Toxic transgenes expressed under the regulation of the nestin promoter and enhancer would be restricted to cancer stem-like cells. Several different species of viruses have also been genetically modified to produce an attenuated virus that, due to the attenuation, becomes tumor selective. HSV, adenovirus, vaccinia virus and poliovirus have all been attenuated in order to yield a tumor-targeted virus\textsuperscript{293-295}. Viral components that influence cell cycle and survival are often deleted in attenuated viruses, such as HSV thymidine kinase, ribonucleotide reductase and ICP34.5. Many OV are engineered to be less capable of replication in normal cells via deletion of immunomodulatory genes (such as the HSV ICP34.5, NS1 of influenza and the C and V protein of paramixoviruses) that help produce a favorable environment for replication of the virus. Such deletions make OV safer clinically, although they can also reduce the potency of the virus against tumor cells.
Reinserting these potent viral genes under tumor-specific promoters can also restrict their expression to cancer cells.

Alternately, viruses can be transductionally targeted to cancer cells by engineering targeting ligands into their capsid, which facilitate transduction and enhance infection into cells that express certain surface molecules. Examples such as EGF and RGD peptide facilitate transduction into tumor cells or tumor-associated vasculature on which the receptors (EGFR, integrins) are expressed exclusively or are markedly upregulated, as compared to normal cells.

**Oncolytic Viral Therapy for Ovarian Cancer**

Oncolytic viral therapy is of interest for treating patients with progressive or refractive ovarian cancer. Eleven different types of viruses have been examined pre-clinically for use against ovarian cancer, most with some-to-robust response in mouse models. Adenovirus, herpes simplex virus, measles virus and vaccinia virus have been most extensively characterized and studied. Pre-clinical studies involving OV for ovarian cancer are summarized in Table 6.
Table 6. Pre-clinical studies using oncolytic viruses against ovarian cancer.

<table>
<thead>
<tr>
<th>Oncolytic Virus</th>
<th>Genetic modification</th>
<th>Targets tumor cells via...</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>E1a CR2 deletion</td>
<td>aberrant Rb pathway</td>
<td>77,296, 297</td>
</tr>
<tr>
<td></td>
<td>E1b deletion</td>
<td>aberrant p53 pathway; aberrant mRNA transport</td>
<td>298,299</td>
</tr>
<tr>
<td></td>
<td>Tropism modification</td>
<td>RGD insert targeting integrins &amp; defective Rb/p16 pathway; chimeric Ad3/5 capsid targeting desmoglein-2 &amp; defective Rb/p16 pathway; retargeted to EGFR via Cetuximab insertion</td>
<td>60,62,300-303</td>
</tr>
<tr>
<td></td>
<td>Transcriptional targeting</td>
<td>tumor-specific promoter-driven E1a (e.g. hTERT, MDR1, SLPI)</td>
<td>304-308</td>
</tr>
<tr>
<td>Echovirus</td>
<td>none</td>
<td>tropism for integrins; defective IFN pathway</td>
<td>309,310</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>ICP3.5 deletion (immunomodulatory gene)</td>
<td>aberrant PKR or PI3k pathways</td>
<td>56,96,311</td>
</tr>
<tr>
<td></td>
<td>ICP10 deletion</td>
<td>activated Ras/MEF/MAPK pathway</td>
<td>312</td>
</tr>
<tr>
<td></td>
<td>ICP6 deletion (viral ribonucleotide reductase)</td>
<td>upregulated expression of ribonucleotide reductase</td>
<td>313,314</td>
</tr>
<tr>
<td></td>
<td>double deletion: ICP34.5 and ICP6</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>addition of membrane fusion capability</td>
<td>syncytial mutant or insertion of hyperfusogenic glycoprotein</td>
<td>57,314</td>
</tr>
<tr>
<td>Maraba virus</td>
<td>attenuated strain</td>
<td>defective IFN pathway</td>
<td>315</td>
</tr>
</tbody>
</table>

(Continued)
Table 6, continued.

<table>
<thead>
<tr>
<th>Oncolytic Virus</th>
<th>Genetic modification</th>
<th>Targets tumor cells via...</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measles virus</td>
<td>attenuated strain grown in tissue culture (Edmonston strain)</td>
<td>tropism for CD46 receptor</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>attenuated strain with addition of monitoring capability</td>
<td>tropism for CD46 receptor and expressing human cacinoembryonic antigen or the sodium-iodine symporter NIS (for monitoring)</td>
<td>317,318</td>
</tr>
<tr>
<td></td>
<td>attenuated strain with reintroduction of wild-type genes</td>
<td>tropism for CD46 with reintroduction of wild-type P/V/C protein which blocks antiviral IFN response pathway activation</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td>tropism modification</td>
<td>incorporation of antibody; FRalpha receptor</td>
<td>320</td>
</tr>
<tr>
<td>Mumps virus</td>
<td>attenuated vaccine</td>
<td>unknown</td>
<td>317,321</td>
</tr>
<tr>
<td>Myxoma virus</td>
<td>none</td>
<td>defective INF/IFN pathway; activated Akt</td>
<td>75,322,323</td>
</tr>
<tr>
<td>Reovirus</td>
<td>none</td>
<td>activated Ras-signaling pathway; also primes anti-tumor immune response</td>
<td>324-327</td>
</tr>
<tr>
<td>Sindbis</td>
<td>none</td>
<td>tropism for LAMR; defective IFN pathway</td>
<td>74,328-331</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>attenuated strain or thymidine kinase(TK)-/Vaccinia Growth Factor(VGF)-deleted</td>
<td>Ras-signaling pathway, defective IFN pathway; upregulated cellular TK; activation of VEGF-signaling pathway</td>
<td>68,332-335</td>
</tr>
<tr>
<td>Vesicular Stomatitis virus</td>
<td>none</td>
<td>defective IFN pathway; defective translational control pathways</td>
<td>71,72,3,36</td>
</tr>
</tbody>
</table>

64
Of these above viruses that have been tested pre-clinically for use against ovarian cancer, several have been examined for use clinically with mixed responses. Clinical trials utilizing OV against ovarian cancer are summarized in Table 7.
Table 7. Clinical trials utilizing oncolytic virus for ovarian cancer patients.

<table>
<thead>
<tr>
<th>Oncolytic Virus</th>
<th>Name</th>
<th>Trial Phase</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Adenovirus Onyx-15</td>
<td>I</td>
<td>no clear response</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>Ad5-delta24-RGD</td>
<td>I</td>
<td>15 with stable disease and 6 with progressive disease; no partial or complete responses</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>Ad5-delta24-GMCSF</td>
<td>compassionate use (Finland)</td>
<td>1 complete response, 1 stable disease and 1 minor response</td>
<td>339</td>
</tr>
<tr>
<td></td>
<td>Ad5/3-delta24-GMCSF</td>
<td>compassionate use (Finland)</td>
<td>1 stable disease</td>
<td>340</td>
</tr>
<tr>
<td>Measles virus</td>
<td>MV-CEA</td>
<td>I</td>
<td>14 stable disease</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td>MV-CEA + MV-NIS</td>
<td>I</td>
<td>NA</td>
<td>NCT 00408590</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>JX-594</td>
<td>I</td>
<td>2 stable disease</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td>GL-ONC1</td>
<td>I/II</td>
<td>NA</td>
<td>NCT 01443260</td>
</tr>
<tr>
<td>Reovirus</td>
<td>Reolysin; Reolysin with paclitaxel</td>
<td>I; I/II</td>
<td>NA</td>
<td>NCT 00602277; NCT 01199263</td>
</tr>
</tbody>
</table>
Oncolytic Viral Therapy with Anti-Angiogenic Agents

The interplay between the tumor and its microenvironment contribute to oncogenesis, aggressiveness of disease, and tumor progression. These interactions can also affect and mediate the response to therapy. OV is also influenced by the tumor microenvironment, as thoroughly reviewed by Wojton\textsuperscript{343}. The tumor microenvironment affects viral infection, replication and spread throughout the tumor.

Tumor vasculature is one major modulator of the effectiveness of OV therapy. The vasculature can affect and be affected by OV therapy in several ways. First, vasculature can be the mechanism of delivery of OV to the tumor if the therapy is administered intravenously. Thus, normalization of the vasculature and better tumor perfusion would yield more effective delivery of the therapeutic. Aberrant and tortuous vasculature, reduced perfusion and elevated interstitial pressure all can act as barriers to OV therapy, especially when delivered to tumors via the intravenous route. Several antiangiogenic agents, including monoclonal antibody against VEGF, bevacizumab, have been shown to “normalize” tumor vasculature and reduce interstitial pressure\textsuperscript{344}. Combining antiangiogenics with OV is of therapeutic interest, and has been studied, with many studies reporting better results with combined therapy (Reviewed in Wojton\textsuperscript{343}). The effect of hypoxia on OV therapy varies with the type of virus and model used, with some viruses (such as adenovirus) having reduced replication and oncolysis\textsuperscript{345-347}, while other viruses (such as HSV) having enhanced replication kinetics\textsuperscript{348,349}. 
Secondly, the OV can infect and lyse the tumor-associated vasculature and spread to nearby tumor cells in that manner; in this way, OV therapy can have a direct antiangiogenic effect. Several reports on this suggest that this direct effect may modulate therapeutic efficacy\textsuperscript{58}. However, studies have also shown that OV therapy can elicit a bystander effect on the tumor vasculature in a pro-angiogenic manner\textsuperscript{350,351}. This pro-angiogenic signaling seems to be modulated by residual tumor cells that are able to escape the initial viral infection and lysis cycle. The cells then find themselves in an environment that is conducive to the growth of new vasculature. Thus the tumor that regrows after the initial tumor regression has greater microvessel density.

Oncolytic viral therapy approaches to treating cancer using BAI1 expression have been employed in several models. Adenovirus expressing BAI1 have been injected intratumorally into subcutaneous and intracerebral models of glioblastoma. This treatment lead to extensive necrosis and reduced tumor vascularity\textsuperscript{42}. Similar disruption of vascularity was seen in a renal cancer model\textsuperscript{48}. Oncolytic viruses have also been developed to express VStat120. Hardcastle et.al. and Yoo et. al. both reported on an oncolytic HSV expressing VStat-120 that targeted glioblastoma in a replication-dependent manner\textsuperscript{98,352}. This virus reduced vessel density in both the subcutaneous and intracranial models of GBM.

Conclusion

Oncolytic viral therapy is a promising potential therapeutic for patients with progressive ovarian cancer. Continued studies and further optimization are needed,
however. It is likely that engineering a virus to target multiple pathway (e.g. oncolysis of tumor cells combined with anti-angiogenic component) will be more potent. Use of OV with chemotherapy is also of interest, as combination strategies are easier to implement clinically when the OV strategy is combined with the standard of care for patients.
Chapter 6. Combining Oncolytic Viral Therapy with Doxorubicin to Treat Ovarian Cancer

Abstract

Purpose. Novel therapeutic regimens are needed to improve dismal outcomes associated with late-stage ovarian cancer. Oncolytic viruses have shown efficacy against ovarian cancer. We studied the application of an oncolytic herpes simplex virus expressing two anti-tumor genes: ICP34.5 under the stem cell-specific nestin promoter, and anti-angiogenic molecule Brain-specific Angiogenesis Inhibitor-1 (BAI1) under the strong viral promoter IE4/5 to treat ovarian cancer. We also studied the use of this viral vector in combination with a second-line standard of care chemotherapeutic drug, doxorubicin.

Experimental Design. The 34.5 Expressing Nestin-driven Vasculostatin-120 Expressing (34.5ENVE) virus was tested for treatment of ovarian cancer in vitro and in vivo. Efficacy of the virus, and its antiangiogenic effects on endothelial cells were assessed in vitro in cancer cell lines and in primary patient ascites samples. Scope of cytotoxic interactions between 34.5ENVE and chemotherapeutic agent doxorubicin were evaluated using Chou-Talalay synergy analysis. Efficacy of oncolytic viral therapy in combination with doxorubicin was evaluated in vivo in the murine xenograft model of progressive human ovarian cancer.
Results. 34.5ENVE showed robust efficacy against ovarian cancer cell lines, and even greater efficacy against ex vivo mouse and patient ascites. When combined with doxorubicin, 34.5ENVE killed synergistically with a robust increase in caspase-3/7 cleavage. The combination of doxorubicin and 34.5ENVE significantly prolonged survival in nude mice bearing intraperitoneal ovarian cancer tumors.

Conclusions. This study establishes the potential for use of oncolytic HSV in combination with doxorubicin for the treatment of late-stage ovarian cancer.

Translational Relevance.

Ovarian cancer is the 5th most deadly cancer in women, and two-thirds of women present with disease that has already metastasized to abdominal organs. Less than 30% of patients presenting in late stages of disease have 5-year survival. While many women show disease regression after standard therapy of carboplatin and/or paclitaxel, nearly 70% recur. Recurrent tumors are often resistant to first-line therapeutics. This may be due to a reservoir of chemotherapy-resistant stem-like cells that survive therapy, and reinitiate aggressive tumor growth. Second line therapies include chemotherapeutic agents such as doxorubicin or topotecan, and anti-angiogenic agents such as bevacizumab. Novel therapies are needed, as many patients present with late-stage disease or recur with chemotherapy-resistant disease. Research that develops and tests therapeutics that are able to target cancer-initiating cells is highly significant.
Introduction.

Epithelial ovarian cancer (OvCa) is the 5th most deadly cancer in women, with over 22,000 new cases and 14,000 deaths in the United States in 2013\textsuperscript{353}. Two-thirds of women present with progressive disease wherein the cancer has already spread to abdominal organs or distant sites\textsuperscript{354}. The five-year relative survival rate for these patients is less than 30\%\textsuperscript{354}. Primary treatment for ovarian cancer involves cytoreductive surgery and a platinum and/or taxane chemotherapeutic regimen\textsuperscript{355}. Unfortunately, standard therapies have shown limited efficacy with nearly 70\% patients presenting with recurrent chemo-resistant disease\textsuperscript{353,356}.

OvCa recurrence is often attributed to a small sub-population of cancer stem-like cells that maintain or rapidly develop resistance to chemotherapy\textsuperscript{2-4,357}. OvCa stem-like cells are critical for re-initiation of tumor growth in pre-clinical models, and are capable of serial propagation of the original tumor phenotype in animals\textsuperscript{23,24}. OvCa stem-like cells isolated from primary patient samples have been shown to display increased nestin expression compared to adherent cells, differentiated cells, and bulk OvCa tumor\textsuperscript{23,24}. Nestin is an intermediate filament first described to be expressed in stem cells within the neural tube at a higher level than differentiated cells, and was described to be critical in the pathway of neural differentiation\textsuperscript{5}. More recently, its expression has been described in stem-like cells in many types of cancer\textsuperscript{8}, and has been found to correlate with poor prognosis\textsuperscript{358}. Thus, a treatment regimen designed to preferentially target nestin-expressing cancer cells may have improved therapeutic efficacy and prolong survival.
The process of angiogenesis is also a key component in enabling ovarian cancer to grow and metastasize (REFS). High levels of intra-tumoral VEGF and VEGF receptor correlate with poor patient prognosis and survival\textsuperscript{359}, and its increased expression contributes to the formation of malignant ascites, a major burden of disease\textsuperscript{32,33,242,244,245,360}. Inhibitors of the VEGF pathway, such as bevacizumab and VEGF Trap have been shown to be effective in women with ovarian cancer in phase II and III clinical trials (reviewed in \textsuperscript{361,362}). We hypothesize that a treatment regimen designed to preferentially target nestin-expressing cancer cells, along with antiangiogenic therapy will have improved therapeutic efficacy and prolong survival.

Oncolytic viral (OV) therapy is a promising biological therapy that preferentially targets tumor cells for lytic destruction, while sparing normal cells\textsuperscript{343}. Many different OVs have been examined for use against ovarian cancer, including oncolytic herpes simplex virus(HSV)-1\textsuperscript{54-58,314}; adenovirus\textsuperscript{60,61,63,305,308,363-367}, reovirus\textsuperscript{326}, and measles virus\textsuperscript{64,65,67,318,320}. Here we examined therapeutic efficacy of 34.5ENVE (\gamma 34.5-Expressed by Nestin promoter and Vasculostatin-120 Expressing), an oncolytic HSV-1 that targets the transcription of the viral ICP34.5 gene to nestin-expressing cells, and encodes for Vasculostatin-120, a secreted antiangiogenic gene for efficacy against ovarian cancer alone and in conjunction with doxorubicin\textsuperscript{98}. ICP34.5 increases viral replication and oncolysis by modulating the host anti-viral immune response\textsuperscript{93,98}.
Materials & Methods

Cell lines and reagents

PA-1, OV-4, OVCAR-3, SKOV3 human ovarian carcinoma cells and Vero (African green monkey kidney cells) were obtained from the ATCC (Manassas, VA). A2780 and A2780-CR (cisplatin resistant) were a gift from Dr. Selvendiran Karuppaiyah (The Ohio State University, Columbus OH). SKOV3.ip1-firefly Luciferase (SKOV3-ip1-fLuc) were a kind gift from Dr. Kah-Wey Peng (Mayo Clinic, Rochester, MN).

Human dermal microvessel endothelial cells (HDMEC) were obtained from ScienCell, and maintained at low passage number in Endothelial Basal Media with supplements (ScienCell, Carlsbad, CA). PA-1 and OV-4 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1x penicillin and streptomycin at 37°C and 5% CO₂. OVCAR-3, SKOV3 and SKOV3-ip1-fLuc were maintained in Minimal Essential Medium supplemented with 20% fetal bovine serum and 1x penicillin and streptomycin at 37°C and 5% CO₂.

Doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO), and dissolved in DMSO, and then further diluted in PBS.

Viruses

Viruses were constructed utilizing an HSV-1 F strain backbone via previously described technology. Briefly, to generate 34.5ENVE, an expression cassette containing HSV ICP34.5 under the regulation of nestin enhancer-driven promoter and antiangiogenic protein Vasculostatin-120 gene under the regulation of the viral Immediate Early 4/5 promoter was inserted into the HSV-1 backbone. (See Figure 1A). This backbone, called HSVQ, is a first generation OV deleted for both copies.
of ICP34.5 and disrupted for ICP6, with a frt/loxP site directly upstream of the disrupted ICP6 gene. The expression cassette was inserted at this frt/loxP site. To generate the revertant 34.5ENVE, the expression cassette containing the ICP34.5 and Vasculostatin-120 gene was subsequently removed. Viruses were propagated in Vero cells. Three days after infection, cells and media were harvested and subjected to three cycles of freezing in liquid nitrogen and thawing at 37C. After freeze-thaw, the lysate was cleared of cell debris by centrifugation at 4000g for 20 minutes. Virus was pelleted by centrifugation at 13,000g for 1 hour at 4C. The titer of the virus was determined via plaque-forming assay in Vero cells, with PFU assessed 3 days after infection. Standard viral production protocols generally yield $10^4$ to $10^5$ PFU per microliter, and viral yields for preparations used in this study fell within that range.

*Cell Viability Assay and Chou-Talalay Analysis*

Cancer cells were seeded into a 96-well plate (1e4 cells/well in 2% FBS media in 100ul volume), and allowed to adhere for 2-4 hours (prior to drug treatment) or for 20-22 hours (prior to virus infection). Cells were treated with drug for 18 hours (followed by wash out) or infected with virus; all treatment and incubation was done in 2% FBS media. Three days after infection or 3.5 days after start of drug treatment, MTT assays (Roche) were carried out according to manufacturing instruction. For combination treatment synergy analysis, treatment scheduled consisted of: seeding cells, treating cells with drug for 18 hours, washing out drug treatment, infecting with virus, incubating for 72 hours at 37C. Viability assay was carried out 3 days after infection. For Chou-Talalay analysis, dose response curves and 50% effective dose
values were first determined for each individual treatment (drug or virus). Fixed ratios of drug and virus were then used to treat the cells, either with dual treatment or with individual treatments (as controls). Using these dose-response curves, CompuSyn software program algorithm assessed what the mathematical interaction was, so called a Combination Index (CI). Combined dose-response curves were fitted to Chou-Talalay lines, which are derived from the law of mass action. CI<1 indicates synergistic interaction, whereas CI>1 is antagonistic and CI=1 is additive. To perform Chou-Talalay analysis, dose response curves and 50% effective dose values were first determined for each individual treatment (drug or virus). Fixed ratios of drug and virus were then used to treat the cells, either with dual treatment or with individual treatments (as controls). Using these dose-response curves, CompuSyn software program algorithm assessed what the mathematical interaction was, so called a Combination Index (CI). Combined dose-response curves were fitted to Chou-Talalay lines, which are derived from the law of mass action. CI<1 indicates synergistic interaction, whereas CI>1 is antagonistic and CI=1 is additive.

Generation of concentrated conditioned media (cCM)

To generate concentrated conditioned media, ovarian cancer cells are seeded on a 10-cm plate at ~90% confluency in 0% FBS media, and infected with OV at a multiplicity of infection (MOI) ~2. Infection proceeds for 1 hour, and then unbound virus is washed away, and fresh 0% FBS media is added, and cells are incubated for 12-14 hours. Media is harvested prior to viral burst, treated with 0.4% human IgG to neutralize contaminating virus and then centrifuged for 1 hour at 13,000rpm to pellet
any virus in the media. The media is concentrated ~100-fold using Amicon Ultra centrifugal concentration filters (Millipore, Billerica, MA), with centrifugation at 4,000rpm for 20 min at 4C.

In Vitro Endothelial Cell Migration Assays

Endothelial cell migration assays were performed using 8µM transwell membranes (Corning Costar, Cambridge, MA) coated on the underside with 0.1% fibronectin (Millipore, Billerica, MA). Concentrated conditioned media stimuli is added to the lower well of the chamber, diluted into 0.05%FBS endothelial cell media to a final volume of 750ul (ScienCell, Carlsbad, CA). HDMEC are serum-starved in 0%FBS media for 4 hours at 37C prior to seeding to increase reactivity. HDMEC are seeded in the upper chamber, onto the membrane (at 5e4 cells/well in 200ul serum-free media). HDMEC are then incubated at 37C at 5%CO2 and migrate for 8-12 hours. Membranes are removed, fixed in 1% glutaraldehyde overnight at 4C, and then stained with 0.5% Crystal violet. Cells remaining on the upper side of the membrane are wiped away with a cotton swab so the only remaining cells on the membrane have migrated through the pores onto the other side of the membrane. Images are obtained at 20x magnification, and quantified by counting 5 fields of view/well, with each treatment done in triplicate. Data is presented as the average of three wells.

Viral Replication Assay and Viral Burst Assay

Cells were seeded onto 6-well plates (3e5 cells/1000ul 2% FBS media), and treated with doxorubicin or mock in triplicate for 18 hours at 37C. Drug was then washed out, and cells were infected at an MOI of 0.05 (for replication assay, to assess changes in viral replication kinetics over time) or 2 (for burst assay, to evaluate
changes in a single viral replication cycle) in 500ul 2%FBS media. Infection proceeded for 1 hour, and then unbound virus was washed away and fresh 2%FBS media was added to each well. Cells were harvested at indicated time points post-infection, and titers were determined by standard plaque assay on Vero cells.

**Immunoblots**

Cells were treated with virus at indicated MOI, and harvested at indicated time points after infection. Cells were pelleted and lysed in RIPA buffer with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). A total of 15ug of protein was separated by 4-20% SDS-PAGE and transferred to PVDF membrane by electroblotting. After blocking with 5% non-fat dry milk (NFDM) in PBS with 0.5% Tween-20 (PBS-T), membranes were incubated overnight at 4C with primary antibody at 1:1000 in 5% NFDM or 5% bovine serum albumin (BSA) in PBS-T. Membranes were washed 5 times in PBS-T, and secondary antibody was added in 5%NFDM at 1:2000, and incubated at room temp for 2 hours. Protein-antibody complexes were visualized using enhanced chemiluminescence (GE Healthcare, Pittsburgh, PA). Immunoblots were probed with rabbit anti-N-terminal BAI1 (Kaur Am J Pathol 2003), mouse anti-human GAPDH (Abcam, Cambridge, MA), or mouse anti-ICP4 (Abcam, Cambridge, MA) antibodies, followed by goat anti-rabbit (Dako, Carpinteria, CA) or sheep anti-mouse (Amersham Biosciences, Pittsburgh, PA) secondary antibodies, and visualized by enhanced chemiluminescence.

**Assay of Caspase Activity**

Cells were seeded onto 96-well plates (1e4 cells/ 100ul 2%FBS media), and were treated with drug, virus or combination (each in triplicate) at indicated
concentrations/MOI. 24 hours later, caspase-3/7 activity was evaluated using Caspase-Glo 3/7 Assay Kit (Promega, Madison, WI) according to manufacturer’s instruction.

Animal experiments

All animal experiments were performed in accordance with the Subcommittee on Research Animal Care of The Ohio State University guidelines, and have been approved by the institutional review board. Six- to eight-week-old female athymic nude nu/nu mice (Charles River Laboratories, Frederick, MD), were used for all tumor studies. Number of mice per group was chosen based on previous studies utilizing this animal model as appropriate to evaluate statistical changes in survival.

Mice were implanted with tumor via intraperitoneal injection of 2e6 SKOV3.ip1-fLuc cells in 500ul sterile saline. Tumors grew for 8 days prior to treatment. Treatment injections with doxorubicin, virus, or mock were also done via intraperitoneal injection of treatment in 100ul sterile saline. End points were predetermined as death, 20% weight gain due to ascities accumulation, development of significant jaundice or wasting (loss of 20% body weight), or development of necrotic subcutaneous lesion at injection site. All endpoints were treated as death.

Ex vivo Ascites Isolation

To isolate ascites from mice with progressive ovarian cancer disease, ascites were isolated via intraperitoneal draining, and red blood cells were lysed using RBC Lysis Solution from MasterPure Complete DNA and RNA Isolation Kit (Illumina, Madison,
Remaining tumor cells and contaminating immune cells were plated in 20%FBS MEM overnight at 37C, and then used for experiments as indicated.

**Luciferase Imaging**

Luciferase imaging was performed using Xenogen IVIS in vivo imaging system (Perkin Elmer, Waltham, MA). Luciferin was diluted and used at in vivo concentration as indicated in manufacturer’s instruction (Perkin Elmer).

**Statistics**

All statistical analysis was performed using GraphPad Prism software. Student’s t-test was used to analyze differences in cell killing, HDMEC transwell migration, and changes in viral titer. A p value of less than 0.05 was considered statistically significant. In the survival analysis, Kaplan–Meyer curves were plotted and compared using the log rank test.

**Results**

*Ovarian cancer cells display elevated nestin expression, and are sensitive to killing by 34.5ENVE oncolytic virus.*

The genetic structures of the viral vectors used in this study have been previously described\textsuperscript{98,369}, and are outlined in Figure 1a. Briefly, rHSVQ1 is an oncolytic HSV derived from wild type strain F which is disrupted for the viral ribonucleotide reductase gene (ICP6), and is also deleted for both copies of γ34.5, the major immunomodulatory protein. 34.5ENVE virus has one copy of ICP34.5 reintroduced into the backbone driven by a modified nestin promoter, and also expresses
Vasculostatin-120 driven by an immediate early viral promoter (called IE4/5)\textsuperscript{49}. A rescue virus, called revertant 34.5ENVE, was generated by removal of the inserted nestin-driven ICP34.5 and IE4/5-driven BAI1 expression cassette from the 34.5ENVE backbone (Supplementary Figure S1). To determine if OvCa cell lines were sensitive to 34.5ENVE relative to revertant 34.5ENVE, the indicated cells were infected with virus at increasing multiplicity of infection (MOI) and cell viability was measured by standard MTT assay. Figure 1b shows sensitivity of OvCa cell lines to killing by both 34.5ENVE and revertant 34.5ENVE. There was a significant increase (2-to-10-fold) in sensitivity to 34.5ENVE-mediated killing as compared to revertant 34.5ENVE (Figure 1c).

\textit{Mouse ascites-derived and patient ascites-derived tumor cells have increased sensitivity to 34.5ENVE.}

To evaluate if increased sensitivity of OvCa cells correlated with nestin expression, we compared sensitivity of SKOV3 cells grown \textit{in vitro} to SKOV3 cells harvested from murine ascites which develop in mice after tumor implantation. Tumor cells isolated from malignant ascites have been described to be more stem-cell like with higher nestin expression\textsuperscript{188,370}. Consistent with published reports, \textit{ex vivo} SKOV3 cells harvested from mouse ascites had a 10.4 and 11.1-fold increased nestin expression compared to adherent SKOV3.ip1 cells grown \textit{in vitro} (Figure 2a). Along with increased nestin expression, the \textit{ex vivo} ascites-derived tumor cells were 20- and 6.1-fold more sensitive to 34.5ENVE relative to revertant 34.5ENVE than SKOV3 cells grown \textit{in vitro} (Figure 2b).
In a parallel experiment, we also analyzed sensitivity of primary patient ascites-derived tumor cells to 34.5ENVE. Ascites from an ovarian cancer patient were isolated and cultured as described\(^{371}\). Quantitative real time PCR for nestin gene expression revealed that these tumor ascites cells had 35-fold greater nestin expression (Figure 2d), as compared to normal hepatocytes grown in vitro. These primary patient ascites-derived tumor cells also showed increased sensitivity toward 34.5ENVE-mediated killing compared to revertant 34.5ENVE (Figure 2e and 2f).

*Antiangiogenic protein Vasculostatin-120 is produced by 34.5ENVE after infection of OvCa, and reduces endothelial cell migration in vitro.*

We examined the ability of OvCa cells infected with 34.5ENVE to produce and secrete Vasculostatin-120 by western blot analysis. Figure 2a shows presence of Vasculostatin-120 in cell lysate of a panel of OvCa cell lines infected with 34.5ENVE. The presence of secreted Vasculostatin-120 was also confirmed by western blot analysis of conditioned medium derived from murine and patient derived ascites cells infected with revertant 34.ENVE, 34.5ENVE or mock infection with PBS (Figure 2B).

To assess the functionality of secreted Vasculostatin-120 produced by infected OvCa cells, we compared the effect of concentrated conditioned media (cCM) from SKOV3.ip1 or primary patient ascites infected with OV or mock on endothelial cells migration in a modified boyden chamber. Briefly, cCM from revertant 34.ENVE, 34.5ENVE or mock infected cells was added to the bottom chamber, and migration of HDMEC towards the bottom chamber was quantified. There was a significant reduction in endothelial cell migration upon treating with Vasculostatin-120-
containing cCM from either infected SKOV-3 (26.4% reduction, p=0.0074) or patient ascites (28.3% reduction, p=0.0309) (Figure 2c-d).

**Therapeutic efficacy of 34.5ENVE against intraperitoneal ovarian cancer cells in vivo.**

To test therapeutic efficacy of 34.5ENVE against ovarian cancer in vivo, mice with established intraperitoneal SKOV3.ip1 ovarian tumors were treated with PBS, or three doses of $5 \times 10^5$ plaque-forming units (PFU) of 34.5ENVE or revertant 34.5ENVE on D+8, D+16 and D+23 after tumor cell inoculation. Kaplan-Meier survival analysis revealed a significant increase in median survival time of mice treated with 34.5ENVE as compared to PBS and revertant 34.5ENVE treated animals. Median survival was 63, 49 and 37 days for 34.5ENVE, revertant 34.5ENVE, and PBS, respectively. (Figure 4a; p<0.001 between PBS and revertant 34.5ENVE, and p=0.0013 between revertant 34.5ENVE and 34.5ENVE.)

IVIS animal imaging of luciferase expression by SKOV3.ip1 cells permitted live imaging of mice to monitor disease progression. There was a significant reduction in luciferase activity (as indicated by RLU via IVIS bioluminescent imaging) in 34.5ENVE-treated cohort 20 days after tumor injection, as compared to PBS treatment (Figure 4b; p=0.0193 between PBS and 34.5ENVE). Interestingly, all mice showed a delayed development of a subcutaneous tumor on the right side of the abdomen, at the site of tumor injection. Since virus treatment was administered via intraperitoneal injection on the left side of the abdomen, this subcutaneous nodule served as an untreated control tumor in each mouse. Eight of eight mice treated with PBS developed aggressive ascites by the time of sacrifice, whereas only 4 of eight
mice treated with revertant 34.5ENVE developed ascites (Figure 4c). Only two of the eight mice treated with 34.5ENVE showed the development of ascites at time of death, while the other six mice were sacrificed due to the growth of the untreated subcutaneous tumor. Gross histology revealed that six of the eight mice treated with 34.5ENVE were free of intraperitoneal metastatic tumor seeding (Figure 4d, tumor nodules indicated with white asterisks).

Doxorubicin synergizes with 34.5ENVE to kill ovarian cancer cells in vitro, and combination therapy prolongs survival in murine xenograft model of human ovarian cancer.

We first tested the sensitivity of OvCa cell lines to doxorubicin by a standard killing assay. All OvCa cell lines tested were sensitive to doxorubicin (Figure 5a). Next, we evaluated sensitivity of doxorubicin treated cells to 34.5ENVE. Cells were treated with doxorubicin for 18 hours followed by drug wash-out, and infection with 34.5ENVE for 72 hours. Percent viable cells were measured by MTT assay. In all 6 ovarian cancer cell lines, treatment of cells with both 34.5ENVE and doxorubicin resulted in more-than-additive cell killing (Figure 5b). Since this suggested synergistic cell killing, we next examined the ability of doxorubicin to synergize with oHSV using the Chou-Talalay synergy analysis method as described\textsuperscript{80,83,377-380}, by pre-treating the cells with doxorubicin (followed by wash-out), and then infecting with 34.5 ENVE. Doxorubicin and 34.5ENVE combination therapy killed the ovarian cancer cells synergistically in all cell lines at most fractions affected, Fa (Figure 5d).

In a parallel experiment we tested sensitivity of primary patient ascites-derived tumor cells to doxorubicin alone, and in combination with 34.5ENVE. Consistent with the
cell line data, strong synergistic cell killing was observed between doxorubicin and 34.5 ENVE (Figure 5e).

*Combination therapy with doxorubicin and 34.5 ENVE does not alter OV replication capacity, but increases caspase-3/7 activation and apoptosis.*

Doxorubicin and 34.5ENVE combination treatment synergistically kills OvCa, but this increase in killing does not appear to be due to an increase in viral output. Viral replication assays and viral burst assays indicate that there is no change in viral replication kinetics (Figure 6a and 6b). A dose-escalation study showed that doxorubicin-treatment did not significantly increase the number of GFP-expressing cells (indicative of viral transgene expression) after 12 hours of infection, indicating that doxorubicin does not affect the ability of the virus to transduce OvCa cells (Figure 6c). Consistent with this, there was no change in HSV-1 receptor expression on cells treated with doxorubicin. However, combination therapy of doxorubicin and 34.5ENVE did robustly increase caspase-3/7 activation in SKOV3 cells over either individual therapy, indicating that the synergistic killing may be mediated via increased apoptosis (Figure 6d). Next, we tested the *in vivo* relevance of this synergistic cell killing of doxorubicin in combination with 34.5ENVE. Mice with established intra-peritoneal tumors were treated with either PBS on D+8, a single dose of 10ug/g of doxorubicin on D+8, a single dose of 5e5 PFU of 34.5ENVE on D+9, or a sequential treatment of 10ug/g doxorubicin on D+8 followed by 5e5PFU 34.5ENVE on D+9 post tumor inoculation. Kaplan-Meier survival analysis revealed a significant increase in survival of mice treated with combination therapy of doxorubicin and 34.5 ENVE (median survival of 58 days) as compared to virus alone.
(median survival 58 days vs. 47 days; p=0.003 between 34.5ENVE only and
doxorubicin with 34.5ENVE) or doxorubicin alone (median survival of 58 days vs. 47
days; p=0.002 between doxorubicin with 34.5ENVE and doxorubicin alone).
Figures & Tables.

Figure 1. Oncolytic viral (OV) therapy is effective against OvCa cell lines.

(A.) Description of viral genomes used in this paper, compared to parental F strain HSV. (B.) Cytotoxicity assay of panel of OvCa cell lines infected with 34.5ENVE (left panel) or revertant 34.5ENVE (right panel) at increasing MOI. Viability assessed after 3d using standard MTT assay. (C.) Bar graph indicating relative sensitivities of OvCa cells to revertant 34.5ENVE as compared to 34.5ENVE.
Figure 1.
Figure 2. Oncolytic viral (OV) therapy is effective against OvCa cells, ex vivo ascites, and patient ascites.

(A) Ex vivo tumor cells isolated from ascites were harvested from established tumors in mouse model, and tested. Freshly-isolated patient ascites were also isolated and tested. Left graph, Ex vivo tumor cells displayed elevated nestin expression. Relative nestin expression was assessed by RT-PCR, and ascites expression was normalized to parental adherent cell line, SKOV3.ip1-fLuc. Right graph, Patient ascites also had an increase in nestin expression, as assessed by RT-PCR and normalized to normal hepatocytes. (E.) Viability assay of two ex vivo ascites isolates infected with OV at increasing MOI, after 3d infection. Viability assessed via MTT. Bar graph displays relative sensitivities of ascites-derived cells to 34.5ENVE, as compared to SKOV3.ip1 sensitivity to 34.5ENVE. (F.) Viability assay of patient ascites-derived tumor cells infected with OV at increasing MOI, after 4d infection. Viability assessed via MTT. Bar graph displays relative sensitivities of ascites-derived tumor cells to revertant 34.5ENVE as compared to 34.5ENVE.
Figure 2.
Figure 3. Vasculostatin-120 is produced by OvCa infected with 34.5 ENVE, and conditioned media from 34.5ENVE-infected OvCa reduces endothelial cell migration in vitro.

(A) Immunoblot of OvCa cell lysates harvested 24 hours after infection with 34.5ENVE, revertant 34.5ENVE (MOI = 0.1) or PBS. Infection with 34.5 ENVE yields a robust infection (as indicated by viral ICP4 protein), and production of Vasculostatin-120. (-), untreated; R, revertant 34.5ENVE; NV, 34.5ENVE. (B) Immunoblot of conditioned media from SKOV3.ip1 or ascites-derived tumor cells, harvested 12 hours after infection with 34.5ENVE, revertant 34.5ENVE (MOI = 2) or PBS. (C.) Endothelial cell migration assay using the the same conditioned media as presented in B. Human Dermal Microvascular Endothelial Cell (HDMEC) migration is reduced in the presence of concentrated conditioned media from SKOV3.ip1 or patient ascites infected with 34.5 ENVE, but not revertant ENVE or PBS. Conditioned media collected 12-13 hours after infection with virus, and then concentrated 100-fold. HDMEC were incubated with CM in bottom chamber of transwell. Images acquired from underside of porous transwell membrane through which HDMEC readily migrate. Number of cells migrated though membrane were quantified and analyzed in bar graphs on right.
Figure 3.
Figure 4. OV, and especially 34.5ENVE, prolongs survival and reduced tumor burden in murine xenograft model of ovarian cancer.

(A.) *In vivo* survival analysis indicates oncolytic virus therapy prolongs survival in murine model of ovarian cancer. Mice were injected with tumor cells at D(-)8, with 8 mice per group. Mice were treated with 3 injections of virus, on D+1, D+8 and D+15. Kaplan-Meyer survival analysis showed a significant increase in survival of mice implanted with human ovarian cancer when treated with OV, with a greater increase in median survival time in 34.5 ENVE-treated cohort as compared to revertant 34.5ENVE-treated (p=0.0013, comparing revertant 34.5ENVE to 34.5ENVE). Median survival of PBS-treated mice was 37 days, revertant 34.5ENVE was 49 days, and 34.5ENVE treatment extended survival to 63 days. (B.) OV therapy reduced tumor burden. Bioluminescent imaging and relative quantification from Day 20 after start of treatment shows a reduction in luminescent signal in mice treated with 34.5ENVE, as compared to PBS-treated mice. (p=0.0193.) (C.) OV therapy reduced presence of ascites at time of death. Graphical representation indicating presence of ascites at time of sacrifice. 34.5ENVE treatment reduced the number of mice with ascites at time of death from 100% in PBS cohort (8/8), to 20% in ENVE-treated cohort (2/8). (D.) Necropsy images at time of sacrifice show tumor burden. Asterisks indicate intraperitoneal tumors. Gross histology indicates that ENVE-treated mice had reduced tumor nodule formation and reduced intraperitoneal spread at time of death.
Figure 4.
Figure 5. Doxorubicin and 34.5ENVE interact in a synergistic capacity to kill OvCa cells, and significantly prolong survival when used together to treat murine model of human ovarian cancer.

(A.) OvCa cell lines and patient ascites are sensitive to doxorubicin killing. Drug treatment applied for 18 hours, then washed out. Viability assessed via MTT 3 days after treatment. (B.) Combination therapy with doxorubicin + 34.5ENVE significantly increased cell death, as compared to either therapeutic alone. (C.) Chou-Talalay analysis of doxorubicin in combination with 34.5ENVE treatment indicates synergistic interaction. In the 6 cell lines and one patient ascites sample tested, doxorubicin synergistically killed with 34.5ENVE in some or all of the Fractions Affected, as assessed by Chou-Talalay analysis (using CompuSyn program). Combination Index (CI): Additive, 0.9-1.1. Synergistic, >0.9. Antagonistic, <1.1. (D.) Kaplan-Meier survival analysis of doxorubicin, 34.5ENVE or combination treatment in the murine model of ovarian cancer. Mice were injected with tumor cells at D(-)8. Mice were treated with one injection of doxorubicin (10ug/g) on D+1, one injection of 34.5ENVE (5e5 PFU/mouse) on D+2, both injections or mock treated with PBS. Kaplan-Meyer survival analysis showed a significant increase in survival of mice with human ovarian cancer when treated with doxorubicin + 34.5 ENVE over either treatment alone. (p=0.0003, comparing ENVE to dox+ENVE, p=0.0002, comparing dox to dox+ENVE) Median survival of PBS-treated mice was 37 days, doxorubicin-treated was 47 days, single dose ENVE-treated was 47 days, and combination therapy of doxorubicin and 34.5 ENVE extended median survival to 58 days.
Figure 5.
Figure 6. Doxorubicin and 34.5ENVE combination therapy induce robust increase in apoptosis as compared to either single treatment via caspase-3/7 signaling.

(A.) Viral replication assay indicates that doxorubicin does not increase viral replication at 3 different time points. SKOV3.ip1 cells were infected at MOI=0.005 for 1 hour, and then cell lysate was harvested at indicated time points. Titration was done on Vero cells in the standard way. (B.) Viral burst assay indicates that doxorubicin does not increase viral particles produced in a single replication cycle. SKOV3.ip1 cells were infected at MOI=1 for 1 hour, and then cell lysate was harvested 24 hours later, after a single replication cycle. Titration was done on Vero cells in the standard way. (C.) Flow cytometry analysis of GFP-expression in cells treated with increasing concentration of doxorubicin. Cells were pretreated with doxorubicin for 18 hours, and the drug was washed out. Cells were infected with 34.5ENVE at MOI=0.05 for 2 hours, followed by wash-out. GFP expression analysis was performed 12 hours post-infection. (D.) Caspase-3/7 analysis of SKOV3 after 24 hours of doxorubicin treatment (18h followed by wash-out), 34.5ENVE treatment (24h) or combination treatment. Values normalized to protein input. p<0.001.
Figure 6.
Discussion.

**Oncolytic HSV expressing ICP34.5 for treatment of ovarian cancer.**

Oncolytic viral therapy has been examined for treatment of many types of cancer, including ovarian cancer. Several different strains of oncolytic HSV, including F-strain derived HSV 1716 and fusogenic Synco-2D, have been shown to be effective against metastatic ovarian cancer in pre-clinical studies\(^5^7,5^8,3^1^4,3^7^2-3^7^4\). Patients with late-stage or recurrent ovarian cancer are currently being recruited for clinical trials to evaluate the use of oncolytic measles virus, or Reovirus with or without paclitaxel (NCT00408590, NCT01199263). However, this is the first report utilizing an oncolytic HSV that has the viral gene ICP34.5 reintroduced. The deletion of the ICP34.5 gene has been shown to significantly reduce growth in even rapidly-dividing cells\(^3^7^5,3^7^6\), but the resultant attenuated virus maintains an enhanced safety profile due to the marked reduction in cytotoxicity\(^3^7^7-3^7^9\). The reintroduction of the ICP34.5 gene under the regulation of a modified nestin promoter produces a more cytolytic virus\(^9^8\). Here we compare the efficacy of 34.5ENVE to revertant 34.5ENVE, which has the ICP34.5 and Vasculostat-120 genes removed.

Our results indicate that OvCa cell lines are sensitive to OV therapy (Fig 1B), and have an increase in sensitivity to 34.5ENVE, as compared to revertant 34.5ENVE. We found that the our panel of OvCa cells lines had varying sensitivity to 34.5ENVE, but most of the OvCa cell lines had similar sensitivity to revertant 34.5ENVE (with the notable exception of OV4). ICP34.5 has a crucial role in regulating the interferon antiviral immune response expression, and its presence and function has been shown to play a pivotal role in gaining control of the host cell anti-viral immune
response\textsuperscript{107}. The variability in sensitivity to 34.5ENVE-mediated cytotoxicity is likely mediated by the effects of the ICP34.5 (or possibly the Vasculostatin-120, although this is less likely). Furthermore, the presence or absence of ICP34.5 expression during HSV infection changes the types of genes that are up- and down-regulated after infection. As reported by Pasieka, in 34.5-wild-type HSV infections, at 12h post infection, most of the genes that are upregulated are metabolic and biosynthesis genes, whereas in 34.5-mutant infections, the majority of up-regulated genes are related to anti-viral immune response pathways\textsuperscript{107}. It has also been proposed that early expression of ICP34.5 is important for augmenting viral infection\textsuperscript{380}. Different cell lines may have variation in their infection kinetics, and this may affect the outcome and sensitivity to OV infection.

We also isolated and tested \textit{ex vivo} ascites from mice with progressive ovarian cancer disease, established using cell line SKOV3.ip1-fLuc. \textit{Ex vivo} ascites are a different tumor cell population than the adherent cells used to produce cancer model due to change in microenvironment. Tumor cells that are present in malignant patient ascites are also very different from the primary tumor. They have undergone EMT, metabolic change, appear more like stem cells\textsuperscript{381-383}. Murine-derived \textit{ex vivo} tumor cells were sensitive to OV-mediated killing, with elevated sensitivity to 34.5ENVE as compared to revertant 34.5ENVE (Figure 1D). These ascites-derived tumor cells had elevated nestin expression as compared to the parental adherent cell line (Figure 1C), which correlates with reports that OvCa cells found floating in malignant human ascites are more stem-cell like. Elevated nestin expression was consistent with the previous reports by Zhang\textsuperscript{24} and Gao\textsuperscript{23} that report the presence
of stem cell populations in ovarian tumors with elevated nestin expression. The ascites-derived tumor cells isolated from the mice also had an increase in 34.5ENVE sensitivity as compared to the parental cell line.

Human patient ascites-derived tumor cells, isolated fresh from an ovarian cancer patient, were also sensitive to both OV, with a dramatic increase in sensitivity to 34.5ENVE (Figure 1E). These cells displayed high levels of nestin expression, even compared to OvCa cell lines (Figure 1C and data not shown). This is the first study, to the knowledge of this author, to evaluate the use of oncolytic HSV against tumor cells derived from freshly-isolated malignant ovarian cancer ascites.

_Oncolytic HSV expressing Vasculostatin-120 for treatment of ovarian cancer_.

Herein, we utilized an oncolytic HSV that expressed Vasculostatin120. Vasculostatin-120 contains the five thrombospondin type I repeats, which modulate the potent antiangiogenic activity of BAI1. In our study, we observed reduced endothelial cell migration _in vitro_, and a reduction in the formation of ascites _in vivo_ in 34.5ENVE-treated populations, as compared to revertant 34.5ENVE or PBS (Figure 2C, Fig 3). We also saw prolonged survival, and a reduction in intraperitoneal seeding to tumor cells with 34.5ENVE treatment (Figure 3). These results align with previous reports that examine the use of thrombospondin-1 mimetic proteins for ovarian cancer with promising results\(^{52,53,384}\). Treatment with mimetics ABT-510 or ABT-898 induced tumor regression, reduced ascites fluid volume and intraperitoneal dissemination, and prolonged survival in an orthotopic, syngenic model of epithelial ovarian cancer\(^{52,53,390}\). Treatment also reduced aberrant tumor-associated vasculature, decreased VEGF expression, and reduced tumor
hypoxia. Furthermore, ABT-510 showed effects against OvCa in vitro, indicating that its effects were not solely mediated via tumor vasculature\textsuperscript{52}. Combination therapy of ABT-510 with paclitaxel or cisplatin resulted in increased tumor cell and tumor vasculature apoptosis, mediated by vasculature normalization\textsuperscript{53}. These reports from the Petrik lab on how TSP-1 mimetics act suggest that the Vasculostatin-120 may be mediating apoptotic effects on the cancer cells, in addition to the vasculature\textsuperscript{52,53,390}. This is something we have not yet evaluated.

\textit{Oncolytic HSV has potential for use in combination with doxorubicin for treatment of ovarian cancer.}

Combination strategies of OV with chemotherapy have been previously examined for use in OvCa. Oncolytic adenovirus has been studied in conjunction with paclitaxel, cisplatin, epirubicin and gemcitabine\textsuperscript{62,76-78,385,386}. Oncolytic HSV has never been tested in combination studies for treatment of OvCa, but it has been show to synergize with DNA-damaging chemotherapy in other cancer models, such as glioblastoma and thyroid cancer\textsuperscript{80,81,83,387}. Low dose etoposide has been shown to synergize with ICP34.5-deleted G47Δ oncolytic HSV when combined to treat glioblastoma cells and glioblastoma stem cells\textsuperscript{387}. The effects were reported to be mediated via enhanced caspase signaling and apoptosis\textsuperscript{387}.

This study is the first to examine the use of doxorubicin with oncolytic HSV for use against OvCa. (Doxorubicin was examined in combination with HSV1716 for treatment of non-small cell lung cancer, where it was found to have an additive effect\textsuperscript{388}). We found that doxorubicin and 34.5ENVE killed OvCa cells lines and patient ascites-derived tumor cells synergistically, with robust cytotoxic effects seen
when doxorubicin and 34.5ENVE were combined, at concentrations where either treatment alone showed little cytotoxic effect (Figure 4B and 4C). However this increased killing did not appear to be mediated by a change in replication kinetics, as indicated by the viral replication assay and viral burst assay (Figure 5A and 5B). Furthermore, doxorubicin treatment did not alter HSV receptor expression on ovarian cancer cells (Figure 5C). However, combination therapy robustly increased caspase-3/7 activation over either individual therapy, indicating that the synergistic killing may be mediated via caspase activation and apoptosis (Figure 5D). Ovarian cancer cells have been shown to be killed via apoptosis after treatment with several different lines of therapy, including doxorubicin\textsuperscript{389-391}. We found that 34.5ENVE also induced caspase-3/7 activation in OvCa cell lines. This is in contrast to glioblastoma cells, which do not undergo an increase in caspase activation following 34.5ENVE therapy (unpublished results, personal communication with B. Kaur and J.Y. Yoo).

Doxorubicin is a second-line therapy that is commonly used for ovarian cancer patients who are resistant to paclitaxel and carboplatin. In other tumor models, DNA-damaging agents, such as etoposide or temozolomide, have been reported to kill cancer cells synergistically when combined with oncolytic HSV\textsuperscript{80,83}. Doxorubicin has not yet been studied in conjunction with oHSV for the treatment of ovarian cancer. We hypothesized that combination therapy would be more efficacious than individual treatment. We found that \textit{in vitro}, 34.5 ENVE induced cell killing of ovarian cancer cell lines, \textit{ex vivo} ascites harvested from mice, and \textit{ex vivo} patient ascites-derived primary tumor cells. 34.5ENVE retained its anti-angiogenic function \textit{in vitro} and \textit{in vivo}. To evaluate the impact of combining 34.5ENVE with doxorubicin, we analyzed
the ability of each agent to kill ovarian cancer cells alone and in combination. Chou-Talalay analysis evaluated the cell-killing capability of doxorubicin with 34.5ENVE to be synergistic. Treatment of tumor-bearing mice with 34.5ENVE and doxorubicin showed improved therapeutic efficacy compared to either agent alone.

In other tumor models, oHSV has been shown to be effective against doxorubicin-resistant cells. Mahller et. al. reported that neuroblastoma cells were composed of a subpopulation of tumor-initiating cells that had elevated nestin expression, were enriched for CD133 expression, and were resistant to doxorubicin. These neuroblastoma tumor-initiating cells were sensitive to an oHSV that expressed nestin-driven ICP34.5. Zhuang et. al. also reported that doxorubicin-resistant, aldehyde dehydrogenase-bright mouse breast cancer stem cells were sensitive to oHSV, and combination therapy with doxorubicin followed by oHSV in vivo resulted in suppression of tumor growth and increased median survival. Zhuang et. al. suggest that the use to oHSV after doxorubicin helps eradicate remaining chemotherapy-resistant stem cell populations. Perhaps the targeting of doxorubicin-resistant OvCa cells mediates some of the synergistic interaction between doxorubicin and 34.5ENVE that we see with our study. The mechanism of synergy requires further examination.

**Conclusion**

In conclusion, we report that doxorubicin, a standard of care for recurrent or refractive OvCa, can be combined with 34.5ENVE to synergistically kill OvCa cells, and combination therapy results in prolonged survival and reduced tumor and
ascites burden in the murine model of human ovarian cancer. This may be a potential combination therapy for use in the clinic for patients with recurrent or refractive ovarian cancer.
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