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THE BIOLOGICAL FUNCTION OF HUMAN EPIDIDYMIS PROTEIN 4 IN EPITHELIAL OVARIAN CANCER

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THE BIOLOGICAL FUNCTION OF HUMAN EPIDIDYMIS
PROTEIN 4 IN EPITHELIAL OVARIAN CANCER

BY

NICOLE ELIZABETH JAMES

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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OF
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ABSTRACT

Epithelial ovarian cancer (EOC) is the most common gynecologic malignancy worldwide. EOC has a notably poor prognosis, owing to the fact that patients are frequently diagnosed at a late stage after the disease has significantly progressed. While many patients typically respond well to frontline platinum-based chemotherapy, the tumor becomes chemoresistant when a recurrence follows within five years. Therefore, there is an urgent need for the discovery of non-invasive early detection biomarkers and novel targeted therapies.

Human Epididymis Protein 4 (HE4) is a secretory protein that is encoded by the whey acidic protein (WAP)-four disulfide core domain protein 2. The WAP domain family is a conserved motif that is inherited by many antiproteases. HE4 was initially found to be a component of the innate immune defenses of multiple epithelia and to function in epithelial host defense, through the promotion of mucosal surfaces first line of defense. HE4 is highly overexpressed in EOC and has been identified as a novel clinical biomarker. Clinical and translational studies have established HE4 as a contributor to tumorigenesis and chemoresistance in EOC. However, the exact processes in which HE4 promotes pathogenesis is unclear. The driving hypothesis of this thesis is that HE4 represents a novel targeted therapy due to its established role in EOC tumorigenesis and suggested function in innate immunity. This evidence underlies the goals of this dissertation which are to elucidate the precise mechanisms of HE4's contribution in EOC pathogenesis and establish HE4's role in tumor immune invasion. It is hoped that results from this investigation will ultimately aid in the

development of a novel targeted therapy against HE4 that can modulate tumor pathogenesis as well as the tumor immune response.

In manuscript I, subtractive hybridization revealed that HE4 significantly suppresses expression of osteopontin (OPN) in peripheral blood mononuclear cells (PBMCs) which ultimately compromised their cytotoxicity against ovarian cancer cells. Ovarian cancer cells exhibited enhanced proliferation in conditioned media from HE4-exposed PBMCs and this effect was attenuated by the addition of recombinant OPN and OPN - inducible cytokines (IL-12 and IFN- γ). In addition, ovarian cancer cells and PBMCs with HE4 downregulation via short hairpin RNA (shRNA) were found to be increasingly more susceptible to cell death.

In manuscript II, subtractive hybridization identified dual specificity phosphatase 6 (DUSP6) as the most upregulated gene upon treatment with recombinant HE4 in PBMCs. Flow cytometry revealed that recombinant HE4 significantly upregulated DUSP6 levels specifically in CD8⁺ (cytotoxic T cell) and CD56⁺ (NK cell) populations. Exposure of these cells to HE4 led to an increase in ERK 1/2 phosphorylation, which was subsequently decreased upon DUSP6 inhibition. These results show that DUSP6 suppression of CD8⁺ and CD56⁺ lymphocyte toxicity is strongly enhanced by HE4. In co-culture of PBMCs and ovarian cancer cells, DUSP6 inhibition attenuated the enhanced proliferation noted upon stimulation with HE4. The effect of DUSP6 inhibition was obliterated in CD8⁺ and CD56⁺ devoid PBMCs.

In manuscript III, the role of DUSP6 and its relationship to HE4 in EOC was further elucidated. Increased DUSP6 levels were observed in ovarian cancer cells overexpressing HE4. siRNA-mediated downregulation of both HE4 and DUSP6

revealed a corresponding decrease of either factor. Treatment with an allosteric DUSP6 inhibitor in combination with chemotherapeutic agents produced synergistic effects on the reduction of cell viability. These effects correlated with alterations in expression of ERK pathway mediated genes. Finally, it was found that DUSP6 was significantly overexpressed in serous EOC patient tissue compared to normal adjacent tissue.

In manuscript IV it was determined from a small-scale proteomics study that 63 proteins were found to interact more strongly with HE4, in HE4 overexpressing clones compared to null vector control. The protein found to exhibit the highest interaction in the HE4 clones was Septin-2, a GTP binding protein. Immunohistochemical analysis of Septin-2 in EOC patient tissue revealed that levels were overexpressed in cancer compared to normal and benign controls. To identify Septin-2's role in EOC, stable knockdown cell lines were constructed using the ovarian cancer cell line SKOV3. Septin-2 knockdown cells demonstrated a significantly lowered proliferation rate compared wild-type (WT) and Plasmid C control cells. To better define the role of Septin-2 in EOC, proteomics was employed. Pathway analysis showed an enrichment in autophosphorylation, citric acid cycle, acetyl CoA/energy, and proteasomal/ubiquitin processes in Septin-2 knockout cells.

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PREFACE

This dissertation adopts manuscript format. It is comprised of an introduction, 4 manuscripts, and a conclusion. The format of each individual manuscript is in accordance with the journal that they were or will be submitted to.

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CHAPTER 1

INTRODUCTION

1.1 Ovarian Cancer Incidence and Overall Survival

Worldwide ovarian cancer has an incidence of 240,000 cases per year and an annual mortality rate of 152,000 [1]. This high mortality rate is largely due to that fact that in many cases ovarian cancer is detected at an advanced disease state. In addition, while the initial response rate to frontline chemotherapy is 60-80%, when the tumor recurs it eventually becomes unresponsive to traditional platinum-based chemotherapeutics[2]. Unfortunately, only a minority of patients with advanced stage disease achieve long term survival, as many patients will develop a recurrence within 12-18 months of completion of their primary treatment regimen [3]. Currently, the five year survival rate for ovarian cancer is only 35% [4], and these dire statistics have not improved significantly in the last 30 years[5].

1.2 Ovarian Cancer Subtypes

Ovarian Cancer is divided into two major subtypes that depend on the tissue of origin. Non-epithelial ovarian cancer includes sex cord stromal, germ cell and non-specified ovarian cancers. Non-epithelial ovarian cancers only represent 10% of all ovarian cancer, [6]while the remaining 90% of cancer comprises epithelial ovarian cancer (EOC). EOC encompasses serous, transitional cell, mucinous, endometrioid, and clear cell ovarian cancer [7]. EOC is generally divided into two subtypes. Type 1 EOC are

considered more genetically stable, exhibit a slower tumor growth, and have disease contained within the ovary upon initial presentation. These cancers respond well to surgical intervention [7]. In contrast, type 2 EOC are characterized by an aggressive growth rate and are usually detected at an advanced stage of IIIC. High grade serous ovarian cancer (HGSOC) is the most common histological subtype of Type II EOC, representing nearly three quarters of all patients diagnosed with ovarian cancer [5]. Seventy percent of the time, HGSOC is diagnosed at an advanced stage, leading to a poor prognosis [5]. Therefore, efforts have been made to develop novel prognostic/diagnostic methods and treatments to combat chemoresistance and improve overall survival for HGSOC.

1.3 Current Ovarian Cancer Therapies

Many women who present with elevated tumor markers and abnormal imaging typically proceed with primary debulking surgery. Initial surgery has three goals: diagnosis, staging and cytoreduction. Diagnosis is important as needle biopsies are not indicated for larger ovarian masses to prevent inadvertent spreading of the disease [8]. If a patient presents with significant comorbidities, clinicians will favor neoadjuvant chemotherapy over surgery. This approach minimizes surgical side effects for patients, as the tumor will be reduced following chemotherapy [8].

For the past 20 years, standard of care for women diagnosed with EOC is a primary frontline regimen of carboplatin and paclitaxel [9]. Carboplatin binds to DNA forming a platinum adduct and causes cell death [10]. Paclitaxel's mechanism of action involves enhancing polymerization of tubulin, which stabilizes microtubules.

This stabilization results in the protection of the microtubule polymer from disassembly, and chromosomes are unable to achieve proper metaphase spindle organization. Ultimately, cells are halted in the G2/M phase of the cell cycle [11]. The overall response rate (ORR) for this combinational first line therapy is greater than 75%. However, the majority of patients experience a recurrence and progression of disease. Once a recurrence occurs post-frontline therapy, the chemotherapy chosen for the patient is based on the platinum-free interval (PFI), which represents the time between the completion of the last platinum-based treatment and the detection of relapse [12]. Patients that have a PFI of six months or less are considered to be platinum-resistant, while patients that have a PFI greater than six months mark are considered platinum-sensitive. This distinction determines the second-line chemotherapy regimen used for the patient. [13]For platinum-sensitive patients experiencing recurrence, doxil or gemcitabine is added to a platinum regimen [12]. Doxil, or pegylated doxorubicin(PLD) is a polyethelyne-glycolate-coated liposomal nanoparticle version of doxorubicin that exhibits enhanced drug delivery [14]. Doxorubicin is an antitumor antibiotic that promotes cell death by intercalation into DNA, disrupting DNA repair mediated by topoisomerase II, and generating free radicals [15], Gemcitabine is a pyrimidine antimetabolite that inhibits tumor cell progression through the G1/S phase, halting DNA synthesis [14]. While platinum-sensitive patients undoubtedly survive longer than patients who are initially platinum refractory, prognosis for these patients is still dismal. Platinum combinatorial therapies with doxil and gemcitabine exhibit a progression-free survival (PFS) of only 11.3 and 8.6, respectively[16].

For platinum-resistant ovarian cancer, a non-platinum monotherapy is used with a non-curative goal of toxicity management, as prognosis in this group is poor. Patients in this group are frequently enrolled in clinical trials as a last attempt to control disease [17]. Topotecan, which works through inhibition of topoisomerase I, is a typical example of a salvage chemotherapy that is used in platinum resistant ovarian cancer [18]. The response rate of patients to this treatment is only 12-18%, and PFS is around 3-4 months[19,20]. Other typical monotherapies for platinum-resistant second line EOC include doxil and bevacizumab [8]. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor (VEGF), a major regulator of angiogenesis. Bevacizumab is approved in the recurrent setting, however it's overall efficacy continues to be studied clinically in different chemotherapy lines and in combination with various treatment regimens [21]. While many large phase III trials report an increase in PFS for patients, this response does not correlate with an increased overall survival [21]. Other approved therapies in the maintenance setting are PARP inhibitors. While these inhibitors are approved for all patients, within this setting it has shown the most substantial benefit for patients who harbor the BRCA mutation—about 20-25% of the patient population [22,23]. Current clinical trials for EOC have largely focused on the immune checkpoint inhibition of programmed death receptor (PD-1) and it's ligand PD-L1, however clinical trial results have suggested only a modest benefit [24]. Therefore, there is still a crucial treatment need for the non-BRCA patient population.

1.4 Detection Methods of EOC

Early detection for EOC is difficult as many symptoms reported by patients, such as bloating and pelvic pain, are common symptoms of benign disease [25]. In addition, the sensitivity and specificity of pelvic examinations for EOC screening purposes within an asymptomatic population are poor. Therefore, diagnosis relies heavily on tumor markers and radiologic imaging [25]. Currently, there has not been an official recommendation for routine screening of asymptomatic women who are not high risk for development of an ovarian malignancy [26].

Cancer antigen 125 (CA 125) is the most commonly used and validated tumor marker for the detection of EOC [27]. However, recently there has been sufficient research dedicated to an improvement of serum biomarkers for early detection of EOC. One biomarker that represents such improvement is Human Epididymis Protein 4 (HE4), which has been shown to have a higher specificity and comparable sensitivity to CA 125 [28]. From these results, the risk of ovarian malignancy algorithm (ROMA) was established, which takes into account a woman's menopausal status and incorporates preoperative serum levels of CA 125 and HE4. The ROMA score exhibits both a higher sensitivity and specificity than CA 125 alone [29]. As HE4 has been extensively studied clinically, its prognostic capabilities have also begun to be examined translationally.

1.5 Molecular Functions of HE4

HE4 is encoded by the Whey Acidic Protein (WAP) 4-disulphide core domain (WFDC2) gene. The WFDC2 transcript was thought to be exclusively expressed in

the epididymis and hence was originally proposed to be a specific marker for this tissue type [30]. WFDC2 is a member of the WAP domain, which is a conserved motif of 50 amino acids, including eight cysteine residues arranged as a 4-disulphide core [31]. While WAP proteins can display a variety of functions, the most comprehensively studied members of this family are the antiproteases secretory leukocyte protease inhibitor (SLPI) and elafin. In addition to antiprotease activities, both exhibit anti-inflammatory activities [32,33]. Due to the familial similarity of HE4, it has been proposed to function similarly to SLPI and elafin; however, this role has not been fully defined. In addition to HE4 overexpression in EOC tissue compared to normal and benign ovarian tissues [29,34], it is also readily expressed in the oral cavity, nasopharynx and respiratory tract [35]. It was suggested that HE4 functions in concert with other WAP domain family members to promote epithelial host defenses of the lung, nasal, and oral cavity; supporting the claim that HE4 plays a role in innate immune defenses [35]. HE4's known molecular functions in EOC pathogenesis, particularly its role in promotion of cell proliferation, chemoresistance, metastasis and steroid biosynthesis, are comprehensively discussed in Chapter 2.

1.6 Problem Statement

Challenges in both treatment and diagnosis of patients has led to strong efforts to elucidate new mechanisms of ovarian cancer pathology that can be used to develop novel targeted therapies, which are so desperately needed for this patient population. HE4 is a secretory protein that is overexpressed in EOC serum and tissue. Extensive studies have also shown that HE4 promotes EOC growth and chemoresistance.

However, the exact mechanisms of HE4 functions in EOC pathogenesis are not completely understood. In addition, while HE4 was initially found to play a role in innate immunity, its function in tumor immunity has yet to be defined. Therefore, further investigation of HE4's mechanistic promotion of tumorigenesis is an important step to determine potential efficacy of a targeted anti-HE4 therapy for the treatment of EOC.

The aim of this thesis research is to:

1. Determine genes most suppressed by HE4 in immune cell populations and determine their involvement in muting the cytotoxic ability of immune cells toward ovarian cancer cells.
2. Determine genes most induced by HE4 in immune cell populations and determine their involvement in muting the cytotoxic ability of immune cells toward ovarian cancer cells.
3. Establish the significance of HE4 regulated genes in EOC pathogenesis.
4. Define novel roles of proteins with an identified association with HE4 in EOC pathogenesis.

1.7 Hypothesis

The overall driving hypothesis of this investigation is that HE4 represents a novel therapeutic target due to its role in the promotion of EOC pathogenesis. While it is known that HE4 has a profound role in EOC diagnosis, its therapeutics capabilities have been largely undefined due to an incomplete identification of its signaling

network in EOC. Although the precise mechanism is unknown, it has been established that HE4 promotes tumorigenesis, chemoresistance, and metastasis in EOC. It has been previously proposed that HE4 plays a role in innate immunity; however, its immune functions in EOC have not been explored. The identification of novel genes and proteins at a global level in both EOC and immune cells could aid in the elucidation of a distinct HE4 signaling network. Thus, information obtained from these studies will ultimately contribute to a comprehensive understanding of the biological function of HE4 in EOC.

In manuscript I, subtractive hybridization revealed that *SPP1*, which encodes for the protein OPN, was the gene most suppressed by HE4 expression in peripheral blood mononuclear cells (PBMCs). Flow cytometry was employed to determine specific immune cell populations within the PBMCs best characterized this relationship. Downstream effectors of the suppressed gene responses were measured via ELISA after stimulation with recombinant HE4. Ovarian cancer cells and PBMCs were then co-cultured and treated with recombinant HE4 to determine how this treatment compared to the effect of untreated PBMCs on ovarian cancer cell viability, cell migration, and proliferation. Immunohistochemistry examined populations of OPN positive T cells in human serous EOC tissue. Finally, HE4 siRNA was employed to determine how its downregulation would affect apoptosis in ovarian cancer cells co-cultured with PBMCs. Results were visualized by propidium iodide (PI) and Annexin V staining.

In manuscript II, subtractive hybridization determined that the gene that was most induced by HE4 in peripheral blood mononuclear cells (PBMCs) was *MKP-3*, which encodes for the protein DUSP6. Flow cytometry allowed for identification of specific immune cell populations within the PBMCs that best characterize this relationship. Flow cytometry and western blot examined levels of ERK activation when cells were treated with recombinant HE4 and a small molecule DUSP6 inhibitor within specific immune cell populations. Cells were treated with recombinant HE4 alone and in combination with DUSP6 inhibition, and the following assessments were made: cell viability, cell proliferation using Ki67 staining, and apoptosis by flow cytometry detection of cells double positive for PI and annexin V. To verify effects the small molecule inhibitor on DUSP6, cell viability, proliferation, and apoptosis experiments were repeated in a co-culture devoid of the previously identified immune populations responsible for upregulation of DUSP6 via HE4.

In manuscript III, the relationship between HE4 and DUSP6 was further elucidated in ovarian cancer cells. To better define DUSP6's role in EOC, immunohistochemistry was performed to determine that levels of DUSP6 expression in patient tissue. Cell viability of ovarian cancer cells was assessed following treatment with a small molecule DUSP6 inhibitor alone and in combination with platinum-based chemotherapeutics to determine synergistic effects. qPCR was used to determine how DUSP6 inhibition alone and in combination with carboplatin or paclitaxel alters expression of extracellular signal-regulated protein kinases (p-ERK) response genes. HE4 and DUSP6 small interfering (si)RNA were employed to determine how decreases of either factor affects the other DUSP6 gene and protein levels were

assessed in HE4 overexpressing clones using qPCR and western blot.

In manuscript IV, Septin-2, a protein previously identified as strongly interacting with HE4, was characterized in ovarian cancer for the first time. Immunohistochemistry was employed to determine Septin-2 expression in EOC tissue. Two stable shRNA knockout Septin-2 ovarian cell lines were developed and proliferation of the control and knockout cell lines were compared by cell counting. Verification of the knockdown was confirmed by quantitative polymerase chain reaction (qPCR) and western blot. Finally, proteomics was utilized to determine global changes in protein levels in the stable Septin-2 knockout cells. Gene ontology pathway analysis was also performed to determine cellular proteins most affected by Septin-2 in EOC.

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CHAPTER 2

REVIEW OF LITERATURE

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Beyond the Biomarker: Understanding the Diverse Roles of Human Epididymis

Protein 4 in the Pathogenesis of Epithelial Ovarian Cancer

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2.1 Abstract

Human epididymis protein 4 (HE4) is an important clinical biomarker used for the detection of epithelial ovarian cancer (EOC). While much is known about the predictive power of HE4 clinically, less has been reported regarding its molecular role in the progression of EOC. A deeper understanding of HE4's mechanistic functions may help contribute to the development of novel targeted therapies. Thus far, it has been difficult to recommend HE4 as a therapeutic target owing to the fact that its role in the progression of EOC has not been extensively evaluated. This review summarizes what is collectively known about HE4 signaling and how it functions to promote tumorigenesis, chemoresistance, and metastasis in EOC, with the goal of providing valuable insights that will have the potential to aid in the development of new HE4-targeted therapies.

2.2 Introduction

Approximately 22,280 new cases of epithelial ovarian cancer (EOC) are diagnosed each year, resulting in 14,240 deaths annually in the United States (1). The 5-year survival rate for stage III ovarian cancer is only 39% (1). These dire statistics are due to the fact that the disease is frequently detected at an advanced stage, which drastically impacts overall patient survival. Initially, many patients respond well to first-line therapy that includes cytoreduction surgery and platinum-based treatment. However, many patients experience a chemoresistant recurrence within the first 2 years following treatment (2). Therefore, there is an urgent need for tools to aid in the early diagnosis of ovarian cancer when the disease is fundamentally curable, as well as

improved treatment options for later stage disease.

Human epididymis protein 4 (HE4) is a secretory protein that is member of the whey acidic protein domain family, bearing a conserved motif found in a number a protease inhibitors (3). HE4 was initially suggested to be involved in the innate immune defense of multiple epithelia and has also been found to function in epithelial host defense (4). In ovarian tissue, HE4 is highly overexpressed in EOC compared normal tissue (5, 6). Clinically, HE4 has been identified as a novel therapeutic biomarker for EOC and has also proven useful in detection of recurrent disease (7) Serum HE4 level predicts EOC with equal sensitivity to the established biomarker CA125 and is less likely to be elevated in benign disease (5). A multicenter study led by our institution established the FDA-approved Risk of Ovarian Malignancy Algorithm (ROMA), which combines menopausal status and serum levels of both HE4 and CA125 to detect and monitor EOC. ROMA demonstrates improved sensitivity and specificity over the Risk of Malignancy Index that uses CA125 alone as a serum based biomarker (6). Recently, it has been reported that HE4 can be detected in EOC patient urine, indicating the possibility that it may be utilized as a non-invasive biomarker (8).

While HE4 has been well studied in the clinical setting, less is known regarding its specific molecular and biological roles in EOC. Several studies have investigated its effect on gene expression in EOC cells, as well as on events associated with aggressive disease. This review will summarize HE4's effect on cell proliferation and tumor growth; invasion, migration, and adhesion; chemoresistance; and steroid biosynthesis (Figure 1). Each section will detail associated pathways and factors that are reported to be involved in these HE4-mediated effects, with the goal of revealing

common themes in signaling pathways affected by HE4 and exposing gaps in our knowledge of HE4 molecular and biological functions.

2.3 Review of Literature

Cell Proliferation and Tumor Growth

Within the past 5 years, a handful of *in vitro* and *in vivo* studies have begun to examine HE4's role in proliferation and tumor growth in EOC. A study by Wang et al. examined the role of HE4 in cell proliferation and found that cells treated with recombinant HE4 formed a statistically greater number of colonies compared with control treated cells (9). Furthermore, cells stimulated with recombinant HE4 exhibited greater cell viability compared with respective controls. In another study by Zhu et al. (10), proliferation rate in two different HE4-overexpressing cell lines was significantly higher than in the control cells. Likewise, Zhu et al. (11) and Lee et al. (12) determined that when HE4 was ablated via shRNA, cell proliferation decreased accordingly. Kong et al. report conflicting results, stating that HE4 inhibits proliferation in ovarian cells (13); however, no other studies support these claims, necessitating further explanation to understand the implications of their results. Several *in vitro* studies suggest that HE4 promotes proliferation through its involvement in cell cycle regulation (11). Silencing of HE4 causes G0/G1 cell cycle arrest and blocks the transition from the G1 to the S phase of the cell cycle. Conversely, when cells are stimulated with recombinant HE4, the number of cells in the G2/M phase is increased, while the number of cells in the G0/G1 phase is reduced (9). These results indicate that HE4 may mediate the cell cycle by promoting the G0/G1 transition. In addition, *in vivo* tumorigenicity studies using HE4 knockdown

clones revealed a marked inhibition in the growth of ovarian tumors in nude mice (14), while injection of HE4-overexpressing cells led to more aggressive tumor growth and an overall higher tumor volume compared with controls (10, 15). Taken together, results from numerous *in vitro* and *in vivo* studies provide compelling evidence that HE4 plays a role in cell proliferation and the promotion of tumorigenesis. A full list of factors associated with HE4-mediated cell proliferation and tumor growth can be found in Table 1A and is outlined in greater detail below.

Associated Pathways and Factors-Cell Proliferation and Tumor Growth

Human epididymis protein 4 has been connected to several oncogenic signaling cascades that play key roles in ovarian cancer progression, including the PI3K/AKT pathway, HIF1 α , and ERK/mitogen-activated protein kinase (MAPK) signaling.

Evidence of HE4's effect on activation of each of these pathways is discussed below.

Protein Kinase B Signaling

AKT has been established as a strong promoter of tumorigenesis, and the PI3K/AKT pathway is one of the most commonly hyperactivated pathways in many types of human cancers (16). Its diverse signaling regulates proliferation, growth, survival, motility, angiogenesis, and glucose metabolism (17). HE4-overexpressing OVCAR3 ovarian cancer cells were found to have a marked increase in activation of protein kinase B (AKT) compared with control cells, while HE4 knockdown in OVCAR3 cells reduced AKT activation (12). Moreover, it was found that HE4-overexpressing

SKOV3 clones had naturally higher gene levels of *AKT3* compared with the null-vector control (18), bolstering the claim that HE4 affects the PI3K/AKT pathway.

Hypoxia-Inducible Factor-1 Alpha (HIF1 α)

Adaptation of malignant cells to hypoxic conditions is a key step in the promotion of tumorigenesis and angiogenesis (19–21), a process that is regulated by the transcription factor HIF1 α . Co-immunoprecipitation revealed an interaction between HIF1 α and HE4 in HE4-overexpressing SKOV3 xenografts. There was also strong colocalization of HE4 and HIF1 α in SKOV3 ovarian xenograft tissue. In addition, when SKOV3 cells were treated with HIF1 α siRNA or 2-methoxyestradiol (a HIF1 α inhibitor), there was a marked decrease in HE4 protein levels (15). It is important to note that 2-methoxyestradiol is not a specific HIF1 α inhibitor as it primarily causes the depolymerization of microtubules, which in turn prevents HIF1 α expression (22). Thus, the specificity of the effect of HIF1 α inhibition on HE4 levels may require further investigation. Although the exact mechanism and significance of the HE4-HIF1 α interaction is not understood, this evidence suggests that HE4 could play a role in regulating HIF1 α functions in angiogenesis.

MAPK Signaling

The MAPK pathway is composed of a family of conserved kinases that mediate essential cellular processes such as migration, growth, proliferation, differentiation, and apoptosis (23). The extracellular signal-regulated kinase (ERK) pathway is the best characterized of all MAPK pathways and is deregulated in approximately one-

third of all cancers. Several studies have shown activation of ERK in response to HE4 treatment or overexpression, or suppression of ERK phosphorylation in response to HE4 knockdown (11,12,18). Using microarray analysis, Zhu et al. determined that seven genes involved in the MAPK pathway (CHUK, GADD45A, IL1A, RPS6KA1, HSPA1B, DUSP1, and JUND) were differentially regulated in response to HE4 overexpression in ES-2 cells (10).

Activation of the MAPK/ERK pathway occurs through EGF binding of its membrane bound receptor, EGFR (24). Using co-immunoprecipitation studies in SKOV3 cells, Moore et al. found that HE4 interacts with EGFR, with a greater degree of immunoprecipitation seen in HE4-overexpressing clones than wild-type cells (15). Furthermore, ovarian xenograft tissue showed colocalization of HE4 and EGFR. In addition, when SKOV3 and OVCAR8 cells were stimulated with growth factors EGF, VEGF, and Insulin, nuclear localization of HE4 was significantly increased. Finally, when EGF was repressed by the small molecule inhibitor Iressa, relative intensity of HE4 staining was decreased in ovarian cancer cell lines. Collectively, these results provide several layers of evidence that HE4 is tied to growth factor signaling and the MAPK/ERK pathway, although further research is needed to elucidate the precise mechanisms involved.

HE4's Role in Proliferation in Other Cancers

Human epididymis protein 4 has been investigated as a putative biomarker in endometrial (25–39), lung (40–52), breast (53, 54), pancreatic (55, 56), and gastric cancer (57). While the majority of these studies examine the value of HE4 as a clinical

biomarker for detecting and monitoring disease, one study investigated the molecular mechanisms of HE4 in pancreatic and endometrial cancer. Lu et al. stimulated both pancreatic and endometrial cancer cell lines with recombinant HE4 and found that cell viability, cell growth, and DNA synthesis was increased prominently in both cancer types (56). They also report that HE4 upregulates gene expression of proliferating cell nuclear antigen (PCNA) and downregulates p21 in both cancer cell lines in a dose dependent manner. PCNA, which is expressed in the late G1/S phase of the cell cycle, is required for DNA repair, replication, cell proliferation, and cell cycle progression (58), while p21 is an important effector of tumor suppressor pathways by promoting cell cycle arrest. Specifically, p21 is able to facilitate p53-dependent G1 growth arrest (59). Therefore, results from this study highlight HE4's role in proliferation in both pancreatic and endometrial cancer and lend support to similar evidence from studies published on EOC.

Invasion, Migration, and Adhesion

Several studies have associated HE4 with metastatic properties, including invasion, migration, and adhesion of ovarian cancer cells. Lu et al. found that adhesion to a fibronectin substrate was twofold greater in SKOV3 cells overexpressing HE4 than in mock cells. In addition, a transwell migration assay demonstrated that the HE4-overexpressing clones had a 1.8-fold greater migration capacity than mock transfected cells. By contrast, immunofluorescence analysis showed that HE4 knockout clones displayed inhibited cell-spreading ability in a statistically significant fashion compared with respective controls. Furthermore, cell invasion, proliferation, and migration were

significantly decreased in these clones (14). In agreement with this study, Ribeiro et al. also found that OVCAR8 ovarian cells treated with recombinant HE4 exhibited 2.07-fold greater invasion capacity and 1.29-fold greater adhesion to a fibronectin matrix compared with untreated controls. Interestingly, there was no change in adhesion to collagen I, IV, laminin I, and fibrinogen matrices, suggesting that HE4 has a specific effect on fibronectin adhesion. Haptotaxis toward a fibronectin substrate also was increased in the ovarian cancer cells treated with recombinant HE4 by 1.72-fold (60).

Zhu et al. used wound healing and transwell invasion assays to show that HE4-overexpressing ES-2 and CaOV3 cells possess enhanced cell migration and invasion capacities. In addition, *in vivo* tail vein injection of HE4-overexpressing ES-2 cells into nude mice resulted in significantly more metastatic lung nodules than mock transfected cells (10). Using the same ovarian cancer cell lines, Zhuang et al. report the importance of HE4 interaction with annexin II (ANXA2) to promote invasion and migration *in vitro* and metastasis *in vivo* (61). Finally, Zou et al. found that knockdown of HE4 in SKOV3.ip1 cells inhibited migration and invasion (62). Taken together, these studies strongly suggest that HE4 plays a prominent role in the promotion of ovarian cancer metastasis. A full list of factors associated with HE4-mediated invasion, migration, and adhesion can be found in Table 1B and is outlined in greater detail below.

Associated Pathways and Factors—Invasion, Migration, and Adhesion

Human epididymis protein 4 appears to interact with numerous molecular pathways that promote metastasis in ovarian cancer. However, it is still not entirely known how HE4 affects signaling pathways and gene expression signatures to promote invasion, migration, and adhesion of ovarian cancer cells. Following is a summary of HE4-mediated molecular pathways that are involved in metastatic events in EOC.

Matrix Metalloproteinases (MMPs)

Human epididymis protein 4 has been associated with MMPs MMP-9 and MMP-2, and Cathepsin B. MMPs are a family of zinc-dependent endopeptidases that are vital for the remodeling of the extracellular matrix (63). They are expressed in almost all types of cancers and are responsible for stimulating angiogenesis, tumor growth, and metastasis (64, 65). Cathepsin B is a lysosomal cysteine protease that has been linked to cancer progression (66), specifically in signaling pathways related to angiogenesis (67). In addition, it can promote MMP activity by degrading MMP inhibitors (68).

Interestingly, silencing of HE4 in ovarian cancer cells led to a decrease in protein levels of MMP-9, MMP-2, and Cathepsin B, suggesting these factors may be involved in HE4-mediated tumor promoting effects (11).

Interleukin-1 alpha (IL1A)

Interleukin-1 alpha is a pro-inflammatory cytokine that is involved in angiogenesis and metastasis. IL1A can directly stimulate the synthesis of VEGF (69) and fibroblastic pro matrix metallic proteinase I (70, 71). IL1A causes resistance to EGFR

inhibitors in both colon and head and neck cancers (72, 73). IL1A was also found to be differentially expressed in three separate microarray studies involving HE4. In two microarrays, IL1A levels positively associated with HE4 levels (10, 74), while in one study their levels were inversely associated (18). While there may be some ambiguity as to how HE4 and IL1A are mechanistically linked, the consistent connection between IL1A with HE4 merits further investigation.

Extracellular Matrix Proteins

Integrins are a family of transmembrane proteins that are vital to ECM adhesion and play important roles in wound healing as well as the pathogenesis of cancer (75–77). Integrin $\beta 5$ (ITG $\beta 5$) gene expression was differentially regulated by HE4 in ES-2 and CaOV3 cells, which was confirmed by positive correlation of ITGB5 and HE4 staining in paraffin embedded ovarian tissue samples (10). This finding suggests that integrin signaling is one mechanism by which HE4 can promote increased adhesion of ovarian cancer cells. However, further research is needed to clarify the mechanisms involved.

In addition to ITG $\beta 5$, three other genes related to ECM modeling—syndecan 1 (SDC1), collagen type 1 alpha 1 (COL1A1), and dystroglycan 1 (DAG1)—were more highly expressed in cells overexpressing HE4 and were downregulated in cells with HE4 knockdown (10). SDC1, also known as CD138, is an essential cell surface adhesion molecule that is responsible for maintaining cell morphology and interactions within the surrounding microenvironment (78). Loss of SCD1 in cancer cells is associated with reduced ECM adhesion and enhanced invasion and cell

motility (79). Another ECM gene found to be affected by HE4 expression levels, COL1A1, is a crucial component of the ECM as it supports cartilage, bone, and tendon tissues in the body and also functions to maintain the rigidity and elasticity of tissues (80, 81). COL1A1 plays an important role in cancer, since tumor cells that express COL1A1 are able to dissociate from their surrounding stromal components, which is essential for tumor growth (81). The final ECM gene found to be affected by HE4 is DAG1, which is a cell adhesion molecule that plays a key role basement membrane assembly (82), muscle integrity (83), and the maintenance of basolateral cell adhesion in numerous epithelial tissues (84). Loss of DAG1 is associated with cancer progression (85). Taken together, these results show that HE4 is strongly interconnected with ECM related proteins, specifically those involved in the ITG β 5 signaling pathway.

Our lab has also determined that HE4 regulates several components of the extracellular matrix (60). We performed microarray analyses comparing untreated OVCAR8 wild-type cells to recombinant HE4 treated cells, and OVCAR8 cells overexpressing HE4 to null-vector control cells. Serpin peptidase inhibitor, member 2 (SERPINB2), gremlin 1 (GREM1), laminin- β 3 (LAMB3), laminin- γ 2 (LAMC2), fibroblast growth factor 5 (FGF5), and tenascin C (TNC) were all found to be significantly upregulated upon treatment with recombinant HE4. These genes encode for extracellular matrix proteins that promote cell migration and adhesion (60). Specifically, we found that HE4 upregulates LAMC2 and LAMB3 proteins in a time-dependent manner, and this increase of both factors in turn leads to an increase in laminin-332 levels (60). Laminin-332, a heterotrimer composed of LAMC2, LAMB3,

and LAMA2, is an important component of the basement membrane in epithelial tissue. Abnormal increases in its levels have been shown to promote increased invasion in cancers (86). Further evidence suggested involvement of the FAK pathway in these events. In addition, activation of matriptase, a serine protease responsible for cleaving laminin-332 in its β chain and regulating its effects on metastatic properties, increased upon *in vitro* exposure to recombinant HE4 (60). This study provides compelling evidence that HE4 is involved in basement membrane invasion and adhesion.

Lewis y Antigen

Human epididymis protein 4 undergoes glycosylation before it is secreted by ovarian cells (87), prompting Zhuang et al. to examine the relationship between HE4 glycosylation status and metastatic properties. Lewis y antigen is a glycosyl antigen that is overexpressed in ovarian cancer and has been associated with chemoresistance and poor prognosis (88–97). They determined that Lewis y antigen was present in HE4 from benign and malignant ovarian tissues, *in vitro* cancer cells, and culture medium. HE4 from ovarian cancer samples contained higher levels of Lewis y antigen than HE4 from benign tissues, and their expression co-localized in ovarian cancer tissue (98). Furthermore, when Lewis y antigen was over expressed, it promoted HE4-mediated invasion and metastasis in ovarian cancer cell lines. Conversely, when Lewis y antigen was blocked, the invasive and metastatic properties of HE4 were significantly decreased (99). Interestingly, overexpression of Lewis y antigen increased tyrosine phosphorylation of EGFR and HER/neu, which promoted cell

proliferation through the PI3K/Akt and Raf/MEK/MAPK pathways (100). Thus, it appears that Lewis y antigen and HE4 affect similar signaling pathways that promote tumor growth and malignancy (101). Taken together, these results show that Lewis y antigen could be a potential therapeutic target to decrease HE4 function in the treatment of EOC.

Heparin Cofactor II (HCII)

SERPIND1 encodes for the protein HCII, which is a serum glycoprotein and protease inhibitor (102). A study in non-small cell lung cancer (NSCLC) showed that HCII promotes cell motility, invasion, and filopodium dynamics through the PI3K/AKT pathway. High HCII expression in NSCLC tissue correlated to an increased recurrence rate and shorter overall survival (103). Furthermore, its levels were upregulated in metastatic brain cell lines compared with non-metastatic parental lines, suggesting an involvement of SERPIND1 in metastatic functions (104). Results from a microarray study by Zhu et al. showed that SERPIND1 was upregulated in HE4-overexpressing cells and conversely downregulated in HE4 knockdown cells. These results were validated *via* qPCR and immunohistochemistry. In addition, they found that 37/50 ovarian cancer samples showed positive expression of both SERPIND1 and HE4, and Spearman correlation analysis confirmed that HE4 and SERPIND1 were positively correlated. Finally, Kaplan–Meier analysis revealed that patients with high levels of HE4 and SERPIND1 had a worse prognosis (74). While these data strongly suggest a connection between HE4 and SERPIND1, which may be related to their roles in

promoting ovarian cancer metastasis, further study of the association between these two proteins is required.

Annexin II

Annexin II is a calcium-dependent, phospholipid-binding protein that is overexpressed in a variety of cancers and is involved in angiogenesis, proliferation, apoptosis, cell migration, invasion, and adhesion (105). High levels of Annexin II activate MAPK signaling, which in turn promotes tumor proliferation (106), invasion (107), and metastasis (108). Zhuang et al. employed mass spectrometry and co-immunoprecipitation to identify Annexin II (ANXA2) as a strong HE4 interacting partner (61). This binding promoted invasion and metastasis in ES-2 and CaOV3 ovarian cancer cells. *HE4* and *ANXA2* gene expression levels were found to be co-dependent, and examination of EOC tissue revealed that both HE4 and Annexin II levels were increased in malignant phenotypes compared with benign and normal ovarian tissues. Both proteins were also more highly expressed in tissues from patients with lymph node metastases than those without. Downregulation of HE4 was found to decrease expression of MKNK2 (MAP kinase-interacting serine/threonine-protein kinase 2) and LAMB2 (laminin, beta-2), two factors associated with MAPK and focal adhesion signaling pathways. When HE4 protein was supplemented, this effect was reversed. Collectively, these results show that HE4 interaction with Annexin II to activate MAPK and focal adhesion signaling is one mechanism by which HE4 may promote ovarian cancer metastasis.

Chemoresistance

Several studies show that HE4 is associated with chemoresistance clinically. The addition of HE4 serum levels in the ROMA score better predicts platinum resistance in patients than CA125 alone (15). Angioli et al. found that HE4 was able to predict chemotherapy response in EOC patients undergoing first-line therapy (109). In addition, higher levels of serum HE4 are reported in women who are resistant to first-line chemotherapy (110). Finally, higher HE4 levels inversely correlate with clinical outcome (111), optimal cytoreduction (112), progression free survival (113), and overall survival (15, 113). While the mechanism underlying HE4's contribution to chemoresistance has not been established fully, a few studies have begun to delineate HE4's role in this process. A full list of factors associated with HE4-mediated chemoresistance can be found in Table 1C and is outlined in detail below.

Associated Pathways and Factors—Chemoresistance

Antiapoptotic Gene Expression

A study performed in our lab by Ribeiro et al. determined that HE4 overexpression promotes collateral chemoresistance to both cisplatin and paclitaxel in SKOV3 and OVCAR8 cells (18). Conversely, CRISPR/Cas9 mediated knockdown of HE4 in SKOV3 cells overexpressing HE4 partially reversed their chemoresistance.

Microarray analysis revealed suppression of cisplatin-induced early growth response 1 (*EGR1*) gene expression in HE4-overexpressing SKOV3 cells compared with null vector-transfected cells (18). *EGR1* is a transcription factor that regulates apoptosis, proliferation, and differentiation through regulating expression of genes such as p53,

BCL2, PTEN, IGF2, PDGF, VEGF, TGFB1, and TNF (114, 115). *EGR1* expression is influenced by MAPK signaling, including phospho-ERK and phospho-p38 (115).

Ribeiro et al. found that p38 was strongly activated in SKOV3 null vector-transfected cells treated with cisplatin, while its activation was suppressed in HE4-overexpressing clones (18), suggesting that HE4-mediated chemoresistance may involve MAPK signaling.

Similarly, a study by Wang et al. showed that HE4 represses carboplatin-induced apoptosis *in vitro*. Recombinant HE4 caused an increase in expression of antiapoptotic protein B-cell lymphoma 2 (BCL-2) and a decrease in expression of pro-apoptotic Bax (Bcl-2 associated X protein) in SKOV3 cells treated with carboplatin (9). This decrease in the Bax/Bcl-2 ratio, in addition to the suppression of EGR1 when HE4 is overexpressed, may contribute to the overall decrease in pro-apoptotic factors that leads to chemoresistance in EOC.

Microtubule Stabilization

Microtubule-associated protein tau, which has been associated with paclitaxel resistance in ovarian (116), breast (117), and gastric cancer (118), was upregulated in SKOV3 cells overexpressing HE4 compared with null-vector cells (18). In addition, HE4-overexpressing cells were found to express significantly higher levels of SEPT3 (Septin 3) mRNA compared with null-vector controls (18). Septins are a family of conserved GTP binding proteins that are associated with microtubules and actin filaments and have an important role in cytoskeletal organization (119). Furthermore,

recombinant HE4 treatment of SKOV3 cells increased β -tubulin levels, indicating that HE4 might promote microtubule stability, leading to paclitaxel resistance.

Kinase Signaling Pathways

Human epididymis protein 4 knockdown has also been shown to lead to a reduction in cell growth and the resensitization of ovarian cancer cells to both cisplatin and paclitaxel (12). Lee et al. found that this effect was due to corresponding decreases of ERK and AKT in HE4 knockouts. Activation of these pathways suppresses apoptotic signaling in tumors, suggesting that HE4's regulation of these pathways may be an important mechanism of chemoresistance (120).

Steroid Biosynthesis

Evidence suggests an association between sex steroids and EOC pathogenesis, which is explained by processes that take place during the menstrual cycle. The ovarian surface epithelium (OSE) plays a critical role in ovulation and postovulatory wound repair. During the menstrual cycle, the OSE proliferates during the pro-estrus/estrus transition. After, ovulation the proliferation rate decreases (121). It is hypothesized that when the OSE is repeatedly exposed to high doses of luteinizing hormone and follicle stimulating hormone during the menstrual cycle, this can promote cell proliferation and increase the likelihood of tumor growth over time (121).

Furthermore, epidemiological data have suggested that ovarian cancer progression, pathogenesis, and etiology are highly dependent on the activity of estrogens (121), and numerous experimental studies have demonstrated the promotive effect of estrogens

on ovarian tumors in mice and human EOC cell lines (122). However, activation of diverse oncogenic pathways in EOC may lead to the eventual downregulation of ER α levels and the overall decrease in ER α related signaling in ovarian cancers, rendering them resistant to anti-estrogen therapies (122). Some evidence exists that HE4 may be involved in this process by regulating steroid signaling in EOC. A full list of factors associated with HE4-mediated steroid biosynthesis can be found in Table 1D and is outlined in detail below.

Steroid Biosynthesis Gene Expression

Two separate microarray pathway analyses identified steroid biosynthesis as a pathway affected by HE4 (10, 74). Important genes that were differentially expressed between HE4-overexpressing and HE4 knockdown cell lines were Forkhead box protein A2 (FOXA2) (74), squalene monoxygenase (SQLE), 7-dehydrocholesterol reductase (DHCR7), 24-dehydrocholesterol (DHCR24), and sterol-4-alpha-carboxylate-3-dehydrogenase (NSDHL) (10). FOXA2, a transcription factor required for normal metabolism (123), promotes cell proliferation, maintains cancer stem cells, and is associated with a higher rate of relapse in triple-negative breast cancer (124). Another gene differentially regulated by HE4, SQLE, is an enzyme required in the later stages of cholesterol synthesis (125). Out of 22 cancer types, SQLE copy number-driven gene expression was highest in breast, ovarian and colorectal cancer (125). Also affected by HE4 levels was DHCR7, one of the terminal enzymes involved in the production of cholesterol from 7-dehydrocholesterol (7DHC). DHCR7 was found to be an important regulatory determinate between cholesterol and vitamin

synthesis, as cholesterol is able to accelerate the proteasomal degradation of DHCR7, which can result in the accumulation of 7DHC and an increased production of vitamin D (126). DHCR24, which was also affected by modulation of HE4 levels, is another enzyme in the cholesterol biosynthesis pathway (127). It interacts physically and functionally with DHCR7 (128) and has a number of different cellular functions including anti-inflammatory and antiapoptotic functions, as well as regulation of oxidative stress and cell differentiation (129). DHCR24 has also been proposed to be involved in tumor progression, as its deregulation has been linked to prostate, ovarian, and urothelial carcinomas (127).

Finally, NSDHL is also involved in cholesterol biosynthesis and produces metabolites that are essential in the conversion of squalene to cholesterol (130). Interestingly, NSLD1 was found to have a role in the control of signaling, vesicular trafficking, and degradation of EGFR and its dimerization partners ERBB2 and ERBB3. A study by Sukhanova et al. showed that NSLD1 knockout *in vivo* leads to a reduction in EGFR activation (131). The results from these microarrays show that modulating HE4 levels results in differential expression of several genes involved in steroid biosynthesis—especially cholesterol—suggesting that HE4 may affect tumor metabolism and ultimately contribute to tumorigenesis.

Estrogen Signaling

In support of the above described pathway analyses, two other studies have shown that HE4 interacts with steroid signaling, specifically estrogen signaling. Lokich et al. showed that ER α expression was reduced in HE4-overexpressing SKOV3 cells,

resulting in increased resistance to tamoxifen and fulvestrant compared with wild-type cells (132). 5-Methylcytosine (5-MC), a methylated form of the DNA base cytosine, is one of the most prominently identified epigenetic modifications, and can cause suppression of ER α gene expression. Deregulation of DNA methylation can result in abnormal gene expression and tumorigenesis (133, 134). Lokich et al. found that 5-MC was readily detected in SKOV3 wild-type and null-vector cells but not in HE4-overexpressing clones, suggesting that HE4 overexpression may have an effect on epigenetic modifications (132). However, methylation of the ER α gene was not specifically examined in this study. It is unclear whether HE4 overexpression would promote increased methylation at the ER α promoter region (even with the presence of global demethylation), which would be expected given the reported suppression of ER α in this study.

Interestingly, Chen et al. reported that when HO8910 ovarian cancer cells were stimulated with estradiol (E2), there was an increase in the expression of HE4 at the mRNA and protein level. This effect was not observed in estrogen-insensitive SKOV3 cells; however, when HE4 was knocked down in SKOV3 cells, their proliferative response to estrogen was restored (135). Collectively with the results shown by Lokich et al, this study suggests that HE4 works to suppress estrogen signaling in ovarian cancer cells, which can contribute to resistance to anti-estrogen therapies. Conversely, it appears that estradiol promotes HE4 expression in estrogen-responsive cells, which could indicate a role for HE4 in the initial tumor promoting effects of estrogen. Further clarification of the effect of HE4 on estrogen signaling may be useful in improving implementation of anti-estrogen based therapies.

2.4 Conclusion

Ovarian cancer is an extremely deadly disease owing to the fact that patients are typically diagnosed at a late stage. Initially, patients respond well to frontline platinum therapy; however, a majority of tumors recur, and the initial chemosensitivity eventually gives way to a broad chemoresistance (136). Available detection methods have improved in recent years with the discovery of HE4 as a diagnostic and prognostic biomarker. However, there has yet to be a breakthrough targeted therapy to combat EOC. While PARP inhibitors are used in the maintenance setting for all patients, this therapy has most significantly benefited BRCA-positive patients, who comprise only 20–25% of patients (137, 138). In addition, inhibitors of immune checkpoints, such as programmed death ligand-1 have demonstrated modest benefit in clinical trials for ovarian cancer (139). Therefore, there is still a crucial need for novel targeted EOC treatments.

Although HE4 is well established as a clinical biomarker for ovarian cancer, it has been largely understudied for its therapeutic targeting potential. However, ongoing research continues to support that HE4 is profoundly involved in the pathogenesis of EOC. The individual studies mentioned in this review provide evidence that HE4 promotes EOC progression through pathways associated with cell proliferation, tumor growth, metastasis, chemoresistance, and steroid biosynthesis. These pathways, along with specific genes that have been shown to be associated with HE4, are summarized in Table 1. This compilation of HE4 regulated factors and pathways will serve as a starting point for scientists to further elucidate specific mechanisms by which HE4 ultimately drives tumorigenesis. In addition, a comprehensive summary of clinical, *in*

vivo, and *in vitro* studies related to each facet of EOC progression and HE4 can be seen in Figure 1. This diagram highlights the progress that has been made to establish HE4 as an attractive therapeutic target, while simultaneously denoting areas of research that are still lacking. The results discussed here suggest that inhibition of HE4 *via* a neutralizing antibody or small molecule inhibitor could provide viable treatment options for patients in dire need of more effective therapies.

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Figure 2.1 Graphical Representation of clinical, in vivo and in vitro studies completed relating to HE4 and EOC.

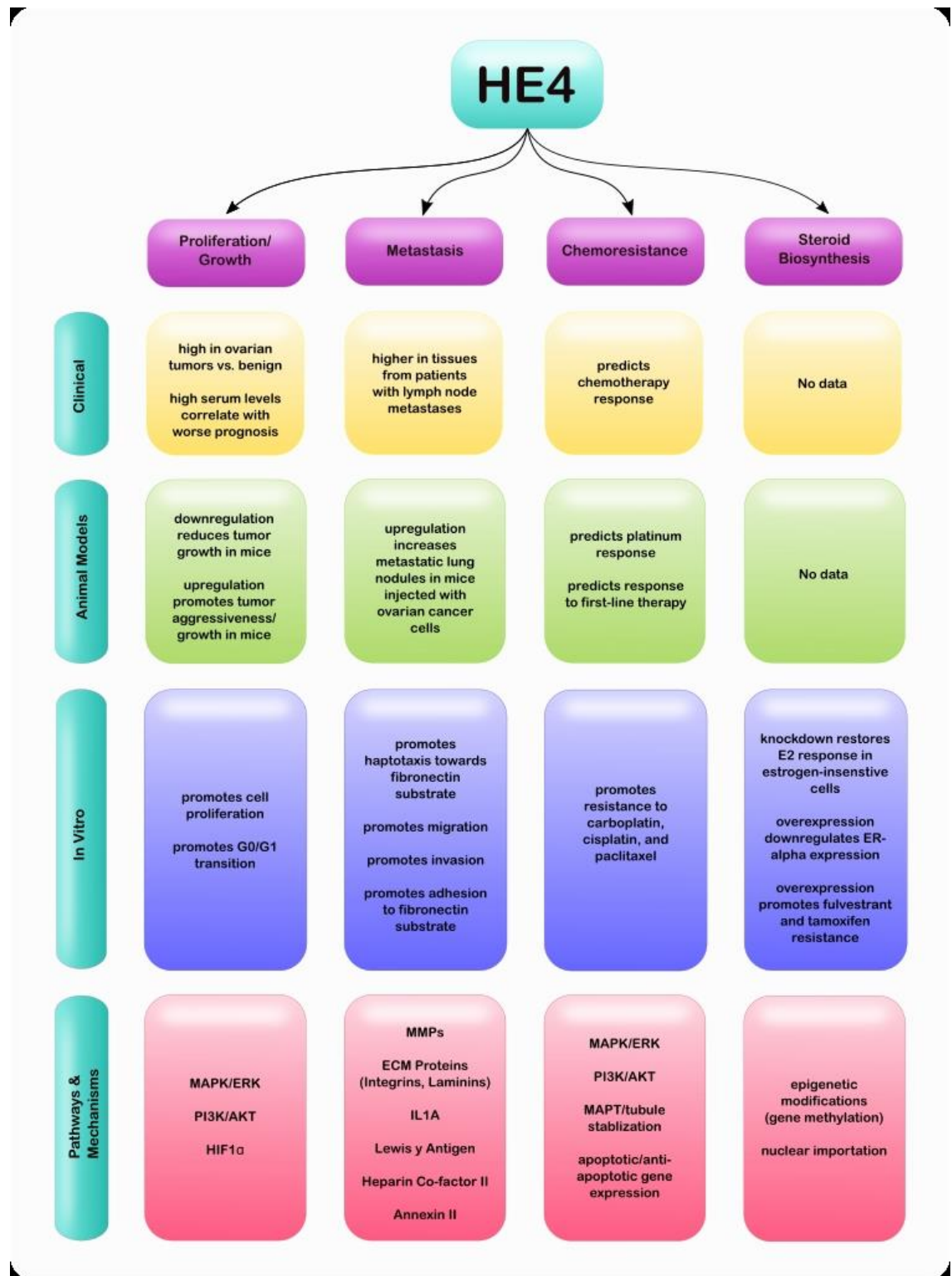


Table 2.1 Summary of factors associated with HE4 in EOC

Gene symbol	Description	Association
(A) Cell proliferation and tumor growth		
AKT	Protein kinase B	<ul style="list-style-type: none"> Upregulated in overexpressing OVCAR3 HE4 cell lines (10) Decreased in response to HE4 knockdown in OVCAR3 cells (10) Upregulated in HE4 SKOV3 clones compared with vector control (16)
HIF1 α	Hypoxia-inducible factor-1 alpha	<ul style="list-style-type: none"> Co-immunoprecipitation with HE4 SKOV3 xenograft tissue (13) Colocalization with HE4 in SKOV3 xenograft tissue (13) Treatment with 2-methoxyestradiol leads to marked decrease in HE4 (13)
ERK	Extracellular signal-regulated kinase	<ul style="list-style-type: none"> Decrease in p-ERK when HE4 was silenced in SKOV3 cells (9) Corresponding decrease and increase in ERK when HE4 was downregulated and overexpressed in OVCAR3 cells (10) Increase in p-ERK in SKOV3 and OVCAR8 cells with recombinant HE4 treatment (16)
CHUK GADD45A	Conserved helix-loop-helix ubiquitous kinase Growth arrest and DNA-damage-inducible protein GADD45 alpha	<ul style="list-style-type: none"> Upregulation when HE4 was induced in ES-2 cells (6)
IL1A RPS6KA1 HSPA1B DUSP1 JUND	Interleukin-1 alpha Ribosomal protein S6 kinase alpha 1 Heat shock 70 kDa protein 1B, Dual specificity protein phosphatase 1 Transcription factor JunD	
EGF/EGFR	Epidermal growth factor/epidermal growth factor receptor	<ul style="list-style-type: none"> Co-immunoprecipitation with HE4 SKOV3 xenograft tissue (13) Colocalization with HE4 in SKOV3 xenograft tissue (13) HE4 overexpressed in OVCAR8 cells when stimulated with recombinant protein (13) HE4 increased when inhibited by IRESSA (13)
VEGF INS	Vascular endothelial growth factor Insulin	<ul style="list-style-type: none"> HE4 overexpressed in OVCAR8 cells when stimulated with recombinant protein (13)
(B) Invasion, migration, and adhesion		
MMP-9 MMP-2 CTSB	Matrix metalloproteinase 9 Matrix metalloproteinase 9 Cathepsin B	<ul style="list-style-type: none"> Downregulated when HE4 is silenced in ovarian cell lines (9)
IL1A	Interleukin-1 alpha	<ul style="list-style-type: none"> Microarray results reveal correlation with HE4 levels (6, 71) One microarray reports an inverse correlation with HE4 (16)
ITGB5	Integrin β 5	<ul style="list-style-type: none"> Differentially regulated by HE4 in ES-2 and CaOV3 cells (6) Correlation with HE4 in paraffin embedded ovarian human tissue (6)
SDC1 COL1A1 DAG1	Syndecan 1 Collagen type 1 alpha 1 Dystroglycan 1	<ul style="list-style-type: none"> Differentially regulated in response to HE4 (71)
LAMB3 LAMC2 GREM1 TNC SERPIND2	Laminin- β 3 Laminin- γ 2 Gremlin 1 Tenascin C Serine peptidase inhibitor member 2	<ul style="list-style-type: none"> Increased expression when stimulated with recombinant HE4 in OVCAR8 cells (57)
LAMA3	Laminin 332 Lewis y antigen	<ul style="list-style-type: none"> Increased in response to HE4 stimulation of LAMB3 and LAMC2 (57) Colocalized with HE4 in human ovarian tissue (98) Immunohistochemistry stained found correlative staining with HE4 (98) Overexpression promoted HE4-mediated invasion and metastasis in <i>in vitro</i> cell lines (99) Knockdown promoted a decrease in invasion and metastatic properties of HE4 (99)
SERPIN D1	Heparin cofactor II	<ul style="list-style-type: none"> Upregulated in HE4-overexpressing clones and downregulated in knockout in <i>in vitro</i> lines (71) Spearman analysis revealed positive correlation with HE4 in human EOC tissue immunohistochemistry staining (71) Poor patient prognosis when levels upregulated in combination with HE4 (71)
ANXA2	Annexin II	<ul style="list-style-type: none"> Mass spectrometry and co-immunoprecipitation identify as strong interacting partner of HE4 (58) Gene levels co-dependent with HE4 (58) Higher along with HE4 in EOC patients with lymph node metastasis than those without (58)
LAMB2 MKN2	Laminin subunit beta-2 MAP kinase-interacting serine/threonine-protein kinase 2	<ul style="list-style-type: none"> Gene levels decreased in presence of HE4 knockdown cell line (58)
(C) Chemoresistance		
EGR1	Early growth response protein 1	<ul style="list-style-type: none"> Suppressed in overexpressing HE4 clones (16)
p38	p38 mitogen-activated protein kinase	<ul style="list-style-type: none"> Activated in NV cells treated with cisplatin and suppressed in overexpressing HE4 clones (16)
BCL2	B-cell lymphoma 2	<ul style="list-style-type: none"> Increased in response to recombinant HE4 <i>in vitro</i> (7)
BAX	bcl-2-like protein 4	<ul style="list-style-type: none"> Decreased in response to recombinant HE4 <i>in vitro</i> (7)
MAPT SEPT3	Microtubule-associated protein tau Septin 3	<ul style="list-style-type: none"> Upregulated in HE4-overexpressing clones (16)
TUBB	β -Tubulin	<ul style="list-style-type: none"> Increased in response to recombinant HE4 <i>in vitro</i> (16)
ERK AKT	Extracellular signal-regulated kinase Protein kinase B	<ul style="list-style-type: none"> Knockdown with HE4 lead to a reduction in cell growth and resensitization to cisplatin and paclitaxel (10)
(D) Steroid biosynthesis		
FOXA2	Forkhead box protein A2	<ul style="list-style-type: none"> Differentially expressed in -overexpressing clones and knockouts (71)
SQLE DHCR7 NSDHL	Squalene monoxygenase Dehydrocholesterol reductase Sterol-4-alpha-carboxylate-3 dehydrogenase	<ul style="list-style-type: none"> Differentially expressed in HE4-overexpressing clones and knockouts (71)
5-MC	5-Methylcytosine	<ul style="list-style-type: none"> Downregulated in HE4-overexpressing clones compared with wild-type SKOV3 cells and null vector (131)
ESR1	Estrogen/estrogen receptor	<ul style="list-style-type: none"> Abolished in HE4-overexpressing clones (131) When stimulated in H08910 cells HE4 increased in gene and protein levels. Effect not observed in SKOV3 cells (131) Increase expression in HE4 SKOV3 knockdown (131)

CHAPTER 3

MANUSCRIPT I

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HE4 Suppresses the Expression of Osteopontin in Mononuclear Cells and Comprises Their Cytotoxicity Against Ovarian Cancer Cells

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I.1 Abstract

Ovarian tumors are known to suppress immunosurveillance and promote immune escape. Here, we examine the role of the secretory glycoprotein HE4 in ovarian cancer immune evasion. Through modified subtractive hybridization analyses of human peripheral blood mononuclear cells (PBMCs), we have characterized gene targets of HE4 and established a preliminary mechanism of HE4-mediated immune failure in ovarian tumors. Upon exposure of PBMCs to recombinant human HE4 *in vitro*, osteopontin (OPN) emerged as the most suppressed gene, while DUSP6 was the most upregulated gene. SKOV3, a human ovarian carcinoma cell line, exhibited enhanced proliferation in conditioned media from HE4-exposed PBMCs, and this effect was attenuated by the addition of recombinant OPN or OPN-inducible cytokines (IL-12 and IFN- γ). Additionally, upon co-culture with PBMCs, HE4-silenced SKOV3 cells were more susceptible to cytotoxic cell death. The relationship between HE4 and OPN was further reinforced through analysis of serous ovarian cancer patient samples. In these biopsy specimens, the number of OPN⁺ T cells correlates positively with progression free survival (PFS) and inversely with serum HE4 level. Taken together, these findings show that HE4 enhances ovarian cancer tumorigenesis by compromising OPN-mediated T cell activation.

I.2 Introduction

Human epididymis protein 4 (HE4) is a member of the whey acidic domain family of proteins (WAP), which are generally regarded as protease inhibitors (1-3). HE4 was first identified in the male reproductive tract but has since been found in select other tissues, such as the kidney, female reproductive tract, breast, and lungs (4,5). In addition, it is highly overexpressed in several human malignancies, including ovarian and endometrial cancer (5-8). HE4's role in normal and malignant tissue is still unclear; however, as a known negative prognostic factor in women with epithelial ovarian cancer, its serum levels correlate with chemoresistance and reduced survival (9-11). Our previous work with HE4 has led to the development of a USFDA approved biomarker tool for evaluation of pelvic masses, coined the Risk of Ovarian Malignancy Algorithm (ROMA) (12-15). The ROMA score incorporates HE4, CA-125, and menopausal status into a calculation to estimate ovarian cancer risk. As a biomarker, HE4 detection and monitoring is already improving patient care. However, it is imperative that we learn more about its function in order to better understand ovarian tumorigenesis and ultimately develop effective therapies for this fatal cancer.

In this present study, we begin to elucidate HE4's role in the interplay between tumor cells and the immune system. We generated cDNA-subtracted libraries of HE4 treated peripheral blood mononuclear cells (PBMCs) and employed a modified subtractive hybridization method to identify differentially expressed genes. This strategy identified osteopontin (OPN) as one of the most prominently suppressed targets in PBMCs following HE4 treatment. OPN is a secretory glycosylated phosphoprotein encoded by the gene *SPPI*. OPN contains an arginine-glycine-aspartate (RGD)

sequence that—via interactions with integrin family members or CD44—triggers downstream signaling events and relays early cell-mediated immune responses (16-18). We observed that HE4-induced OPN suppression mitigated the cytotoxicity of PBMCs against cultured human ovarian cancer cells *in vitro*. Further, the expression levels of OPN in stromal infiltrating T cells in biopsy samples from serous ovarian cancer patients showed direct association with patient progression free survival (PFS). Together, our data demonstrates that HE4 inhibits the immune function of PBMCs, most prominently T cells, via suppression of OPN production.

I.3 Materials and methods

Subtractive hybridization and TA-cloning.

Primary human PBMCs were obtained under the auspices of Women & Infants Hospital IRB approval from a single volunteer. Approximately 5×10^7 of PBMCs were obtained from 40 mL of heparinized total blood. The cells were suspended in 5 mL of serum free RPMI1640 medium (#31800022; Invitrogen, Carlsbad, CA, USA) and incubated with or without $0.01 \mu\text{g} / \text{mL}$ (approximately 270 pM) of rHE4 (MBS717359; MyBiosource, San Diego, CA, USA) for 6 hours, and total RNA was isolated using TRIzol reagent (Invitrogen). The dose of HE4 ($0.01 \mu\text{g} / \text{mL}$, 270 pM) was chosen as a comparable concentration to serum levels in patients with various types of ovarian tumors (19). Around 300 μg of total RNA was isolated in this scale of preparation. The RNA was stored at -80 degrees until messenger RNA (mRNA) isolation. Blood draws were repeated at a minimum of 7-day intervals until the amount of total RNA collected reached 1 mg. Next, mRNA was purified using oligo dT coated

magnetic beads (Takara-Clontech, Mountain View, CA, USA). Approximately 10 μ g of mRNA was isolated from the 1 mg of total RNA, from which subtractive cDNA libraries were constructed using PCR-SelectTM cDNA Subtraction Kit (Takara-Clontech, Mountain View, CA, USA), following the manufacturer's instructions. Briefly, the tester and driver cDNAs are synthesized from poly A⁺ RNA generated from control and HE4 treated PBMCs. The tester and driver cDNAs are each digested with a restriction enzyme, Rsa I, to yield shorter, blunt-ended molecules. The tester cDNA is then subdivided into two portions, and each is ligated with a different cDNA adaptor. The ends of the adaptor do not contain a phosphate group, so only one strand of each adaptor attaches to the 5' ends of the cDNA. The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in. The differentially expressed genes were identified through two steps of hybridizations followed by two steps of PCR. The PCR products of the differentially expressed genes were cloned into a pUC19-TA vector. The clones containing the inserts were selected by blue/white selection and were amplified by colony PCR using M13 primers.

Cell culture

The human ovarian cancer cell lines SKOV3 and OVCAR8 were obtained from ATCC (Manassas, VA, USA). RPMI1640 (#31800022; Invitrogen) and DMEM supplemented with 1.0 mM of sodium pyruvate (#31600034; Invitrogen) were used for culturing PBMC and SKOV3, respectively. Conditioned media was obtained from 24-hour PBMC culture with or without 0.01 μ g / mL (270 pM) of rHE4. Residual

rHE4 in the conditioned media was deprived as follows. Five mLs of media was incubated with 10 μg (100 μL) of anti-human HE4 antibody (sc-293473; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at 4 degrees. Then, 100 μL packed volume of protein G coated sepharose beads (GE Healthcare Life Science, Pittsburg, PA, USA) was added and incubated for 4 hours at 4 degrees. After the incubation, the sepharose beads were removed by centrifugation and the supernatants were processed through a sterile 0.2 μm pore syringe filter. The conditioned media were used without any dilution. For the cell-mediated cytotoxicity assay, 2×10^5 target cells (SO or shHE4 transfected SKOV3) were seeded in each well of 6-well plates, and then were incubated overnight with complete media. The next day, cells were placed in serum free media for another overnight incubation to induce quiescence, and then 1×10^7 of the effector cells (PBMC) mixed with propidium iodide (Invitrogen) were added to each well. Some of the wells contained 5 pg / mL of rIL-12 (219-IL-005; R&D Systems, Minneapolis, MN, USA), 20 pg / mL of rIFN- γ (SRP3058; Sigma-Aldrich, St. Louis, MO, USA) or 0.05 μg / mL of rHE4 (ab132299; Abcam) in combinations as indicated in Figure 4 (lower panel). The ovarian cancer cell lines were morphologically normal and kept growing up to 72 hours in serum deprived DMEM. In order to avoid unexpected effects of unknown constituents in the serum, all experiments were performed under serum free condition. shRNA for human HE4 (TR318721; Origene, Minneapolis, MN, USA) were transfected into SKOV3 using Lipofectamine 2000TM (Invitrogen) following the manufacturer's instructions. In other cases, cells were treated with 20 pg / mL recombinant OPN (ab92964; Abcam) or 0.01 μg / mL rHE4.

Quantitative Real-Time PCR

RNA was isolated from PBMCs of healthy donors using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). qPCR was performed using Premix Ex-TaqTM II (Clontech-Takara) probes for OPN, IL-12B and IFN- γ . All reactions were normalized using GAPDH as an endogenous control. Amplification data were analyzed using the $\Delta\Delta$ Ct method.

Flow cytometry

FITC-labeled anti CD3 (HIT3a), CD14 (M5E2), CD19 (HIB19) and CD56 (B159) antibodies were obtained from BD Biosciences (Billerica, MA, USA). Alexa Fluoro[®] 647-labeled anti OPN antibody (EPR3688) was obtained from Abcam. After staining for cell surface markers (CD3, CD14, CD19 and CD56), the cell membrane was permeabilized by 0.2 % Triton X-100 and 0.2 % digitonin, and then stained for OPN. Flow cytometric analysis was performed with FACSCanto system and FACSDiva software (BD Biosciences).

ELISA

ELISA kits for OPN, IL-12AB, IFN- γ and HE4 were obtained from R&D Systems. The assays were performed following the manufacturer's instructions.

Viability and migration assays

1×10^3 / well SKOV3 cells were seeded in a 96-well culture plate. After overnight incubation with serum free medium, conditioned media was added to the quiescent cells that were cultured for 24, 48 and 72 hours. The cell viabilities at each time point were evaluated using CellTiter-Blue[®] (Promega, Madison, WI, USA). Cell migration assays were performed using InnoCyte[™] Cell Migration Assay (EMD Millipore, Taunton, MA, USA). 5×10^4 SKOV3 cells were seeded in the upper chamber of a 96-well plate with the lower chamber containing the PBMC-conditioned media. Migration activities were accessed after incubating the cells for 24 hours in a CO₂ incubator at 37 °C.

Immunohistochemistry

SKOV3 cells were seeded at 0.5×10^4 / chamber in a 4-chamber slide. After overnight incubation with serum free medium, conditioned media was added to the quiescent cells and the cells were cultured for 48 hr. The cells were fixed with 2 % formaldehyde and permeabilized by 0.2 % TritonX-100. The slides were then incubated with a mouse monoclonal anti-Ki67 antibody (clone B56; PD Pharmingen, Franklin Lakes, NJ, USA) overnight at 4°C in a humidified chamber. The slides were then incubated with an ALP conjugated anti-mouse IgG (H+L) secondary antibody (Bio-Rad, Hercules, CA, USA) for 30 minutes at room temperature. Bound antibody was detected using the ALP substrate kit (Vector Laboratories, Burlingame, CA, USA) and lightly counterstained with veronal acetate buffered 1% methyl green solution, pH 4.0 (Vector laboratories). Permount[™] (Fisher Scientific, Ottawa, Ontario, Canada) was

used as the mounting media and sections were cover slipped. The immunohistochemical studies were repeated four times on samples prepared from different cultures. The proportion of Ki67 positive cells was calculated according to the following formula: $100 \times (\text{the number of Ki67-positive nuclei} / \text{total number of nuclei})$. Each image was analyzed at least four times to obtain an average labeling index.

Western blotting

Cellular contents of HE4 in SKOV3 cell lines transfected with shRNA against HE4 were assessed by western blotting. Antibodies against HE4 were obtained from Origene (TA326648). Anti-actin antibody (clone 2G2; EMD Millipore) was used for detection of the internal loading control. The results were visualized with SuperSignal™ West Pico chemiluminescent substrate (ThermoFisher Scientifics) and analyzed with the UN-SCAN-IT gel software for Windows (Version 6.1; Silk Scientific Inc.).

Confocal immunofluorescent microscopy

Formalin-fixed, paraffin-embedded tissue sections were cut to a thickness of 4 μm . For heat-induced epitope retrieval, deparaffinized sections in 0.01 M citrate buffer were treated three times at 90 °C for 5 minutes using a microwave oven. After blocking with 10% normal horse serum, sections were incubated with rabbit anti-OPN antibody (FL-314; Santa Cruz Biotechnology) or mouse anti-CD3 (PS-1; Abcam) overnight at 4 °C, washed with PBS and incubated with DyLight 488 goat anti-rabbit

IgG (DL1488; Vector Laboratories) or DyLight 594 horse anti-mouse IgG (DL2594; Vector Laboratories) secondary antibodies for 1 hour at room temperature in the dark. Slides were washed again with PBS and cover-slipped with DAPI-containing mounting medium (Vector Laboratories). Confocal images were acquired with a Nikon C1si confocal (Nikon Inc. Mellville, NY, USA) using diode lasers 402, 488 and 561. Ten fields/sample were randomly selected based on DAPI staining and counts were performed for CD3 and OPN using a 40x objective. Counts are expressed as # of positive cells/mm². All donors of the biopsies and the PBMCs provided written informed consent. The study was approved by the Women & Infants Hospital ethics committee.

Statistics

Data were expressed as average \pm SEM of at least three independent experiments. An unpaired, two-tailed Student t-test was used to determine significance. Multiple treatments were analyzed by using one-way ANOVA followed by Ryan's multiple comparison test. Spearman's rank correlation test was used to assess the immunofluorescent staining on biopsy specimens. Differences between groups were considered statistically significant when $p < 0.05$.

I.4 Results

Differential expression of PBMC genes after HE4 exposure

To identify differentially expressed genes after HE4 exposure, modified subtractive hybridization was performed. PCR products of the differentially expressed genes were

cloned into pUC19-TA vectors to create a differential cDNA library. PCR products from 252 HE4-induced and 253-HE4 suppressed gene colonies were sequenced resulting in the identification of 211 induced genes and 208 suppressed genes. Among the identified genes, 23 induced and 15 suppressed sequences showed no significant similarity (NSS) to known genes in available nucleotide databases. Among the 208 suppressed genes, OPN emerged as the most frequently identified gene (6 times out of 253 sequences, 2.4%; Table 1).

HE4 reduces OPN expression in PBMCs

HE4-induced suppression of osteopontin in PBMCs was then confirmed via three modalities: flow cytometry, quantitative PCR (qPCR), and ELISA using PBMCs from four individual donors. First, PBMCs were cultured with recombinant human HE4 (rHE4; 0.01 $\mu\text{g} / \text{mL}$) for 24 hours and collected for flow cytometry analysis. Protein expression of OPN in CD3⁺ PBMCs (T cells) was found to be significantly reduced with HE4 exposure ($48.8 \pm 1.0\%$ vs $37.4 \pm 1.0\%$; $p < 0.05$; Figure 1A). PBMCs were harvested after a 6-hour incubation with rHE4 (0.01 $\mu\text{g} / \text{mL}$), revealing a 0.70 ± 0.03 -fold reduction in OPN mRNA production (Fig 1B). PBMCs were then exposed to rHE4 (0.01 $\mu\text{g} / \text{mL}$) for 24 hours and concentrations of OPN in the cell lysates and the culture supernatants were measured by ELISA. The concentrations of OPN in lysates (159.82 ± 3.14 vs 103.61 ± 3.23 pg / mL , $p < 0.01$) and culture supernatants (53.37 ± 3.14 vs 30.08 ± 3.48 pg / mL , $p < 0.01$) were also decreased with HE4 exposure (Figure 1C).

HE4-mediated IL-12 and IFN- γ reduction in PBMCs is reversible with supplementation of OPN

In lipopolysaccharide-stimulated macrophages, OPN has been shown to enhance IL-12 production and suppress IL-10 production, thereby promoting Th1 activity (17, 18). In order to estimate the impact of HE4 on PBMCs, transcriptional expression and protein levels of Th1 related cytokines IFN- γ and IL-12 were evaluated. Cells were incubated with either: (a) vehicle control, (b) 0.01 $\mu\text{g} / \text{mL}$ rHE4 or (c) 0.01 $\mu\text{g} / \text{mL}$ of rHE4 and 20 pg / mL rOPN for 6 hours and cell lysates and/or culture supernatants were taken for qPCR and ELISA. As shown in Figure 2A, relative expressions of IL-12B and IFN- γ mRNA were decreased (61% and 69% respectively) upon treatment with rHE4. This suppression was partially reversed by the addition of recombinant OPN (rOPN) to culture conditions. Protein expression, as determined by ELISA, is shown in Figure 2B. IL-12 concentrations, both in lysates and culture supernatants, were reduced after HE4 exposure (4.81 ± 0.17 to 2.05 ± 0.08 pg / mL in cell lysate and 7.17 ± 0.26 to 3.56 ± 0.20 pg / mL in supernatant). The addition of rOPN resulted in a nearly complete reversal of HE4-mediated IL-12 suppression. Similarly, IFN- γ concentrations in the cell lysates and supernatant decreased significantly with rHE4 treatment (from 35.55 ± 1.03 to 14.41 ± 1.10 pg / mL and from 19.92 ± 0.82 to 11.10 ± 0.59 pg / mL , respectively) and the addition of rOPN again caused recovery of the cytokine levels.

Conditioned media from HE4-treated PBMCs enhanced the viability, proliferation, and invasiveness of ovarian cancer cells

In order to assess the effects of PBMC-produced soluble factors on cancer cell activity, SKOV3, an immortalized ovarian cancer cell line, was incubated with the conditioned media from PBMC cultures (2×10^6 / mL density) with or without rHE4. SKOV3 cells cultured with HE4-treated PBMC media showed significantly higher viability than cells cultured with the HE4-depleted PBMC conditioned media at 48 hours (1773.84 ± 436.38 vs. 3081.17 ± 348.03 , $p < 0.01$) and 72 hours (3146.67 ± 494.87 vs. 4568.84 ± 407.74 , $p < 0.01$; Figure 3A). Next, a cell migration assay was employed to determine whether conditioned media from rHE4-exposed PBMCs affects ovarian cancer migration as a surrogate of metastatic capability. The SKOV3 cells that were incubated with HE4-exposed PBMC media showed more extensive migration than control cells (RFU of 1147.21 ± 365.09 vs. 3138.14 ± 419.66 , $p < 0.01$, Figure 3B). Immunohistochemistry using anti-Ki67 was performed to evaluate the proliferation of SKOV3 cells in the presence of rHE4-exposed PBMC media or vehicle-exposed conditioned media. The proliferation rate of tumor cells in HE4-exposed PBMC conditioned media was higher than control media (63.8 ± 18.1 vs 39.9 ± 7.6 %, $p < 0.01$, Figure 3C). These findings suggest that PBMCs alter their soluble factor release under the influence of rHE4, thus enhancing the viability, proliferation and migration capabilities of the cultured ovarian cancer cells.

HE4 inhibition increases ovarian cancer susceptibility to PBMC-mediated cytotoxicity
In order to evaluate the impact of native (tumor-cell produced) HE4 on PBMCs, SKOV3 cells were co-cultured with PBMCs after stable transfection with HE4 specific shRNA (shHE4) or a scrambled oligonucleotide control plasmid (SO). Clones

of shRNA transfected cells were tested for their phenotype by western blotting and ELISA (Figure S5). After a 2-hour incubation at 37 °C, the effector cells were washed away and the target cells were analyzed by flow cytometry. The silencing of HE4 in SKOV3/PBMC co-cultures led to a significant increase in IL-12 and IFN γ concentrations (Table 2). As shown in Figure 4, HE4 silencing also increased tumor cell susceptibility to PBMC cytotoxicity, an effect that was reversed by the addition of rHE4. Furthermore, this “rescue” by rHE4 was at least partially abrogated by the addition of recombinant IL12 (rIL-12) or recombinant IFN- γ (rIFN- γ) to the culture conditions. These findings suggest that the native HE4 production by ovarian cancer cells is critical to cell-mediated cytotoxicity resistance.

Ovarian cancer patient prognosis correlates to the number of intra- and peri-tumoral CD3⁺ T cells and stromal OPN-producing cells

Twenty biopsies from high-grade serous ovarian cancer patients were evaluated by dual fluorescent stain with antibodies against CD3 and OPN (Table 3). In the tumor segments of the biopsy specimen, some CD3⁻ tumor cells showed high OPN expression, while in the stromal area of the biopsy the principal OPN⁺ cells were CD3⁺ T cells (Figure 5A). A significant portion of the stromal CD3⁺OPN⁺ cells was accompanied by strong OPN staining in their cytosols or the surrounding areas (Figure 5B). In order to investigate the clinical relationship of HE4, OPN and CD3, numbers of T cells (CD3⁺) and total OPN⁺ cells were correlated with pre-operative serum HE4 level (available for 13 patients) or PFS duration (available for 16 patients). The numbers of CD3⁺ infiltrating T cells, both in the tumor and stroma, were directly

proportional (tumor, $r = 0.541$, $p = 0.03$; stroma, $r = 0.512$, $p = 0.02$) to the patients' PFS duration (Figure 5C). Additionally, the number of OPN⁺ cells, both in tumor and stroma, were in inversely proportional (tumor; $r = -0.635$, $p = 0.019$, stroma; $r = -0.582$, $p = 0.037$) to serum levels of HE4. Moreover, the number of OPN⁺ cells in the stroma (but not in the tumor) were directly proportional to the patients' PFS duration ($r = 0.711$, $p = 0.002$; Figure 5D). These findings suggest that tissue infiltrating T cells play a critical role in the suppression of ovarian cancer progression.

I.5 Discussion

HE4 is known to be highly overexpressed in ovarian cancer, but its causal relationship to ovarian tumorigenesis has not been firmly established. Emerging studies suggest that HE4 overexpression promotes ovarian tumor growth and imparts strong resistance against the most commonly used chemotherapeutics (20-24). Accordingly, serum HE4 level is an early predictor of platinum resistance (9, 23), and ovarian cancer patients that experienced greater HE4 reduction during neoadjuvant chemotherapy exhibited improved overall survival (24). Our study has shown a novel role for HE4 in the inhibition of immune cell activity through OPN suppression. We identified the gene for OPN, *SPP1*, as the most prominently suppressed gene in PBMCs in response to HE4 exposure *in vitro*. Additionally, HE4 was found to downregulate OPN production in CD3⁺ T cells. It is important to note that the changes in OPN expression in T cells after HE4 exposure are quite modest according to the flow cytometric analysis, and this raises the question of whether these small differences translate into functional consequences. However, the changes in OPN levels determined by qPCR and ELISA

(Figure 1B and C) appear much more robust. These findings suggest that the secretion of OPN is an important factor to include in the assessment of the biological response to HE4. In accordance with this hypothesis, we confirmed suppressed secretion of OPN-induced cytokines IL-12 and IFN- γ in the rHE4 exposed PBMCs. HE4's inhibition of immune cell function was further clarified by our co-culture experiments showing reduced antitumoral cytotoxicity.

OPN is primarily considered a pro-tumorigenic protein. In various types of cancers, serum OPN levels are directly proportional to degree of malignancy and inversely proportional to patient survival (25-27). OPN also plays a critical role in tumor formation and growth by promoting cancer cell survival, proliferation, metastasis, and angiogenesis (28, 29). On the other hand, some studies describe anti-tumor effects of OPN (30-33). Among them, Crawford et al., with elegantly designed cancer cell inoculation experiments using OPN null mice, demonstrated that host-derived OPN acted as a chemoattractant to enhance the host defense activity of macrophages, whereas tumor-derived OPN inhibited macrophage function to enhance the growth or survival of cancers (30). In our study, the number of OPN⁺ cells in stroma (mainly CD3⁺ T cells), but not in tumor (mainly CD3⁻ tumor cells), correlated positively to patients' PFS durations. The dichotomic function of OPN presented by Crawford et al. may serve as an explanation of the findings in the present study.

In summary, this study is the first to implicate HE4 in ovarian cancer immune escape and provide the rationale for targeting HE4 to restore normal tumor immune editing. We are currently working to identify small molecules and/or neutralizing antibodies to further validate the utility of HE4 inhibition as a novel immunotherapeutic in the

treatment of ovarian cancer. However, several barriers remain in the achievement of this objective. For example, PBMCs in ovarian cancer patients may already be exposed to a chronically high level of HE4, which may have differing effects than the acute exposure performed in our study. Secondly, due to multiple complicated steps in the subtractive hybridization procedure, this study stands on the data from a single donor. The benefit of this experimental strategy lies in perspicuous outcomes; however, it also introduces inherent limitations in interpretation of the results. To begin to circumvent this pitfall, we validated the HE4-mediated downregulation of OPN using flow cytometry, qPCR, and ELISA in PBMCs from four healthy donors. This issue will be further addressed in subsequent studies on HE4. Lastly, it is important to note that OPN is known to play a role in humoral immunity (34-36). Further studies are required to fully understand the role of HE4 and OPN in humoral immunity in relation to ovarian cancer. Additionally, as we showed in Table 1 that PBMCs modulated a variety of genes in response to HE4 exposure. It is therefore very likely that other factors, besides osteopontin, are also contributing to in the inhibitory effect of HE4 on the immune system. Further analysis of the functions of these genes, and how they are associated with HE4, is warranted.

I.6 References

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Figure I.1 HE4 downregulates expression of OPN in PBMCs.

(A) Two-color flow cytometric analysis of PBMC following a 24-hour incubation with 0.01 $\mu\text{g} / \text{mL}$ of rHE4 (HE4) or vehicle (CTR). 2D-scatterplots of OPN (Alexa Fluor 647) and CD3 (FITC) are shown. The numbers on the scatterplots represent mean \pm SEM % of each quadrant. (B) OPN transcription in response to a 6-hour incubation with 0.01 $\mu\text{g} / \text{mL}$ rHE4 (HE4) or vehicle (CTR) were evaluated by real time PCR. A bar graph represents relative expression levels against control. (C) OPN concentrations of PBMC lysates and culture supernatants after a 24-hour incubation with 0.01 $\mu\text{g} / \text{mL}$ of rHE4 (HE4) or vehicle (CTR) were evaluated by ELISA. All the experiments were done with PBMCs from four individual donors and repeated 3 (A), 9 (B) and 10 (C) times. The mean is shown in the bar graphs; error bars represent SEM ($n > 10$). * $p < 0.05$, ** $p < 0.01$.

Figure I.1

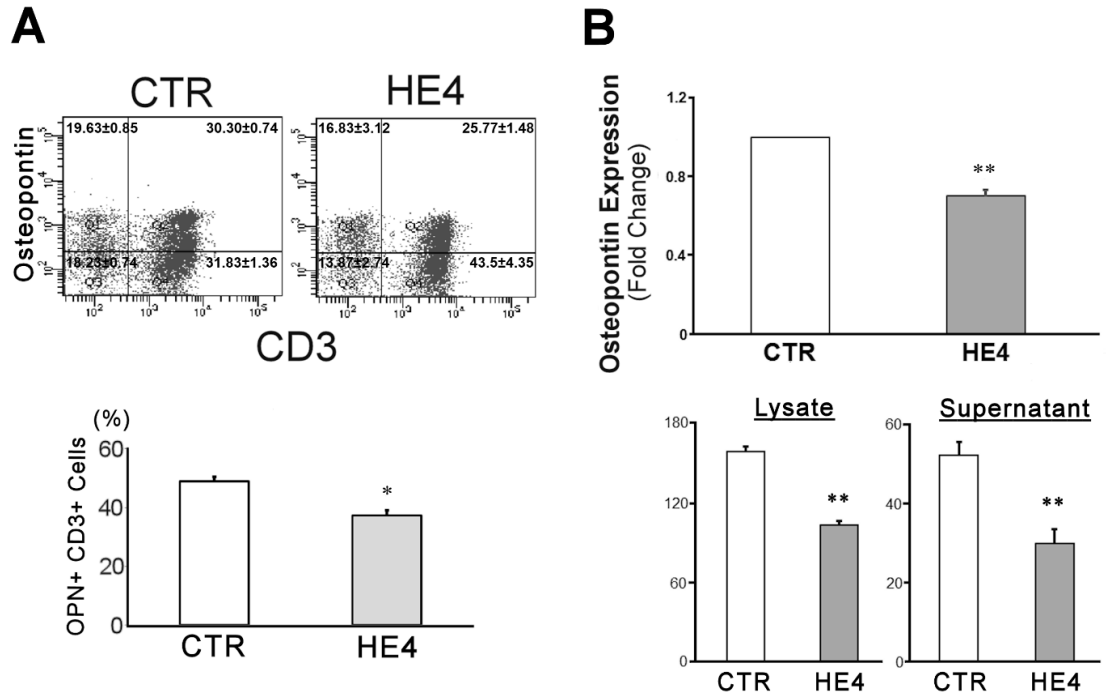


Figure I.2 HE4 suppresses expression and secretion of IL-12 and IFN- γ by PBMCs.

(A) PBMCs were incubated for 6 hours in serum free media under the indicated conditions (vehicle, 0.01 $\mu\text{g} / \text{mL}$ rHE4 and rHE4 + 20 pg / mL of rOPN). After a 6- hour incubation, transcription levels of IL-12B (p40) and IFN- γ were evaluated by real time PCR. A bar graph represents relative expression levels against control. (B) The concentrations of IL-12AB (p70) and IFN- γ in the cell lysates and the culture supernatants from 24-h incubation under the same conditions were measured by ELISA. All the qPCRs and ELISAs were done with PBMCs from four individual donors. Each assay was repeated 4 times (qPCR) or 10 times (ELISA). The mean is shown; error bars represent SEM. * $p < 0.01$.

Figure I.2

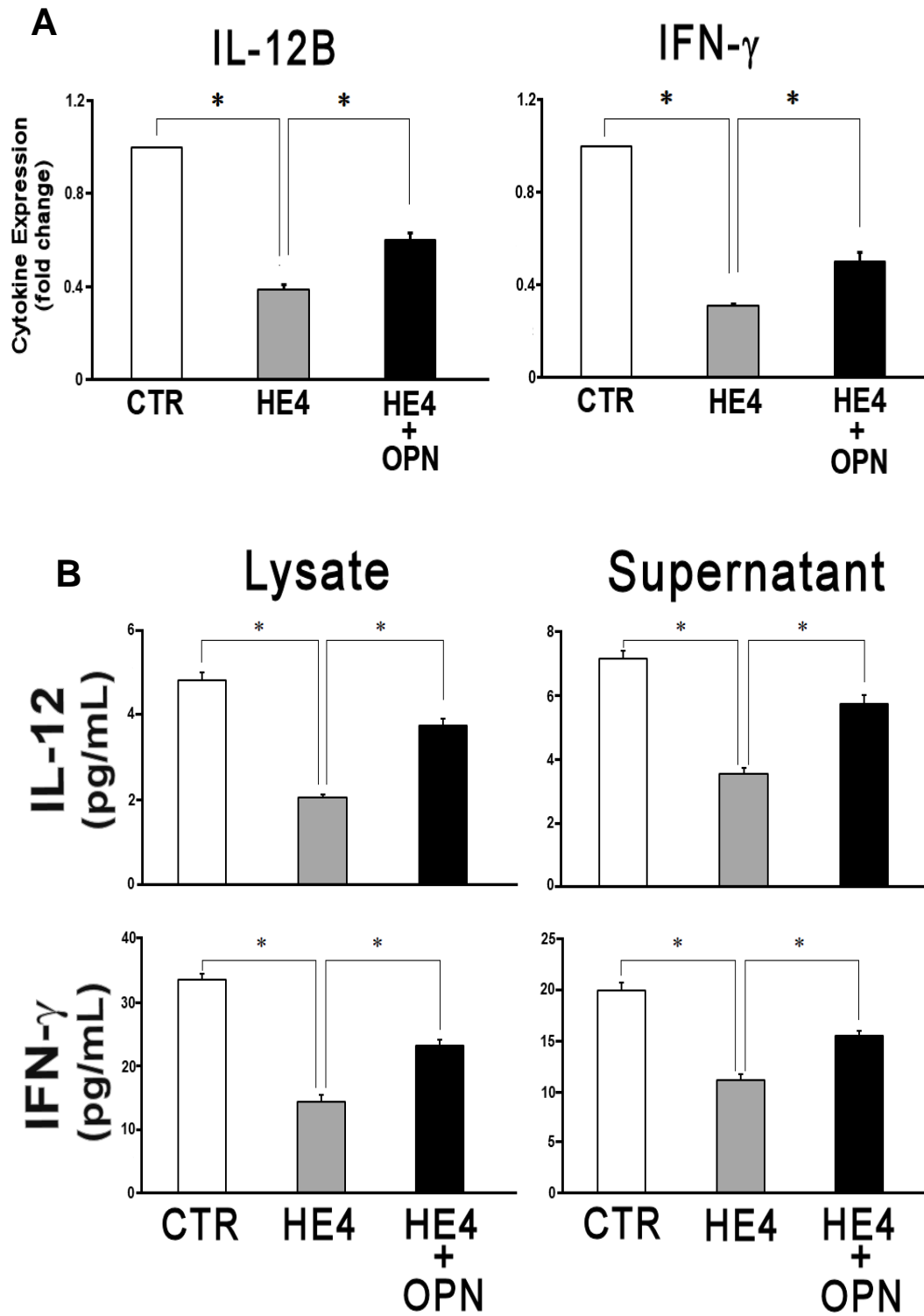


Figure I.3 Responses of SKOV3 and OVCAR8 human ovarian cancer cell lines to PBMC conditioned media.

(A) Cells were incubated with conditioned media from the PBMC culture treated with vehicle (blue line) or rHE4 (red line). The cell viabilities were assessed at 24, 48 and 72 hours after treatment (n = 10 for 0 hours, n = 8 for 24, 48 and 72 hours). (B) Cell migration activities with conditioned media were assessed at 24 hours of incubation (n = 10). (C) Ki67 immunohistochemistry staining was performed on SKOV3 / OVCAR8 cell lines incubated with the PBMC conditioned media for 24 hrs. Ki67⁺ cells are identified with red nuclear staining (upper panel). Bar graph (lower panel) represents the percentage of Ki67⁺ cells in total countable cells under 200x fields (n = 6). Scale bar: 50 μ m. The mean is shown; error bars represent SEM (n = 10). * p < 0.05, ** p < 0.01.

Figure I.3

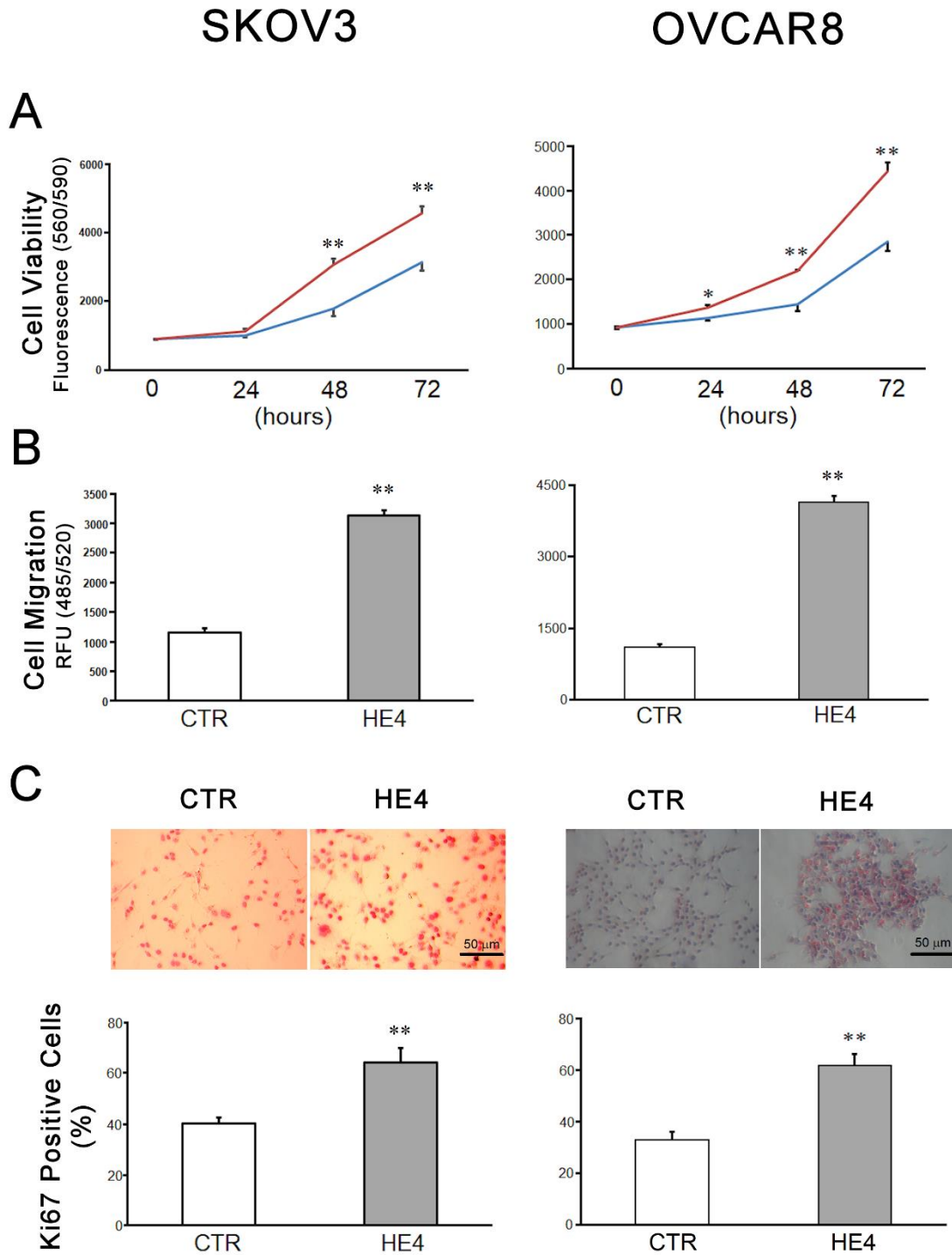


Figure I.4 Flow cytometric analysis of the cytotoxicity of mononuclear cells against SKOV3 tumor cells.

Cell membranes of SKOV3 (target) cells were labeled with DiOC18₍₃₎ fluorescent dye and then incubated with PBMCs in the presence of propidium iodide (PI) as a marker of cell death. After washing away the non-adherent cells (PBMCs), the PI positive tumor cells were quantitated via flow cytometry (upper panel). The numbers on the histograms represent mean percentage of each bisection. The bar graph (lower panel) represents percentages of PI positive (dead / dying) cells in various culture conditions. The mean is shown; error bars represent SEM (n > 10). * p < 0.01, **p < 0.05.

Figure I.4

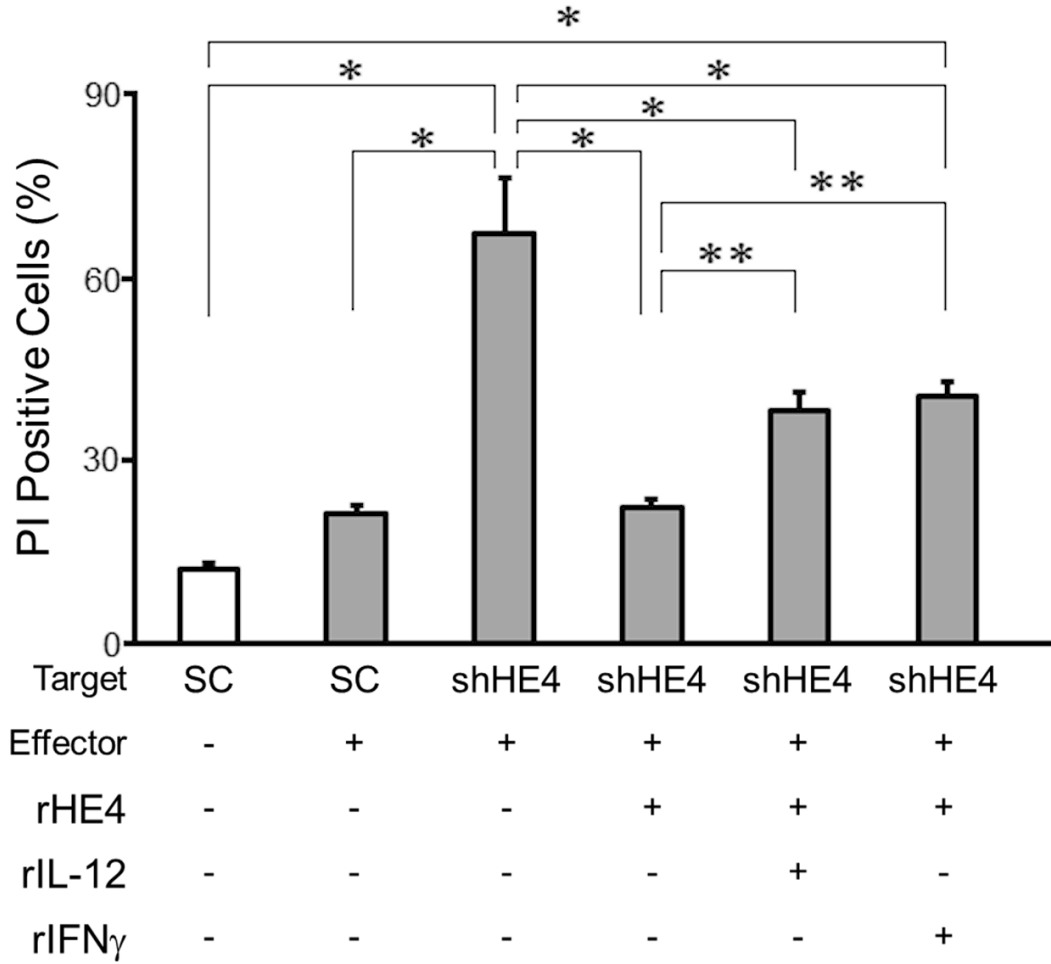
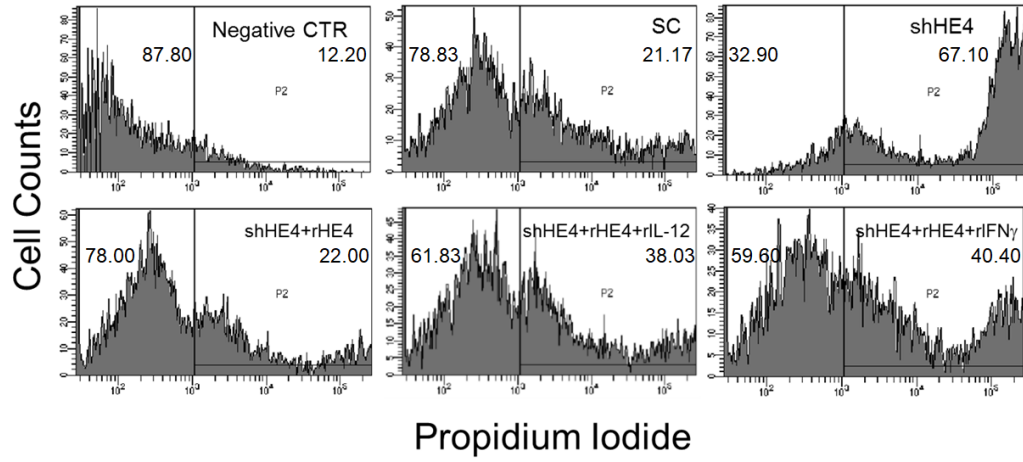


Figure I.5 Confocal immunofluorescent analysis of CD3 and OPN expression in biopsy samples.

Twenty biopsies (listed in Table 3) were evaluated. (A) Stromal and tumoral CD3⁺ cells and OPN⁺ tumor cells are indicated by arrowhead. A biopsy from a benign serous tumor (Benign) and an uninvolved section of oophorectomy (Normal) were utilized as a negative control (B). Enlarged image depicting image co-staining of stromal and tumoral CD3⁺/OPN⁺ T cells in their cytosol or the surrounding area (C, D). Graphic representations of Spearman's rank correlations between the numbers of CD3⁺ or OPN⁺ cells and clinical parameters. CR; corrected ranks.

Figure I.5

Figure I.5 a & b

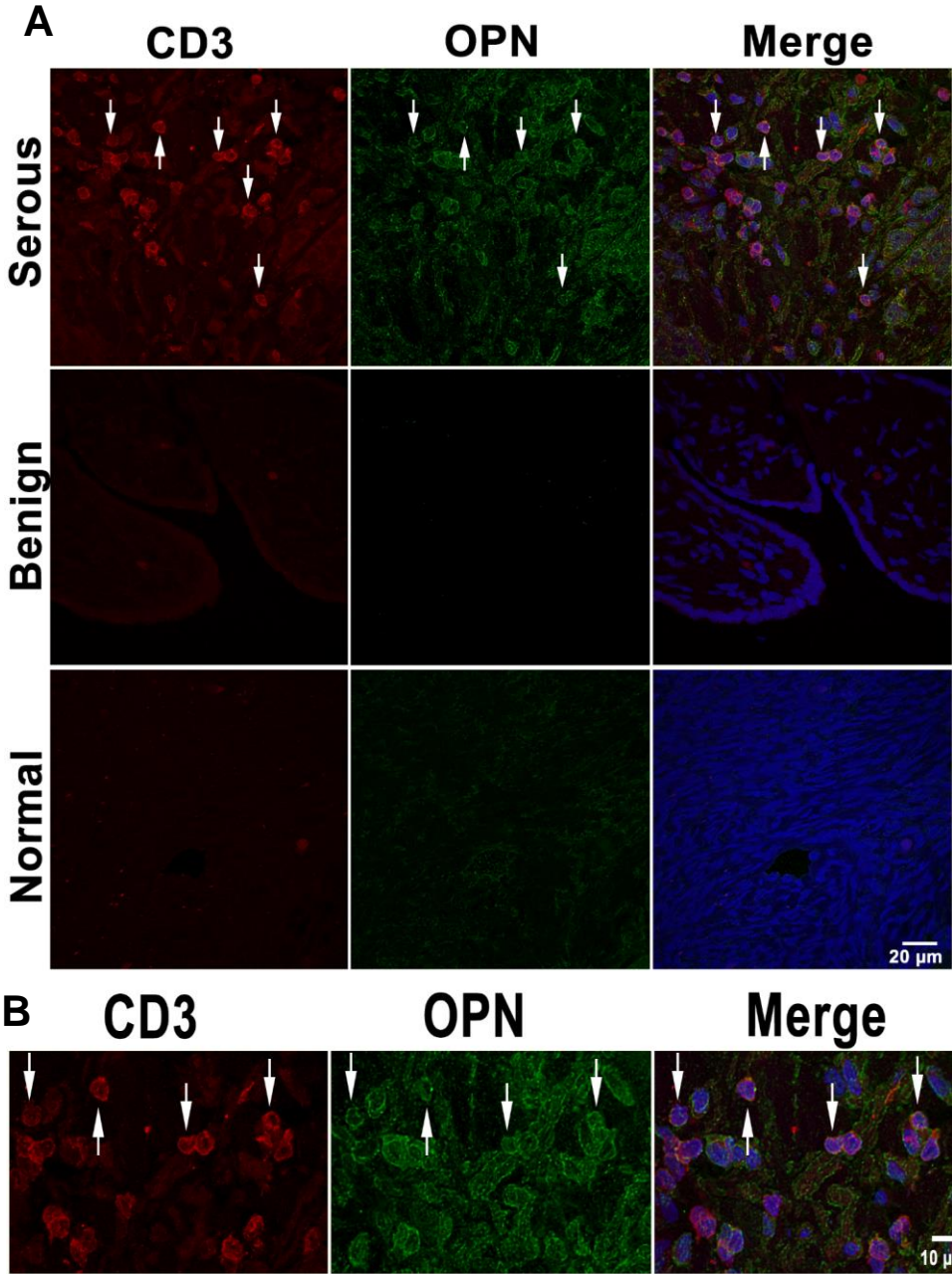


Figure I.5 c & d

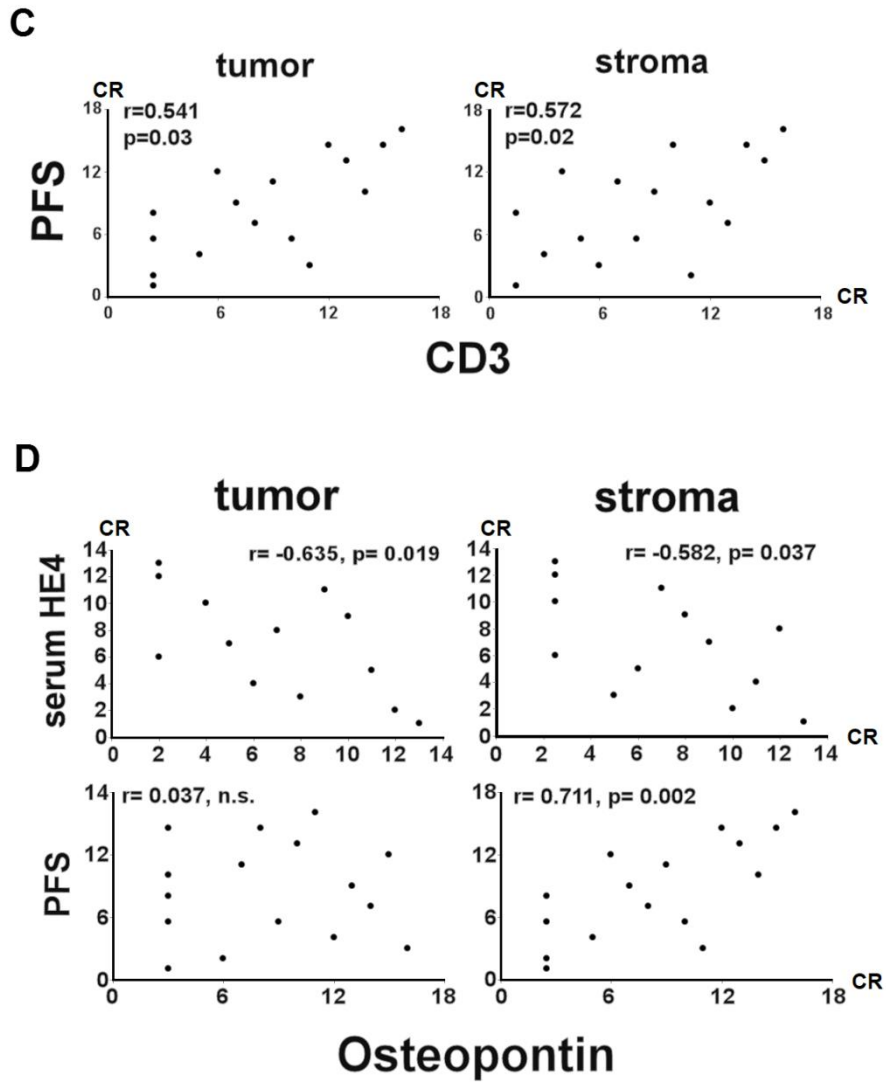


Table I. 1 Genes suppressed in response to HE4

Genes suppressed in response to HE4		
Frequency	ID	gene name
15	NSS	no significant similarity
6	NM_001040058	secreted phosphoprotein 1 (SPP1), transcript variant 1
3	NM_015574	ankyrin repeat domain 17 (ANKRD17)
3	NM_001693	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2 (ATP6V1B2)
3	NM_000206	interleukin 2 receptor subunit gamma (IL2RG)
3	NM_022818	microtubule-associated protein 1 light chain 3 beta (MAP1LC3B)
3	NM_001243121	phosphodiesterase 4A (PDE4A)
3	NM_080792	signal regulatory protein alpha (SIRPA)
3	NM_015131	WD repeat domain 43 (WDR43)
2	NM_001025604	arrestin domain containing 2 (ARRDC2)
2	NM_001164755	aspartate beta-hydroxylase (ASPH)
2	NM_032408	bromodomain adjacent to zinc finger domain, 1B (BAZ1B)
2	NM_002985	chemokine (C-C motif) ligand 5
2	AC132216	chromosome 11, clone RP13-786C16
2	NC_018926	chromosome 15, alternate assembly CHM1_1.1
2	NM_001291549	cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A)
2	NM_014280	DnaJ (Hsp40) homolog, subfamily C, member 8 (DNAJC8)
2	XM_011535514	eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)
2	XM_011518416	family with sequence similarity 120A (FAM120A)
2	NM_020447	family with sequence similarity 219 member B (FAM219B)
2	NG_029887	golgin A3 (GOLGA3)
2	NM_002107	H3 histone, family 3A (H3F3A)
2	NM_001128619	leucine zipper protein 6 (LUZP6)
2	NM_002463	MX dynamin-like GTPase 2 (MX2)
2	NM_004687	myotubularin related protein 4 (MTMR4)
2	NM_001251855	phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5)
2	NM_000437	platelet-activating factor acetylhydrolase 2, 40kDa (PAFAH2),
2	NR_049751	reticulon 3 (RTN3)
2	NM_001198719	retinoblastoma binding protein 7 (RBBP7)
2	NM_001028	ribosomal protein S25 (RPS25)
2	XM_011534644	serine/threonine kinase 10 (STK10)
2	NM_001242933	sorting nexin 1 (SNX1)
2	XR_241300	splicing factor 3b, subunit 1, 155kDa (SF3B1)
2	NM_181892	ubiquitin-conjugating enzyme E2D 3 (UBE2D3)
2	NM_006007	zinc finger, AN1-type domain 5 (ZFAND5)
1		159 genes

Table I. 2 Concentrations of IL-12, IFN- γ and HE4 in co-culture medium

Concentrations of IL-12, IFN- γ and HE4 in co-culture medium

Target	Effector	IL-12 (ng/mL)	INF- γ (ng/mL)	HE4 (pM)
SC	-	-	-	534.15 \pm 41.81
SC	+	14.84 \pm 0.48	177.20 \pm 1.07	639.01 \pm 50.38
shHE4	-	-	-	174.12 \pm 18.55*
shHE4	+	31.95 \pm 0.68**	417.74 \pm 3.54**	237.91 \pm 34.24**

SC; SKOV3 with scrambled oligo, shHE4; SKOV3 with HE4 shRNA

mean \pm SE is shown (n = 10)

*p<0.01 vs. SC, **p<0.01 vs.SC + Effector cells

Table I.3 Clinical Parameters of Donors

Table 3		
Clinical parameters of donors		
Sample ID	pre-OP sHE4* (pmol/mL)	PFS** (months)
S10-10110	174	10
S10-10726	na	16
S10-15910	na	25
S10-17790	376	12
S10-18470	529	22
S10-4387	462	31
S10-5618	na	9
S10-5842	na	na
S10-6697	150	na
S10-7183	1232	na
S11-1189	3289	8
S11-2223	550	28
S11-2493	591	37
S11-2684	3255	24
S11-3415	na	na
S11-622	na	38
S11-6675	4702	16
S11-6721	na	64
S11-7794	410	38
S11-8032	623	21
* pre-operation serum HE4		
** progression-free survival		
na; not available		

CHAPTER 4

MANUSCRIPT II

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HE4 Sabotages Cytotoxic Mononuclear Cells Inducing Dual Specificity

Phosphatase 6 Secretion

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II.1 Abstract

Objective

Selective overexpression of Human epididymal secretory protein 4 (HE4) points to a role in ovarian cancer tumorigenesis but little is known about the role the HE4 gene or the gene product plays. Here we examine the role of the secretory glycoprotein HE4 in ovarian cancer immune evasion.

Methods

Through the modified subtractive hybridization analyses of human peripheral blood mononuclear cells (PBMCs), we have characterized gene targets of HE4 and established a preliminary mechanism of HE4-mediated immune failure in ovarian tumors.

Results

Dual specificity phosphatase 6 (DUSP6) emerged as the most upregulated gene in PBMCs upon *in vitro* exposure to HE4. CD8⁺ cells and CD56⁺ cells found to be sources of the upregulated DUSP6. The HE4 exposure enhanced Erk1/2 phosphorylation specifically in these cell populations and the effect was erased by co-incubation with DUSP6 inhibitor, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI). In co-culture with PBMC, HE4-silenced SKOV3, a human ovarian carcinoma cell line, exhibited enhanced proliferation with exposure to the external HE4; this effect was partially attenuated by adding BCI to the culture. Additionally, the reversal effects of BCI were erased in the co-culture with CD8⁺ / CD56⁺ cell deprived PBMC.

Conclusion

Taken together, these findings show that DUSP6 is a suppressor of the cytotoxicity of the CD8⁺ and CD56⁺ lymphocytes and HE4 enhances tumorigenesis of ovarian cancer through the compromised cytotoxicity of the CD8⁺ and CD56⁺ cells by upregulation of self-produced DUSP6, which acts as an autocrine factor.

II.2 Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women, and the deadliest gynecologic cancer. The American Cancer Society estimates that in 2017, there will be an estimated 22,440 new cases of EOC and 14,080 deaths in the United States [1]. Only 15% of patients are diagnosed at an early stage when the disease is fundamentally curable, keeping the 5-year survival rate at a dismal 46% [2]. Recurrence following initial treatment is common, occurring in approximately 80% of cases, and all patients with recurrent disease eventually succumb to their illness [3]. These dire statistics highlight the need for continued research into improved diagnostic and treatment options for EOC.

Despite continued efforts, there remains a lack of effective treatments for EOC. Standard first-line therapy consists of debulking surgery followed by taxane-platinum chemotherapy[3]. Other targeted therapies are also employed, including the antiangiogenic drug bevacizumab and the PARP inhibitor olaparib; however, these treatments have not led to an improvement in overall survival [4]. One promising new area of investigation lies in understanding how tumors develop immune tolerance and evade elimination by cytotoxic lymphocytes. Immune checkpoint molecules such as PD-1, CTLA4, TIM3, IDO, and others, suppress T cell activation and help tumor cells escape targeting and elimination by the immune system [5]. Nivolumab, a monoclonal antibody against PD-1, is expressed on T cells and suppresses their activation upon binding of its tumor cell associated ligands, PDL1/PDL2, has greatly improved survival for metastatic melanoma patients [6]. PD-1 has also been studied in relapsed platinum-resistant EOC; however, overall response rates for EOC do not exceed 15%

[7]. This inefficacy of immune checkpoint inhibitors is likely due to compensatory immune suppressive pathways [8,9], or activation of oncogenic pathways that promote immune tolerance [5]. Overall, we require a greater understanding of factors that contribute to immune evasion in EOC in order to develop treatments that reactivate the body's immune response to tumors.

Human epididymis protein-4 (HE4) is a member of the whey acidic four-disulfide core protein family [10]. It is elevated in tumor tissue and serum of EOC patients, and is used as part of the Risk of Ovarian Malignancy Algorithm (ROMA)—along with CA125 and menopausal status—for the diagnosis and management of EOC [11, 12]. ROMA shows greater sensitivity and specificity for the detection and monitoring of EOC than the Risk of Malignancy Index, which uses CA125, pelvic sonography, and menopausal status [12]. HE4 also has the advantage of presenting fewer false positives than CA125 in the case of benign gynecologic disorders [11, 13]. *In vitro* and *in vivo* studies have shown that HE4 promotes multiple aspects of ovarian cancer aggression, including growth and proliferation; invasion, migration, and adhesion; chemoresistance, and anti-estrogen resistance [14–23]. Clinically, patients with high levels of serum HE4 have greater chemoresistance and worse prognosis [22, 24–26]. We hypothesized that HE4 may also promote immune evasion in EOC. We began to test this hypothesis by determining HE4-mediated gene expression in peripheral blood mononuclear cells (PBMCs), and went on to evaluate the effect of HE4 and one of its targets, DUSP6, on immune cell function and cytotoxicity against ovarian cancer cells.

II.3 Methods

Subtractive hybridization and TA-cloning

5×10^7 PBMCs from single donor were suspended in 5 mL of serum free RPMI1640 medium (Invitrogen, 31800) and incubated with or without 0.01 $\mu\text{g/mL}$ of rHE4 (Abcam, ab184603) for 6 hours. Then, total RNA was isolated using TRIzolTM Reagent (Invitrogen, 15596018). Next, mRNA was purified using MagnosphereTM UltraPure mRNA Purification Kit (Takara-Clontech, 9186). From the 5 μg of mRNA, subtractive cDNA libraries were constructed using PCR-SelectTM cDNA Subtraction Kit (Takara-Clontech, 637401) following the manufacturer's protocols. PCR products of the differentially expressed genes were cloned into a pUC19-TA vector. Top 10 competent cells (Invitrogen, C404003) were transformed with the clones and were seeded on a Xgal/IPTG containing LB/ampicillin plates. The colonies of clones containing the inserts were selected by blue/white selection and were amplified by direct colony PCR using LA Taq[®] DNA polymerase (Takara-Clontech, RR002A) and M13 primers.

Cell culture

Primary human PBMCs were obtained under the auspices of Women & Infants Hospital IRB approval from total blood of four individual volunteer by density gradient centrifugation using Histopaque[®]-1077 (Sigma-Aldrich, 10771). The human ovarian tumor cell line, SKOV3, human NK cell line, NK-92MI, human T cell line, TALL-104 and H9 were obtained from ATCC (HTB-77, CRL-2408, CRL-11386 and HTB-176, respectively). RPMI1640 was used for culturing PBMC and lymphocyte

lines and DMEM (Invitrogen, 31600) were used for SKOV3. Conditioned media were obtained from a 24-hour PBMC culture. Residual rHE4 in the conditioned media was deprived as follows. Five mL of media was incubated with 10 μg (100 μL) of anti-human HE4 antibody (Santa Cruz Biotechnology, sc-293473) for 1 hour at 4 degrees. And then, 100 mL packed volume of protein G coated sepharose beads (GE Healthcare Life Science, 17061801) were added to the media and incubated for 4 hours at 4 degrees. After the incubation, the sepharose beads were removed by centrifugation and the supernatants were processed through sterile 0.2 μm pore syringe filter. For the cell-mediated cytotoxicity assay, 1×10^6 target cells (SKOV3) were seeded on 6-well plates, and then were incubated overnight with complete media. The next day, cells were placed in serum free media for another overnight incubation and then 5×10^6 / mL of the effector cells (PBMCs) were added to the quiescent target cells. After a 12-hour incubation, the effector cells were washed away and harvested target cells were stained with 1 μg / mL of propidium iodide with or without Alexa Fluor® 488 labeled annexin V (Invitrogen, V13241). Some of the wells contained 0.01 $\mu\text{g}/\text{mL}$ of rHE4 and 1 μM of DUSP6 inhibitor, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI; Sigma-Aldrich, B4313). All experiments were performed under serum free condition.

Flow cytometry

FITC-labeled anti CD3, CD4, CD8, CD14, CD19 and CD56 antibodies were obtained from BD Biosciences (555916, 561005, 560960, 555397, 555412 and 562794, respectively). Alexa Fluor® 647-conjugated anti DUSP6 antibody was obtained from

Abcam (ab200751). Alexa Fluor[®] 647-conjugated anti phosphor-p44/42 MAPK antibody was obtained from Cell Signaling Technology (13148). After staining for cell surface markers (CD3, CD14, CD19 and CD56) the cell membrane was permeabilized by 0.2 % Triton X-100 and 0.2 % digitonin, and then stained for DUSP6 or phosphor-p44/42-MAPK. Flow cytometric analysis was performed with FACSCanto system and FACSDiva software (BD Biosciences).

Quantitative Real-Time PCR

RNA was isolated from cells using TRIzol (Invitrogen, A33250) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, 18080093). qPCR was performed using Premix Ex-Taq[™] II (Clontech-Takara, 639676) probes for DUSP6. All reactions were normalized using GAPDH as an endogenous control. Amplification data were analyzed using the $\Delta\Delta C_t$ method.

ELISA

ELISA kits for HE4 and DUSP6 were obtained from MyBioSource (MBS280223 and MBS073193, respectively). The assays were performed following the manufacturer's instruction.

Western blotting

Phosphorylation of Erk1/2 in NK-92MI, TALL-104 and H9 cell lines were assessed by western blotting. Antibodies against phosphorylated and total Erk1/2 MAPK were

obtained from Cell Signaling Technology (9101 and 4695). The results were visualized with SuperSignal™ West Pico chemiluminescent substrate (ThermoFisher Scientifics, 34080) and analyzed with the UN-SCAN-IT gel software for Windows (Silk Scientific Inc.).

HE4 silencing with shRNA

shRNA for human HE4 (Origene, TR318721) were transfected into SKOV3 using Lipofectamine® 2000 (Invitrogen, 11668) following the manufacture's instruction. Individual single cells were selected by culturing under the pressure of 5 µg / mL of puromycin (Research Products International, 58-58-2).

Cell viability assay

1 x 10³ / well SKOV3 cells were seeded in a 96-well culture plate. After overnight incubation with serum free medium, 5 x 10⁶ /mL of effector cells (PBMCs) were added to the quiescent cells. The cell viabilities were evaluated at 24, 48 and 72 hours using fluorescent based CellTiter-Blue® (Promega, G8080) and Spectra Max Gemini EM fluorescent micro plate reader (Molecular Devices).

Immunohistochemistry

0.5 x 10⁴ / chamber of SKOV3 cells were seeded in a 4-chamber slide. After overnight incubation with serum free medium, 5 x 10⁶ /mL of effector cells (PBMCs) were added to the quiescent cells and the cells were cultured for 48 hrs. Ki67 positive cells were counted in twenty of 200x fields. A mouse anti-Ki67 monoclonal antibody was

purchased from BD Biosciences (550609). An alkaline phosphatase (ALP) labeled anti-mouse IgG secondary antibody and an ALP substrate kit were obtained from Vector laboratories (AP-2000, SK-5100).

Depletion of CD8⁺ and CD56⁺ cells from PBMCs

CD8⁺ and CD56⁺ cells were removed from PBMC using magnetic CD8 and CD56 MicroBeads (Miltenyi Biotec, 130-045-201 and 130-050-401) with autoMACS cell separator (Miltenyi Biotec, 130-092-545). Briefly, 5×10^7 of PBMC was suspended in 60 μ L of separation buffer (PBS, pH 7.2 with 0.5 % BSA and 2 mM EDTA), and then, 20 μ L each of CD8 and CD56 MicroBeads were added to it, followed by 15 minutes incubation at 4 degrees. After washing, resuspended the cells in 500 μ L of the separation buffer and proceed to magnetic separation using autoMACS[®] Columns (Miltenyi Biotec, 130-021-101). Unlabeled cells that pass through were collected and combined with total effluent from washed column.

Statistics

Data were expressed as average \pm SEM of at least four independent experiments. An unpaired, two-tailed Student t-test was used to determine significance. Multiple treatments were analyzed by using one-way ANOVA followed by Ryan's multiple comparison test. Differences between groups were considered statistically significant when $p < 0.05$.

III.4 Results

Differential expression of PBMC genes after HE4 exposure

To identify differentially expressed genes after HE4 exposure, modified subtractive hybridization was performed. PCR products of the differentially expressed genes were cloned into pUC19-TA vectors to create a differential cDNA library. PCR products from 250 each of HE4-induced and HE4-suppressed gene colonies were sequenced resulting in the identification of 209 induced genes and 206 suppressed genes. Among the identified genes, 20 induced and 13 suppressed sequences showed no significant similarity (NSS) to known genes in available nucleotide databases. Among the 209 induced genes, dual specificity phosphatase 6 (DUSP6) emerged as one of the most frequently identified genes (3 times out of 250 sequences, 1.2%; Table 1).

HE4 induces DUSP6 expression in PBMCs

HE4-induced upregulation of DUSP6 in PBMCs was then confirmed via three modalities: quantitative PCR (qPCR), ELISA and flow cytometry. First, PBMCs were harvested after a 6-hour exposure with recombinant human HE4 (rHE4; 0.01 $\mu\text{g}/\text{mL}$), revealing a 1.60 ± 0.13 -fold increase ($p < 0.01$) in DUSP6 mRNA production (Figure 1A). The concentrations of DUSP6 in PBMC lysates (9.38 ± 0.62 vs 15.62 ± 0.97 ng/mL, $p < 0.01$) and culture supernatants (0.77 ± 0.10 vs 1.43 ± 0.14 ng / mL, $p < 0.01$) after a 24-hour exposure to rHE4 were also increased (Table 2). PBMCs were then cultured with rHE4 for 24 hours and collected for flow cytometry analysis. Protein expression of DUSP6 in CD3⁺ PBMCs (T cells) was found to be significantly increased with HE4 exposure (34.4 ± 0.6 % vs 47.0 ± 3.2 % of total CD3⁺ cells; $p <$

0.05; Figure 1B left panel). The DUSP6 expression in CD56⁺ cells (NK/T cells, NK cells) was also increased to a lesser extent (34.1 ± 2.3 % vs 41.7 ± 1.7 % of total CD56⁺ cells; $p < 0.05$; Figure 1B right panel). In order to identify a T cell subset involved in the HE4 responsive induction of DUSP6, two-color flow cytometry using anti-DUSP6 antibody and anti-CD4 (helper T cell) or CD8 (cytotoxic T cell) antibodies were performed. As shown in Figure 2, after a 24-hour exposure to rHE4, CD8⁺ T cells (9.9 ± 0.8 % vs 1.9 ± 0.1 %; $p < 0.01$) but not CD4⁺ T cells (15.6 ± 1.4 % vs 15.4 ± 1.5 %) showed significant DUSP6 induction. These finding suggested that the CD8⁺ and CD56⁺ cytotoxic mononuclear cells were responsible for the HE4 responsive DUSP6 induction.

CD8⁺ and CD56⁺ cytotoxic lymphocytes are targets of HE4 induced DUSP6

In order to identify effector cells for the HE4 induced DUSP6, two-color flow cytometry using antibodies against phosphor-Erk1/2 (pErk1/2) and CD4, CD8, CD14, CD19 and CD56 were performed. Significant decreases of pErk1/2⁺ populations were observed in CD8⁺ (30.2 ± 2.4 % vs 4.3 ± 0.2 % in total CD8⁺ cells; $p < 0.01$) and CD56⁺ (32.3 ± 4.0 % vs 5.4 ± 0.6 % in total CD56⁺ cells; $p < 0.01$) cells after a 24 hours rHE4 (0.01 $\mu\text{g/mL}$) exposure, and the decreases were abrogated by co-treatment with 1 μM of DUSP6 inhibitor, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI) in both CD8⁺ cells and CD56⁺ cells (23.3 ± 0.7 % and 30.5 ± 2.6 %, respectively; Figure 3A). Next, CD56⁺ NK cell line (NK92MI), CD8⁺ cytotoxic T cell line (TALL-104) and CD4⁺ helper T cell line (H9) were incubated with the conditioned media from a 24- hour PBMC culture with or without rHE4 and BCI for 1

hour. The lysates of the cells were used for western blotting to evaluate Erk1/2 phosphorylation. As shown in Figure 3B, 1-hour incubation with the HE4 exposed PBMC conditioned media suppressed Erk1/2 phosphorylation in NK92MI cell (0.67 ± 0.07 -fold vs CTR, $p < 0.01$) and TALL-104 cell (0.56 ± 0.10 -fold vs CTR, $p < 0.01$) but not in H9 cell (1.01 ± 0.03 -fold vs CTR). The rHE4 responsive pErk1/2 suppressions were abrogated by the PBMC conditioned media from co treatment with rHE4 and BCI in both NK92MI (0.90 ± 0.04 -fold vs CTR) and TALL-104 (0.89 ± 0.06 -fold vs CTR). These findings suggested that the HE4 induced DUSP6 acts as an autocrine suppressor for Erk1/2 MAPK in CD8⁺ and CD56⁺ cytotoxic lymphocytes.

HE4 attenuates ovarian cancer susceptibility to PBMC mediated cytotoxicity

In order to evaluate the impact of HE4 on PBMC cytotoxicity against cancer cells, the human ovarian tumor cell line, SKOV3, was co-cultured with PBMCs (5×10^6 / mL density). To minimize the effect of native HE4 produced by tumor cell, the SKOV3 cells were stably transfected with HE4 specific shRNA (shHE4). The effector cells (PBMCs) were washed away, and the target cells (SKOV3) were analyzed by three independent modalities: cell viability, Ki67 immunostaining, and flow cytometry for propidium iodide (PI) and annexin V. First, SKOV3 cells co-cultured with PBMC suspensions containing 0.01 $\mu\text{g/mL}$ of rHE4 showed significantly higher viability than cells cultured with the rHE4 free suspensions at 24 (1222.70 ± 29.48 vs. 1517.98 ± 34.32 , $p < 0.01$), 48 (2038.38 ± 55.94 vs. 3508.64 ± 164.98 , $p < 0.01$) and 72 hours (1983.33 ± 100.41 vs. 2935.89 ± 116.47 , $p < 0.01$), and the increased viabilities were partially abrogated by adding 1 μM of BCI to the culture (1295.68 ± 39.87 , $2667.27 \pm$

95.13 and 2424.50 ± 105.70 , at 24, 48 and 72 hours, respectively; Figure 4A). Second, immunohistochemistry using anti-Ki67 was performed to evaluate the proliferation activities of SKOV3 cells in the presence of PBMCs with or without rHE4 and BCI for 24 hours. The number of Ki67 positive tumor cells in rHE4-containing PBMC suspension was higher than the cells in rHE4-free suspension, and the increased activity was partially attenuated by adding BCI to the culture (27.6 ± 1.7 %, 68.5 ± 2.6 % and 48.9 ± 2.3 %, respectively; Figure 4B). Finally, after a 6 -hour incubation at 37 degrees, the effector cells were washed away and the target cells were analyzed by 2-color flow cytometry using PI and Alexa Fluor[®] 488 labeled annexin V. As shown in Figure 4C, SKOV3 / PBMC co-cultures with rHE4 led to a significant decrease in populations of PI / annexin v double positive dying cells (24.3 ± 1.2 % vs. 13.4 ± 0.8 %, $p < 0.01$), and the tolerance of the target cells was partially reversed by adding BCI to the culture (18.1 ± 0.6 %, $p < 0.01$ vs. CTR and HE4). These findings suggest that HE4 enhances tolerance of cancer cells against immunocompetent mononuclear cells via up-regulation of DUSP6 in PBMCs. In order to confirm involvement of CD8⁺ / CD56⁺ cytotoxic lymphocytes in the HE4 induced immunomodulation, the co-culture study was repeated using PBMCs deprived of CD8⁺ / CD56⁺ cells. As shown in Figure 5A-C, all the effects of BCI shown in Figure 4 were erased in the CD8⁺ / CD56⁺ cell free co-cultures, suggesting that the cytotoxic lymphocytes play a pivotal role in the immunoediting by DUSP6 up-regulation in response to exposure to HE4.

II.5 Discussion

Several studies from our laboratory and elsewhere have revealed multidimensional roles for HE4 in the pathogenesis of ovarian cancer, including the promotion of tumor growth, chemoresistance, anti-estrogen resistance, invasion, migration, and adhesion [14–23]. In this present study, we have begun to delineate another vital function of HE4 in disrupting immune cell function, which has implications for immune system targeting of tumor cells. DUSP6, which we found to be upregulated by rHE4 treatment in CD8⁺ T cells and CD56⁺ NK cell subsets of PBMCs, is likely one key mediator of this effect in these immune cell subsets.

DUSP6 is a member of the DUSP family that dephosphorylates threonine and tyrosine residues on MAPK substrates. It specifically dephosphorylates ERK, a member of the MAPK family that also includes p38 and JNK. MAPKs are activated by growth factors, cytokines, integrin ligands, and stress signals to regulate growth, survival, apoptosis, and immune response in diverse cell types. Interestingly, DUSP6 is expressed at low levels in resting cells and is actually stimulated by ERK activation, promoting a negative feedback loop on ERK activity [27]. This early response of DUSP6 to ERK activation could explain the apparently contradictory activation of ERK by HE4 in cancer cells [14, 16, 17, 23] and our results showing that HE4 upregulation of DUSP6 expression leads to suppression of ERK phosphorylation in PBMC subsets.

Several reports reveal a role for DUSP6 in development, organogenesis, and cancer [27]. However, its effect on cancer progression is highly dependent upon the type of cancer and even the stage. For example, in pancreatic cancer, it is upregulated in early

stages but is often completely diminished as the tumor progresses towards the invasive ductal carcinoma state [28]. In lung cancer, it has been shown to act as a tumor suppressor [29]. Conversely, it is upregulated in glioblastoma and HER2-positive breast cancer [30, 31]. One report found that its downregulation in ovarian cancer results in hyperactivation of ERK and subsequent chemoresistance [32]. These discrepancies are likely due to variable deregulation of ERK signaling and compensatory pathways that are highly context dependent [27]. In contrast to the roles of the tumor producing DUSP6 on the tumorigenesis, the functions of DUSP6 originated from immune cells have rarely been evaluated.

Even less is known regarding the role of DUSP6 in immune cell function. Other members of the DUSP family, including DUSP1, DUSP2, and DUSP10, are known to have roles in immune response [27], and a few reports suggest that DUSP6 does as well. Elevated DUSP6 was shown to cause downregulation of ERK phosphorylation in CD4⁺ T cells in elderly individuals, who have suppressed immune responses [33]. Another report confirmed this age associated rise in CD4⁺ T cell DUSP6 expression, and found that young immunosuppressed patients with end stage renal disease have DUSP6 levels comparable to elderly healthy individuals [34]. One study found that DUSP6 downregulates ERK activity in CD4⁺ T cells and increases their regulatory T cell functions [35]. Together, these reports suggest that higher levels of DUSP6 contribute to immune suppression. It has also been shown that DUSP6 is downregulated in T cells upon IL-2 withdrawal [36], and IL-2 was found to upregulate DUSP6 gene expression in T cells [37]. Since IL-2 stimulates cytotoxic T cell expansion and activation as well as that of immune suppressive regulatory T cells

[38], it remains to be determined how the IL-2 responsiveness of DUSP6 plays into its apparent effect on immune suppression, and how this relates to tumor immune response.

Although much remains unknown regarding the specific effects of DUSP6 on cancer progression and tumor immunity, our findings begin to reveal some novel insights. We report for the first time that HE4-mediated upregulation of DUSP6 in CD8⁺ T cell and CD56⁺ NK cell subsets of PBMC cells leads to the inhibition of their cytotoxic activity against SKOV3 ovarian cancer cells. While DUSP6 has been connected to immune function of CD4⁺ T cells, our results reveal that the subsets of lymphocytes affected by DUSP6 are context dependent. Further investigation into the inhibitory effects of DUSP6 in these different populations will be illuminating. Moreover, we have begun to establish HE4 as a critical regulator of immune cell function, which deepens our understanding of the mechanistic role HE4 plays in ovarian cancer pathogenesis.

II.6 References

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Figure II.1 HE4 upregulates expression of DUSP6 in PBMCs.

(A) DUSP6 transcription in response to a 6-hour incubation with 0.01 $\mu\text{g}/\text{mL}$ rHE4 (HE4) or vehicle (CTR) were evaluated by triplicated trials of real time PCR using PBMCs from four individual donors. (B) Two-color flow cytometric analysis of PBMC following 24-hr incubation with 0.01 $\mu\text{g}/\text{mL}$ of rHE4 (HE4) or vehicle (CTR). 2D-scatterplots (upper panel) of DUSP6 (Alexa Fluor 647) and CD3 or CD56 (FITC) are shown. The lower panel shows bar graph from flow cytometric analyses using PBMCs from four individual donors. The mean \pm SEM are shown. * $p < 0.05$.

Fig. II.1

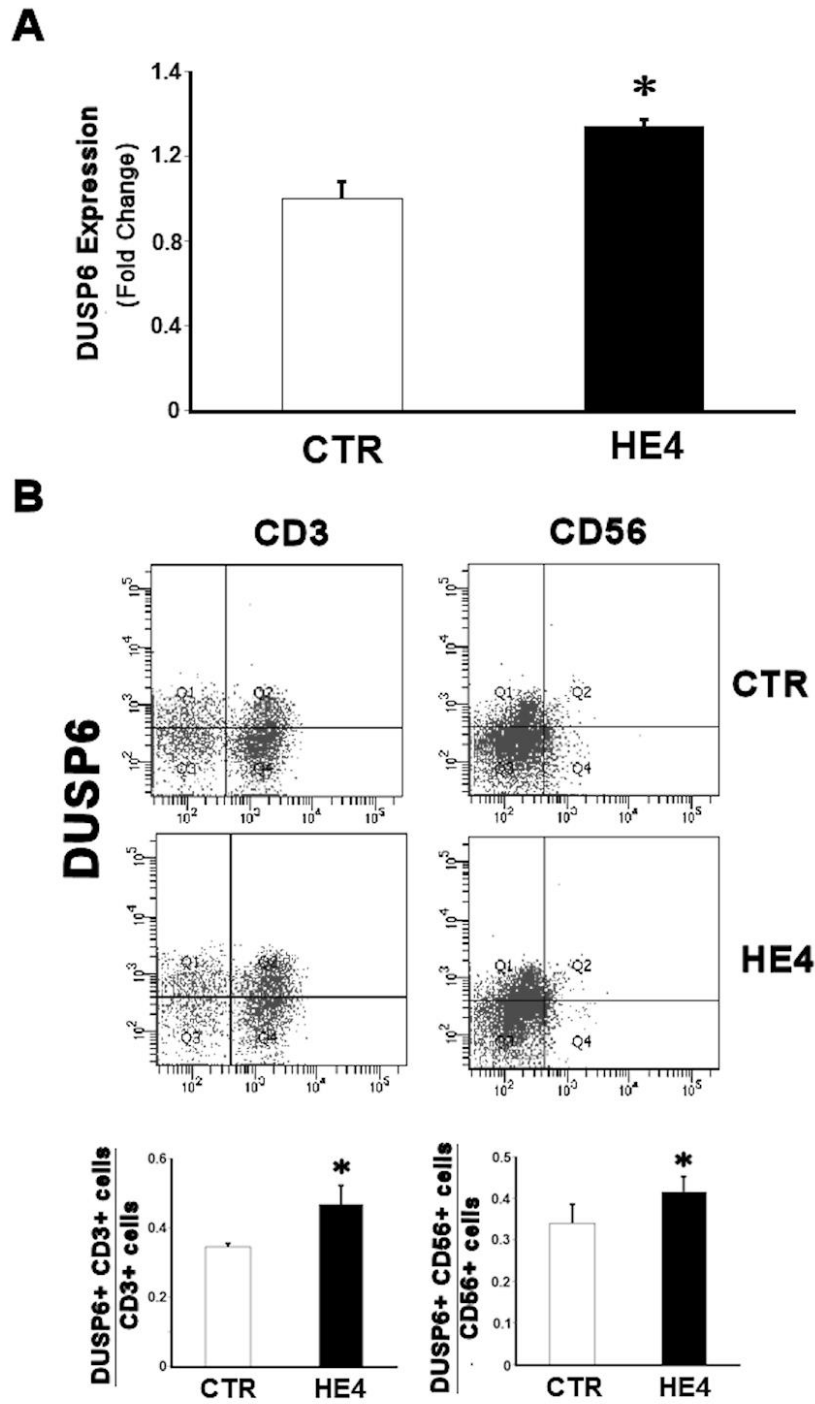


Figure II.2 HE4 upregulates expression of DUSP6 in peripheral CD8⁺ T cells.

Two-color flow cytometric analysis of PBMCs following 24-hour incubation with 0.01 µg/mL of rHE4 (HE4) or vehicle (CTR). 2D-scatterplots (upper panel) of DUSP6 (Alexa Fluor 647) and CD4 or CD8 (FITC) are shown. The lower panel shows a bar graph from flow cytometric analyses using PBMCs from four individual donors. The mean using ± SEM are shown. *p<0.01.

Fig. II.2

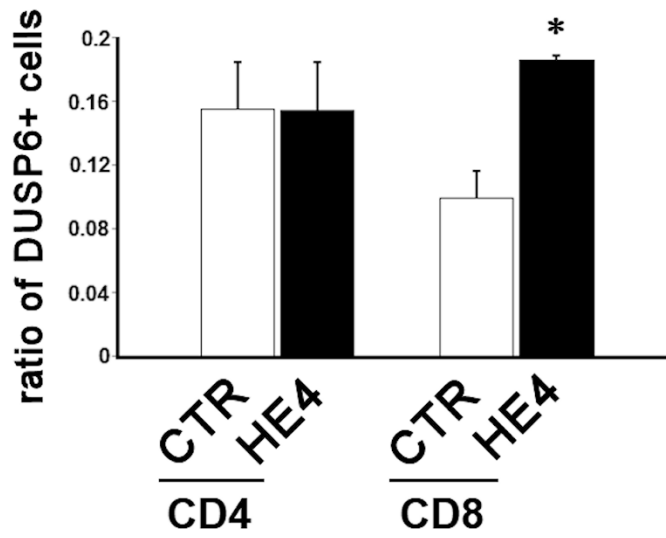
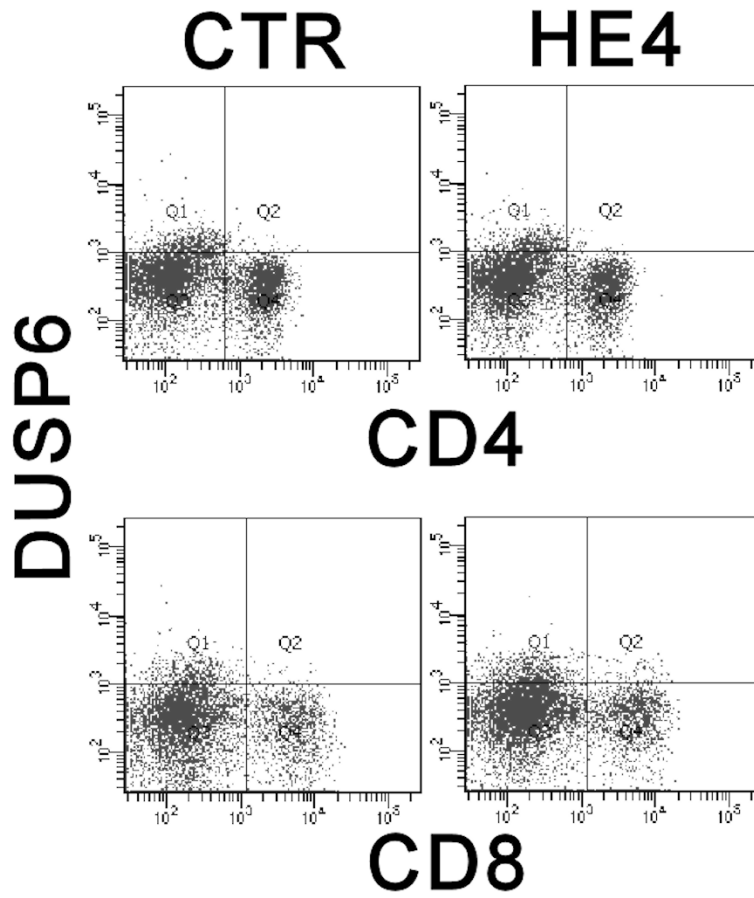


Figure II.3 HE4 suppresses Erk1/2 phosphorylation in CD8⁺ and CD56⁺ cells via DUSP6 induction.

(A) Two-color flow cytometric analysis of PBMC following a 24-hour incubation with rHE4 (0.01 µg /mL) and BCI (1 µM) as indicated. 2-D scatterplots of phosphor-Erk1/2 (Alexa Fluor 647) and CD8 or CD56 (FITC) are shown. Mean ± SEM from analyses with four individual donors are shown in the bar graph. (B) Immunoblotting for phosphor-Erk1/2 in CD56⁺ NK92MI, CD8⁺ TALL-104 and CD4⁺ H9 cells following a 1-hour incubation with the conditioned media from a 24-hour PBMC culture with rHE4 (0.01 µg/mL) and BCI (1 µM) in the indicated combinations. Blots of total Erk1/2 are shown as loading controls. Bar graph represents the relative band densities to controls. Mean ± SEM are shown (n=4). *p < 0.05, **p < 0.01.

Fig.II.3

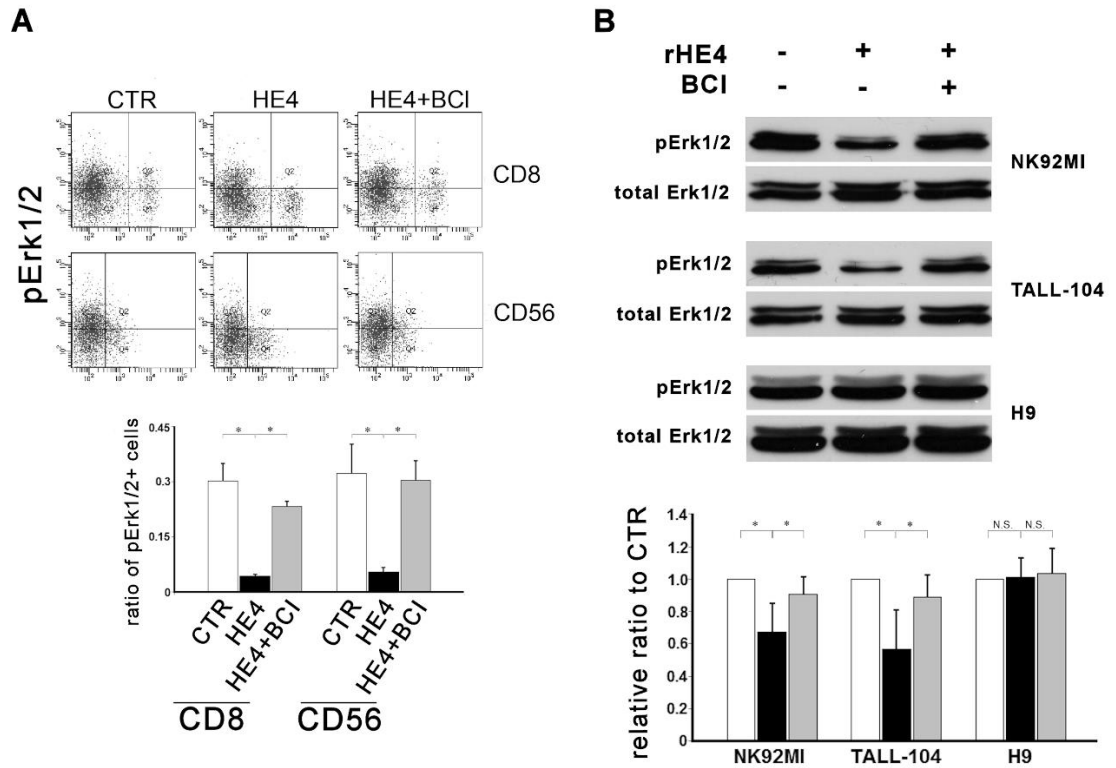
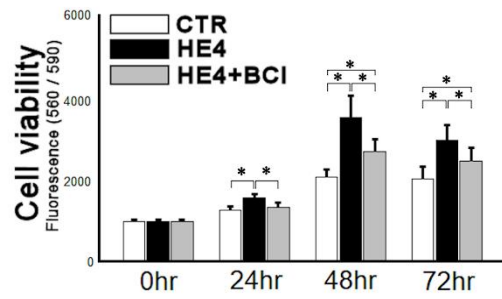


Figure II.4 Responses of SKOV3 cells to co-culture with PBMCs

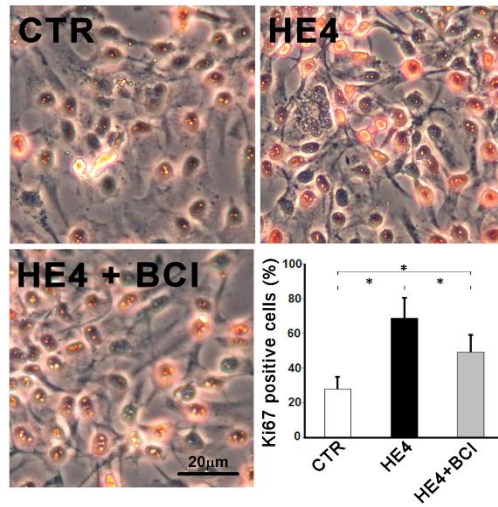
(A) Cells were co-cultured with PBMC (5×10^6 / mL) with rHE4 (0.01 μg /mL) and BCI (1 μM) in indicated combinations. The cells viabilities were assessed at 24, 48 and 72 hours of the culture (n = 10). (B) Ki67 immunohistochemistry staining was performed on SKOV3 cells co-cultured with PBMC for 24 hours. Ki67⁺ cells are identified with red nuclear staining. Bar graph represents the percentage of Ki67⁺ cells in total countable cells under 200x fields (n = 20). (C) Two-color flow cytometric analysis of SKOV3 following 6-hour PBMC co-culture with of rHE4 (0.01 μg /mL) and BCI (1 μM) as indicated. 2D-scatterplots of propidium iodide and annexin V (Alexa Fluor[®] 488) are shown. Bar graph represents the percentage of propidium iodide / annexin V double positive cells in total cells (n = 4). Mean \pm SEM are shown in the bar graphs. *p < 0.01.

Fig.II.4

A



B



C

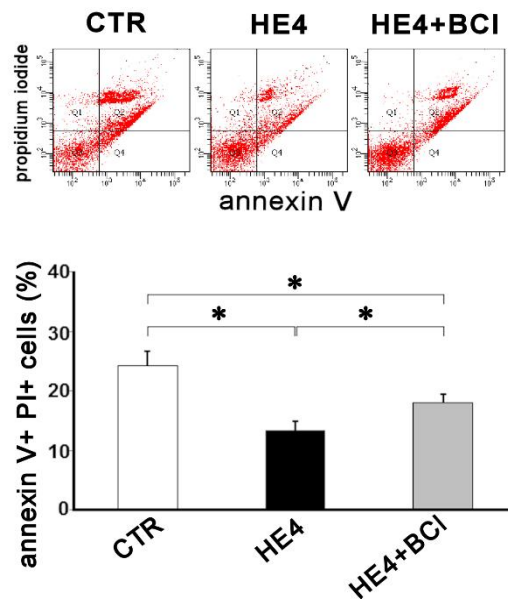


Figure II.5

Responses of SKOV3 cells to co-culture with CD8⁺ / CD56⁺ cell free PBMCs

(A) Cells were co-cultured with CD8⁺ / CD56⁺ cell-free PBMCs with rHE4 (0.01 µg /mL) and BCI (1 µM) in indicated combinations. The cells viabilities were assessed at 24, 48 and 72 hours of the culture (n = 10). (B) Ki67 immunohistochemistry staining was performed on SKOV3 cells co-cultured with CD8⁺ / CD56⁺ cell-free PBMCs for 24 hrs. Ki67⁺ cells are identified with red nuclear staining. Bar graph represents the percentage of Ki67⁺ cells in total countable cells under 200x fields (n = 20). (C) Two-color flow cytometric analysis of SKOV3 following 6 -hour CD8⁺ / CD56⁺ cell free PBMCs co-culture with of rHE4 (0.01 µg/mL) and BCI (1 µM) as indicated. 2D-scatterplots of propidium iodide and annexin V (Alexa Fluor[®] 488) are shown. Bar graph represents the percentage propidium iodide / annexin V double positive cells in total cells (n = 4). Mean ± SEM are shown in the bar graphs. *p < 0.01.

Fig.II.5

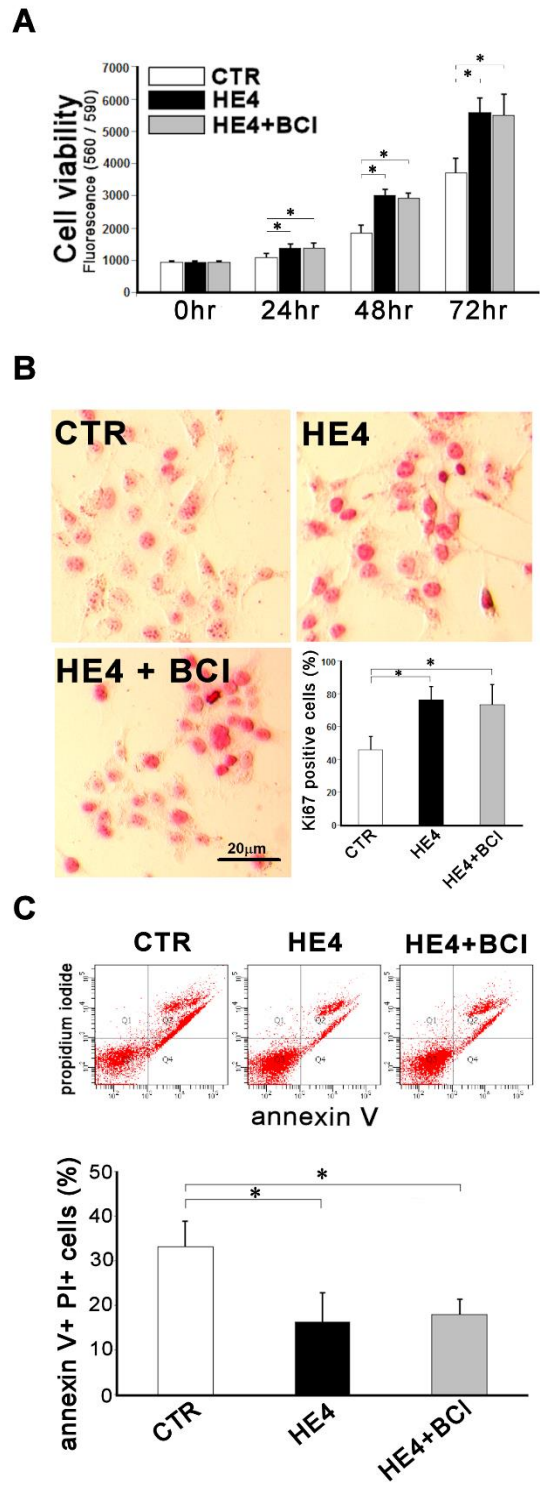


Table II.1 Genes induced in response to HE4

Frequency	ID	gene name
23	NSS	no significant similarity
3	NG_033915	dual specificity phosphatase 6 (DUSP6)
3	XM_017002424	capping actin protein of muscle Z line alpha sub unit 1 (CAPZA1)
3	NM_001402	eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)
3	XM_017000674	FGR proto-oncogene, Src family tyrosone kinase (FGR)
3	NM_001261446.1	thioredoxin reductase 1 (TXNRD1)
3	NM_021109	thymosin beta 4, X-linked (TMSB4X)
3	BC006364	tubulin folding cofactor D
2	AK223032	beta actin variant
2	AC008397.7	chromosome 19 clone CTC-251H24
2	NM_001170330	chromosome 4 open reading frame 3 (C4orf3)
2	AY430097	DAZ associated protein 2 (DAZAP2)
2	NM_001005360	dynamin 2 (DNM2)
2	NG_002350.4	eukaryotic translation elongation factor 1 alpha 1 pseudogene 5 (EEF1A1P5)
2	NM_004468.4	four and a half LIM domains 3 (FHL3)
2	NM_001077488	GNAS complex locus (GNAS)
2	NM_001321232	histocompatibility (minor) HA-1 (HMHA1)
2	NM_000206.2	interleukin 2 receptor, gamma (IL2RG)
2	NM_001127605.2	lipase A, lysosomal acid (LIPA)
2	NM_012335.3	myosin IF (MYO1F)
2	XM_011541520	notch 2 (NOTCH2)
2	NM_001165412	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1)
2	NM_020820.3	phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1 (PREX1)
2	NM_001251855	phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5)
2	NM_201384.2	plectin (PLEC)
2	NM_002952	ribosomal protein S2 (RPS2)
2	NM_001007.4	ribosomal protein S4, X-linked (RPS4X)
2	NM_000655	selectin L (SELL)
2	NM_004252	SLC9A3 regulator 1 (SLC9A3R1)
2	NM_022733.2	small ArfGAP2 (SMAP2)
2	NM_001278206	solute carrier family 43, member 3 (SLC43A3)
2	NM_025250.2	tweety family member 3 (TTYH3)
2	BC050652.1	zinc finger, DHHC-type containing 16
2	NM_004773	zinc finger, HIT-type containing 3 (ZNHIT3)
2	XM_011516569	zyxin (ZYG)
1		154 genes

Table II.2 DUSP6 concentration in cell lysates and culture media of PBMCs

Cell lysates*		Culture media**	
CTR	HE4	CTR	HE4
9.38 ± 0.62	15.62 ± 0.97***	0.77 ± 0.10	1.43 ± 0.14***

*I 2.5 mg/mL of total protein(ng/mL)

**in 5 mL media of 5 x 10⁶ PBMC culture

The mean ± are shown, n = 10 / each group, *** < 0.01 vs CTR

CHAPTER 5

MANUSCRIPT III

In Preparation for Submission to Oncotarget

Inhibition of DUSP6 Sensitizes Ovarian Cancer Cells to Chemotherapeutic Agents Via Regulation of ERK Signaling Response Genes

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III.1 Abstract

Dual Specificity phosphatase 6 (DUSP6) is a phosphatase that deactivates extracellular-signal-regulated kinase (ERK). Since the ovarian cancer clinical biomarker human epididymis protein 4 (HE4) has been shown to interact with the ERK pathway, the objective of this study was to determine the relationship between DUSP6 and HE4 and begin to elucidate the role of DUSP6 in epithelial ovarian cancer (EOC). Western blot and quantitative PCR following knockdowns showed that HE4 and DUSP6 levels were reduced with knockdown of the other protein in SKOV3 and OVCAR8 ovarian cancer cells. Furthermore, DUSP6 levels were upregulated in cells overexpressing HE4. Since HE4 has been shown to promote chemoresistance in EOC, the effect of DUSP6 on chemotherapeutic response was evaluated. MTS assay revealed a significant decrease in cell viability with pharmacological inhibition of DUSP6 using BCI in cells treated with carboplatin or paclitaxel, compared to treatment with single-agent chemotherapy alone. Quantitative PCR was used to evaluate gene expression responses to BCI, recombinant HE4 (rHE4), carboplatin, paclitaxel, and combinatorial treatments. DUSP6 inhibition with BCI altered expression of ERK pathway response genes, including early growth response protein 1 (EGR1) and c-Jun. Expression of EGR1, a strong promotor of apoptosis, was higher in ovarian cancer cells co-treated with BCI and paclitaxel or carboplatin than in cells treated with chemotherapeutic agent alone. Alternatively, the expression of c-Jun, a proto-oncogene, decreased with co-treatment of BCI and paclitaxel or carboplatin. The effect of BCI on the expression of these two genes opposed the effect of rHE4 on their expression. Finally, expression levels of DUSP6 in EOC tissue were evaluated by

immunohistochemical staining and quantification of mean and maximum intensity or integrated optical density (IOD). Levels of DUSP6 were noted to be significantly upregulated in serous EOC tissue compared to adjacent normal tissue, and a positive correlation between HE4 and DUSP6 levels was observed by Spearman Rank correlation. Unpaired 2-tailed student t-test was employed to determine statistical significance of results. In conclusion, DUSP6 inhibition sensitizes ovarian cancer cells to chemotherapeutic agents and alters gene expression of ERK response genes. The ability to detect HE4 levels in EOC patients coupled with the established co-dependence of DUSP6 with HE4, indicates that DUSP6 could plausibly function as a novel therapeutic target in EOC.

III.2 Introduction

Epithelial ovarian cancer (EOC) remains the most common and deadly gynecologic cancer, responsible for 240,000 diagnoses and 152,000 deaths worldwide each year [1]. The 5-year survival rate remains at 35% [2], which is largely due to difficulty with early diagnosis, coupled with the frequency of chemoresistant recurrences [3].

Although a majority of EOC is initially responsive to chemotherapy, once the disease recurs, chemoresistance inevitably develops and the patient eventually will succumb to their illness [4]. Therefore, there is a need for improved diagnostic approaches, as well as novel treatment targets to combat chemoresistance.

Human epididymis protein 4 (HE4) has been established as a novel clinical biomarker for EOC. Inclusion of preoperative levels of HE4 into the diagnostic Risk of Ovarian Malignancy Algorithm (ROMA) results in demonstrably improved specificity and sensitivity in detection and monitoring of the disease over Cancer Antigen 125 (CA 125), pelvic sonography, and menopausal status [5]. Research has also shown its mechanistic involvement in promoting EOC pathogenesis, including the promotion of proliferation, chemoresistance, anti-estrogen resistance, adhesion, invasion, and migration [6–16]. One oncogenic pathway that has been shown to interact with HE4 in several studies is the extracellular signal regulated kinase (ERK) pathway. Several reports indicate that ERK activation is enhanced with HE4 treatment or overexpression, while ERK activation is reduced with HE4 knockdown [8, 14, 15]. Our lab has revealed a more complicated response of ERK to recombinant HE4 treatment; specifically, we have observed downregulation of ERK phosphorylation at early time points, and upregulation at later time points [8]. Although the exact

mechanism of HE4 interaction with the ERK pathway is not clarified, it is well established that HE4 mediates ERK activation in EOC.

Dual specificity phosphatase 6 (DUSP6) is a key negative regulator of ERK signaling via dephosphorylation of ERK at serine/tyrosine residues. ERK activation upregulates gene expression of DUSP6, which promotes a negative feedback loop on ERK activation [17]. DUSP6 has been shown to have differing effects on tumor progression depending on the tumor type. In pancreatic cancer, it is initially upregulated, but diminished at later stages, and is considered a tumor suppressor [18]. It is also considered a tumor suppressor in lung cancer [19]. However, in glioblastoma and HER-2 positive breast cancer, it has been shown to be upregulated [20, 21]. In gastric cancer, DUSP6 inhibition can overcome chemoresistance [22], and it has also been characterized as a therapeutic target in acute lymphoblastic leukemia [23]. One study in ovarian cancer suggested that it may act as a tumor suppressor [24]. The goal of the present study was to determine the relationship between HE4 and DUSP6 in EOC and begin to elucidate the role of DUSP6 in EOC.

III.3 Methods

Cell Culture, Treatments, and siRNA Knockdowns

SKOV3 and OVCAR8 cells were obtained from ATCC and cultured in Dulbecco Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin, in a humidified incubator at 37°C/5% CO₂. Cells were plated at sub-confluent density the day before treatments. Cells were treated with 3.75 μM BCI (Sigma, B4313), 20 nM recombinant HE4 (My BioSource, MBS355616), 100-

500 μ M carboplatin (Sigma Aldrich, C2538), 10 nM paclitaxel (Sigma Aldrich, T7402), or control treatments (.037% DMSO and/or H₂O) for indicated time points. Knockdowns were performed using siRNA directed against DUSP6 (Santa Cruz, sc-39000), HE4 LNA GapmeRs (Exiqon, 300600 Design ID 414262-1), control non-targeting siRNA (Santa Cruz, sc-37007) or Negative Control GapmeRs (Exiqon, 300610). Five μ L lipofectamine 2000 (Invitrogen, 52887) was incubated at room temperature in 100 μ L serum/antibiotic free DMEM. Meanwhile, siRNA was incubated separately in 100 μ L serum/antibiotic free medium at a concentration of 2 μ M for 5 minutes. The tubes were combined and incubated at room temperature for 20 minutes. The complex was added to cells cultured in DMEM with serum but no antibiotic to a final concentration of 100 nM. Cells were collected or underwent additional treatments after 48 hours.

Western Blot

Western blot was performed as previously described [9]. GAPDH was used as a loading control. Antibodies and dilutions used are as follows:

DUSP6 (MyBioSource, MBS8516662, 1:500)

HE4 (Santa Cruz, sc-293-473, 1:200)

GAPDH (Cell Signaling, 2118, 1:2000)

Phospho-ERK (Cell Signaling, 1:2000)

ERK (Cell Signaling, 1:2000)

Densitometry

Image J “analyze gel” function was used to perform densitometry analysis of western blot images in 8-bit TIFF format. Band densities were normalized to GAPDH, and the lowest value was set to 1 for plotted graphs.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described [9]. Validated primers for *DUSP6*, *EGR1*, and *c-JUN* were purchased from realtimeprimers.com. Custom primer sequences (Invitrogen) are as follows:

18s rRNA (F) – CCG CGG TTC TAT TTT GTT GG

18s rRNA (R) – GGC GCT CCC TCT TAA TCA TG

Cell Viability Assay

Cells were seeded at 2 000 cells/well in 96-well plates and treated as described above. After 48 h, cell viability assays were performed by adding 10 µl/well of CellTiter 96® Aqueous One Solution Cell Proliferation MTS Assay (Promega, G3580), incubating at 37°C/5% CO² for 2 h, and reading absorbance at 492 nm. Results are displayed as percent survival of vehicle treated cells.

Immunohistochemistry

Immunohistochemical staining of an ovarian cancer microarray (US Biomax, OV802a) and patient tissues from the Women & Infants Pathology Department was performed as previously described [35], using antibodies for HE4 (Santa Cruz, sc-

293473), DUSP6 (MyBioSource, MBS8516662). Confocal microscopy was performed by an independent imaging technician at the Rhode Island Hospital Digital Imaging Core Facility with a Nikon C1si confocal (Nikon Inc. Mellville, NY, USA). Two to three fields/sample were randomly selected based on DAPI staining, and minimum, mean, and maximum gray values were determined for each field. For the tumor microarray, normal adjacent tissues were used to set the threshold for positive staining. Integrated optical density (IOD) was calculated in serous samples using the mean values multiplied by the total area.

Statistics

Where statistics are shown, $n \geq 3$ biological replicates. Error bars represent standard deviation (STDEV) for quantitative PCR and MTS results, and standard error of the mean (SEM) for immunohistochemistry results. P-values were determined by unpaired, 2-tailed Student *t*-test. For correlation analysis, Spearman rank test was used to determine R value. Differences were considered statistically significant when $p < 0.05$.

III.4 Results

HE4 and DUSP6 Levels Are Co-Dependent in Ovarian Cancer Cell Lines

We first confirmed the upregulation of DUSP6 by HE4 by examining mRNA and protein levels in SKOV3 and OVCAR8 ovarian cancer cells stably overexpressing HE4 (clone 1 and clone 5, respectively) or their null vector (NV) counterparts. DUSP6 mRNA was upregulated by HE4 overexpression (1.2)-fold ($p < 0.05$) and (3.9)-fold

($p < 0.05$), in SKOV3 and OVCAR8 cells, respectively (Figure 1A-B). To determine the reciprocity of the relationship between HE4 and DUSP6, we performed transient siRNA knockdown of DUSP6 and LNA GapmeR knockdown of HE4. We observed that knockdown of HE4 protein resulted in a corresponding downregulation of DUSP6, and knockdown of DUSP6 resulted in a corresponding downregulation of HE4 (Figure 1C-F).

Inhibition of DUSP6 Sensitizes Ovarian Cancer Cells to Chemotherapeutic Drugs

Next, we wanted to begin to determine the function of DUSP6 in ovarian cancer cells. Since one well-known role of HE4 in EOC is the promotion of chemoresistance, we treated SKOV3 and OVCAR8 cells with a DUSP6 inhibitor (BCI) alone or in combination with paclitaxel or carboplatin, the standard of care chemotherapeutic agents in EOC. Treatment of cells with BCI alone resulted in a small but significant reduction in cell viability as determined by MTS assay – 86.3% and 84.7% in OVCAR8 and SKOV3, respectively. In both cell lines, co-treatment with BCI and carboplatin resulted in a synergistic effect on cytotoxicity compared to either treatment alone. Carboplatin alone treatment resulted in 89.8% and 86.8% survival in OVCAR8 and SKOV3 cells, respectively, while BCI with carboplatin resulted in 33.9% and 50.2% survival in OVCAR8 and SKOV3 cells, respectively. In OVCAR8 cells, a synergistic effect was noted with BCI and paclitaxel treatment as well, with survival reducing from 51.4% with paclitaxel alone to 25.3% with BCI and paclitaxel (Figure 2A-B).

DUSP6 Inhibition Alters Expression of ERK Pathway Responsive Genes

In order to determine how regulation of ERK signaling by BCI versus rHE4 might affect downstream gene expression, we treated cells with BCI alone or in combination with rHE4, paclitaxel, or carboplatin, and examined expression of the ERK pathway response genes EGR1 and c-Jun. EGR1 is a transcription factor involved in promoting apoptosis in many cancers [25–28], and has been shown to be involved in cisplatin resistance in esophageal and ovarian cancers [29, 25]. We have previously shown that HE4 suppresses EGR1 gene upregulation in response to cisplatin treatment of SKOV3 cells [8]. On the other hand, c-Jun is an AP-1 transcription factor involved in promoting cell survival and growth [30, 31]. Treatment with BCI modestly upregulated EGR1 expression in both cell lines, while treatment with rHE4 downregulated EGR1 expression—a result that is in agreement with our previous study showing HE4 suppresses cisplatin-mediated upregulation of EGR1. The effect of BCI on EGR1 expression was more apparent with rHE4 co-treatment, where it reversed the downregulation of EGR1 by rHE4. Furthermore, co-treatment with BCI and either paclitaxel or carboplatin upregulated expression of EGR1 compared to treatment with either chemo drug alone. These results show that BCI opposes the effects of HE4 on EGR1 expression and promotes EGR1 expression while suppressing c-Jun expression in cells exposed to chemotherapy drugs (Figure 3A-D).

DUSP6 Levels Are Upregulated in EOC Tissue Compared to Adjacent Normal Tissue, and Correlate with HE4 Tissue Levels

To verify the clinical relevance of our findings, we performed immunohistochemistry of DUSP6 in an EOC tumor microarray and compared levels in serous adenocarcinoma samples (n=40) to levels in normal adjacent tissue (NAT; n=7). Mean intensity of DUSP6 was 545 (+/- 24.5) in EOC samples, and 432 (+/-19.6) in NAT (p=0.005). Moreover, maximal intensity was significantly greater in serous EOC samples than NAT. Maximum intensity was 1653 (+/-75.3) for EOC and 900 (+/- 110.3) for NAT (p=0.016), indicating that some areas of EOC exhibited particularly strong staining for DUSP6 (Figure 4A). Representative images are shown in Figure 4B.

In order to determine if a correlation exists between HE4 levels and DUSP6 levels in EOC, we co-stained for both proteins in the ovarian tissue microarray, and calculated correlations for mean intensity values and integrated optical density (IOD). Spearman Rank correlation test revealed a positive correlation between DUSP6 and HE4 mean intensities (R=0.45, p=0.0038) and IOD values (R=0.64, p=0.00001) (Figure 4C-D). Together, these results suggest that DUSP6 may be involved in promoting tumorigenesis in EOC, and corroborate our results indicating a relationship between HE4 and DUSP6.

III.5 Discussion

In this study, we have determined that HE4 and DUSP6 levels are co-dependent in ovarian cancer cells, and that these two proteins interact and are correlated in patient

tissue. Future studies are needed to elucidate the exact mechanistic relationship between DUSP6 and HE4. Studies by us and others have confirmed that HE4 activates ERK in ovarian cancer cells [8, 14, 15], while DUSP6 is a known negative regulator of ERK signaling [17]. Interestingly, despite the fact that HE4 and DUSP6 have opposing roles on ERK activation, they appear to produce similar effects on biological function of tumor cells. Our results show that activation of ERK by the DUSP6 inhibitor BCI as opposed to HE4 produces very different effects on gene expression and cellular functions such as chemotherapy response.

The two ERK responsive genes we have characterized show opposite expression patterns with BCI treatment. EGR1 is activated by ERK via the transcription factor ELK-1, and EGR1 is itself a transcription factor that activates expression of pro-apoptotic genes [32]. A previous study by our lab showed that HE4 overexpression in SKOV3 cells suppresses cisplatin-mediated upregulation of EGR1 [8]. Here, we observe that HE4 downregulates EGR1 expression, which is consistent with these previous results. Conversely, BCI treatment opposes the effect of rHE4 on EGR1 expression, indicating differing effects downstream of ERK activation by these two treatments. C-Jun, which is also an ERK responsive gene, is regulated oppositely as EGR1. rHE4 treatment upregulates expression of c-Jun, which is consistent with its role as a promoter of tumor growth and proliferation [6, 12, 13, 33, 34]. Meanwhile, BCI again opposes this effect in BCI and rHE4 co-treated cells. Furthermore, BCI suppresses chemotherapy-mediated increases in c-Jun levels. The effects of BCI on EGR1 and c-Jun together may contribute to the overall increased efficacy of BCI and chemotherapy treatment over chemotherapy alone.

The role of DUSP6 in EOC is not well studied. One report showed that DUSP6 appears to function as a tumor suppressor in EOC [24], but our results suggest the opposite effect. Therefore, further study is needed to fully elucidate the role of DUSP6 and determine if its function is context dependent. In general, DUSP6 remains an interesting protein, in that it has opposing roles in different tumor types. In some cancers, it appears to act as a tumor suppressor, while in others it acts to promote tumorigenesis and aggressive behavior [19–24]. Our results are consistent with a recent study by Wu et al. (2018) showing its involvement in cisplatin resistance in gastric cancer [22]. The authors observed an increase in phospho-ERK with BCI treatment, but a downregulation of the ERK-response genes RPS6KA1, EGR1, MMP2, MMP9, MYC, and ELK3. Furthermore, they found that BCI treatment enhanced cisplatin sensitivity in gastric cancer cells and in vivo xenografts. In our study, we observed different effects of DUSP6 inhibition on ERK-response genes depending upon gene function—namely, upregulation of the tumor suppressor EGR1 and downregulation of the proto-oncogene c-Jun. Collectively, our study and the one by Wu et al. illustrate that the relationship between ERK activation and downstream gene activation is not straightforward and appears to be highly context-dependent. Therefore, although BCI serves to increase ERK activation, it has different effects on ERK response genes, which serve to enhance chemotherapy efficacy.

In conclusion, this study highlights a novel function of DUSP6 in EOC and reveals that it may be involved in regulating chemoresponse. Targeting HE4 and/or DUSP6 in EOC may be an effective method of reversing chemoresistance and improving long-term response rates in select patient populations.

III.6 References

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Figure III.1 HE4 and DUSP6 Levels are Co-Dependent in Ovarian Cancer Cell Lines
DUSP6 mRNA levels are higher in SKOV3-C1 (A) and OVCAR8-C5 (B) cells overexpressing HE4 than in null vector (NV) cells. * $p < .05$ (C) HE4 protein levels are reduced in cells with DUSP6 knockdown. (D) DUSP6 protein levels are reduced with HE4 knockdown.

Fig.III.1

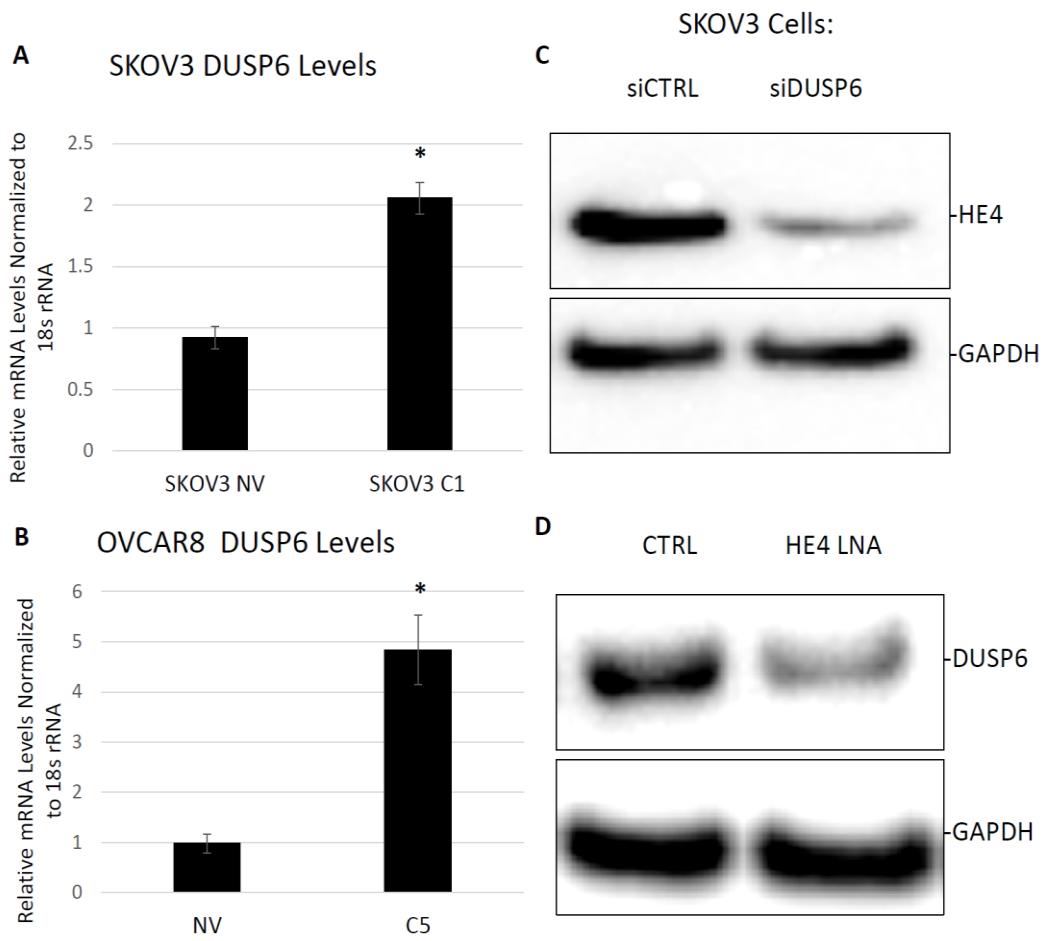


Figure III.2 Inhibition of DUSP6 Sensitizes Ovarian Cancer Cells to
Chemotherapeutic Drugs.

(A) SKOV3 cells exhibited reduced viability when co-treated with the DUSP6 inhibitor BCI and either paclitaxel or carboplatin compared to either chemotherapeutic agent alone. (B) SKOV3 cells exhibited reduced viability when co-treated with the DUSP6 inhibitor BCI and either paclitaxel or carboplatin compared to either chemotherapeutic agent alone. Error bars represent standard deviation of 3 biological replicates in a single experiment. * $p < 0.05$, *** $p < .0005$, **** $p < .00005$

Fig.III.2

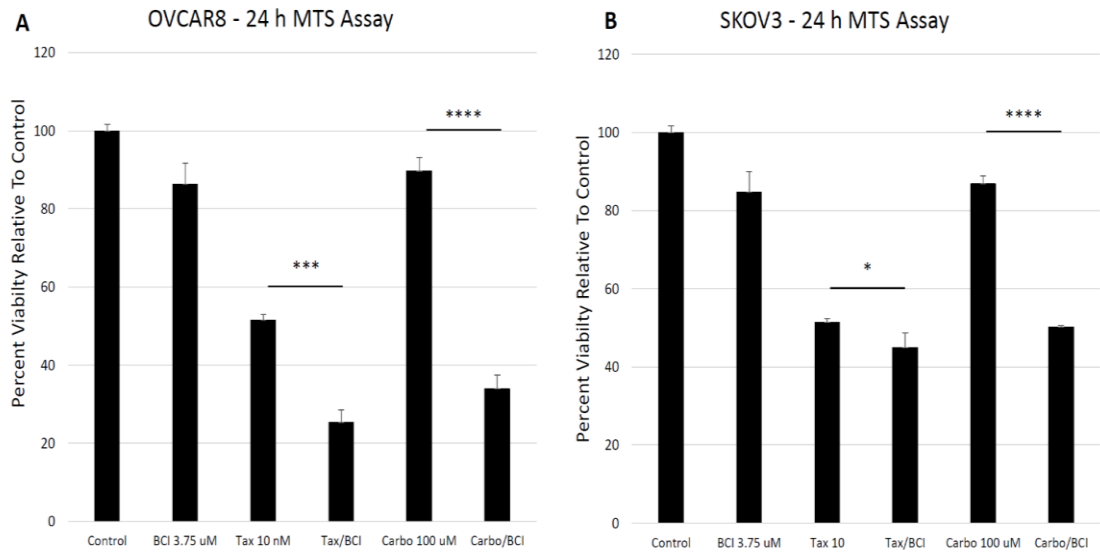


Figure III.3 DUSP6 Inhibition Alters Expression of ERK Pathway Responsive Genes.

(A-B) BCI opposes the effect of rHE4 on EGR1 levels in OVCAR8 and SKOV3 cells.

EGR1 mRNA levels are higher in cells co-treated with BCI and chemotherapeutic

drugs than in cells treated with chemotherapy alone. (C-D) BCI opposes the effect of

rHE4 on EGR1 levels in OVCAR8 and SKOV3 cells. JUN mRNA levels are lower in

cells co-treated with BCI and chemotherapeutic drugs than in cells treated with

chemotherapy alone. n=2-3 independent experiments.

Fig.III.3

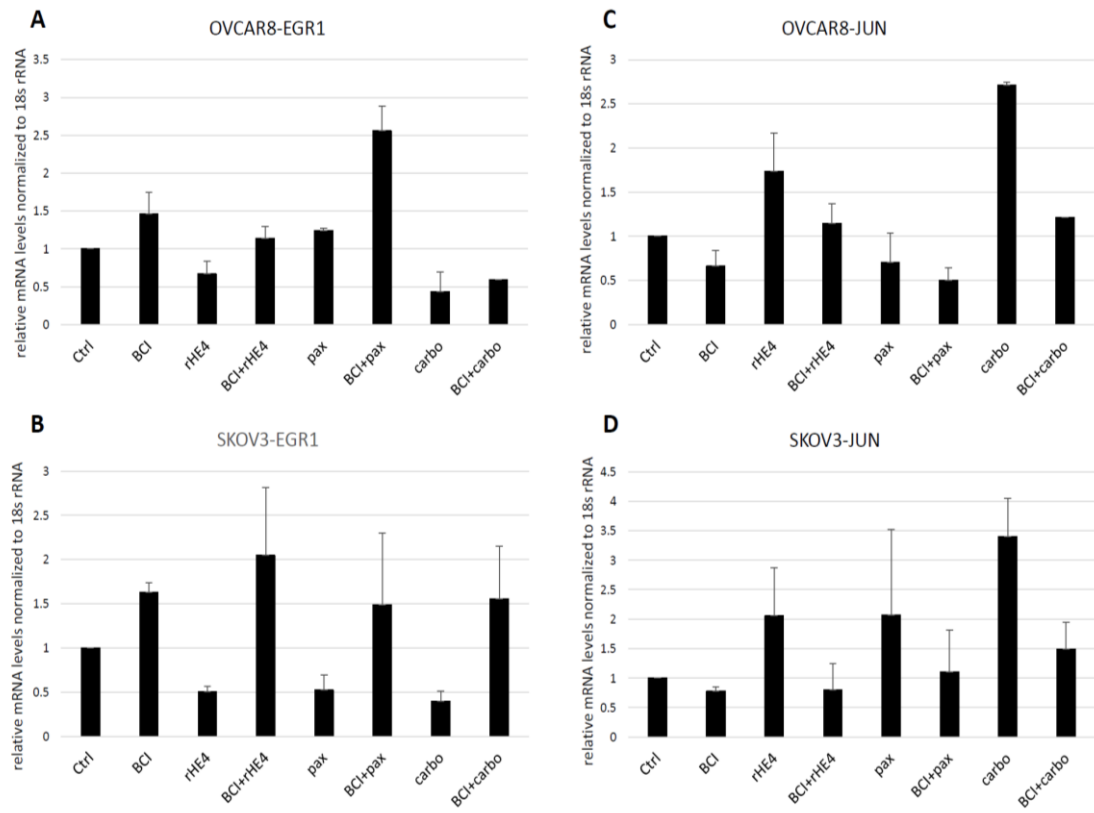


Figure III.4

Figure 4. DUSP6 Levels are Higher in EOC Tissue than Normal Adjacent Tissue, and Correlate with HE4 Tissue Levels.

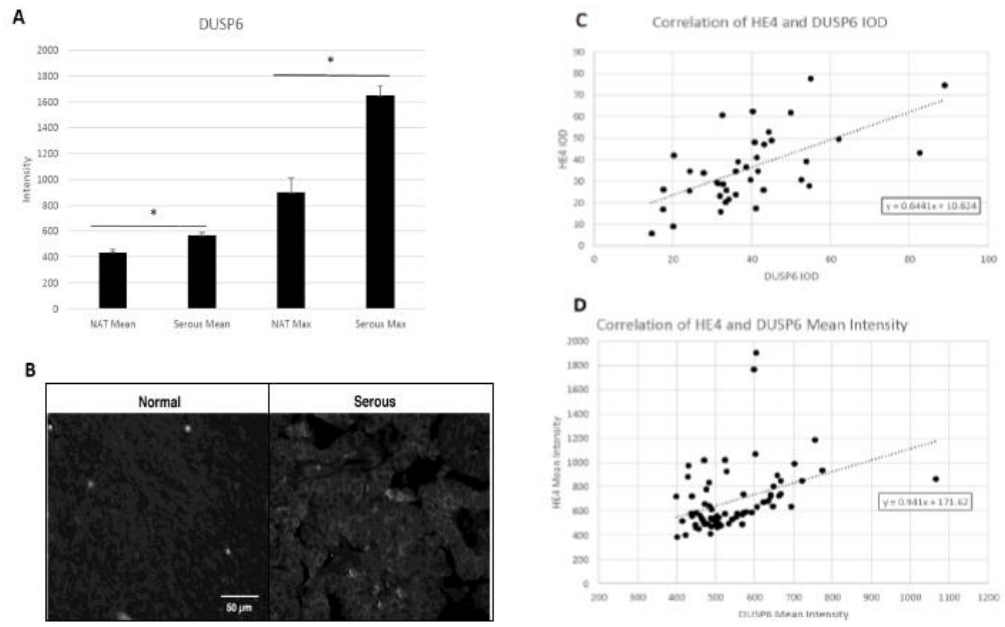
(A) DUSP6 mean and maximum intensity staining is higher in serous EOC tissue (n=40) than in normal adjacent tissue (NAT) (n=7). Error bars represent deviation.

*p<0.05 (B) Representative images of NAT and serous EOC DUSP6 staining. (C)

Correlation of DUSP6 and HE4 mean intensity. (D) Correlation of DUSP6 and HE4

integrated optical density (IOD). Graph excludes one outlier data point for clarity (data is included in Spearman Rank Correlation calculation).

Fig.III.4



CHAPTER 6

MANUSCRIPT IV

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Septin-2 is Overexpressed in Epithelial Ovarian Cancer and Mediates Proliferation via Regulation of Cellular Metabolism Proteins

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IV.I Abstract

Epithelial Ovarian Cancer (EOC) is associated with dismal survival rates due to the fact that patients are frequently diagnosed at an advanced stage and eventually become resistant to traditional chemotherapeutics. Hence, there is a crucial need for new and innovative therapies. Septin-2, a member of the septin family of GTP binding proteins, has been characterized in EOC for the first time and represents a potential future target. Septin-2 was found to be overexpressed in serous and clear cell human patient tissue compared to benign disease. Stable septin-2 knockout clones developed in an ovarian cancer cell line exhibited a significant decrease in proliferation rates. Comparative label-free proteomic analysis of septin-2 knockout cells revealed differential protein expression of pathways associated with the TCA cycle, acetyl CoA, proteasome and spliceosome. Further validation of target proteins indicated that septin-2 plays a predominant role in post-transcriptional and translational modifications as well as cellular metabolism and are the first to suggest the potential novel role of septin-2 in promoting EOC tumorigenesis through these mechanisms.

IV.2 Introduction

Epithelial Ovarian Cancer (EOC) is the most lethal gynecologic malignancy [1]. In 2018, there will be an estimated 22,240 new cases of EOC diagnosed and 14,070 deaths in the United States. While EOC accounts for only 2.5 % of all female cancers, it is responsible for 5% of all cancer deaths due to low disease survival rates [2]. These dire statistics are attributed to the fact that the majority of patients are diagnosed at an advanced stage. In addition, while patients generally respond well to frontline platinum-based chemotherapy, chemoresistant recurrences are common [3]. Therefore, there is a strong need for novel early detection methods and targeted therapies for EOC patients.

Septin-2 is a member of the septin family, a conserved family comprised of 13 GTP binding proteins [4]. Septins, which are structurally observed as rods and filaments, are vital to a number of cellular processes, including cytokinesis, vesicle trafficking, and exocytosis [5]. They are considered to be a fourth component of the cytoskeleton due to their association with actin, microtubules, and membranes [6]. Septins have been identified as having a role in neurodegenerative disease, since they were detected in brain tissue from patients with Alzheimer disease [7]. In addition, they have been reported to be involved in bacterial infections, Parkinson's disease, and male infertility [8].

In more recent years, emphasis has been placed on investigating the role of septins in tumorigenesis [9]. Due to their natural function in scaffolding and membrane compartmentalization, it is plausible that they could also play a role in the organization of membrane associated proteins involved in diverse tumorigenic

signaling pathways [6]. Septin-9 is the best studied septin family member in relationship to cancer, and its methylation status is utilized as a biomarker in colorectal cancer [10]. However, there have also been numerous studies linking septin-2 to neoplasia. Thus far, septin-2 has been specifically implicated in Hodgkin's lymphoma and biliary tract, gastric, hepatocellular, and breast cancer [11–15], but its role in EOC has not yet been investigated.

In this study, we begin to elucidate septin-2's function in EOC. As septins have been shown to have diverse roles in tumorigenesis, this is the first step in specifically defining septin-2's contribution to EOC pathogenesis. To establish the clinical relevance of septin-2 in EOC, we first sought to compare levels of septin-2 in various histological pathologies of EOC versus benign disease. Furthermore, we present for the first time a global analysis of septin-2 mediated proteomics in EOC and describe signaling pathways most affected by septin-2 depletion. The results from this study lay the framework for future mechanistic studies to determine the precise role of septin-2 in EOC.

IV.3 Methods

Cell Culture

SKOV3 wild type (SKOV3WT) and OVCAR8 wild type (OVCAR8WT) cell lines were obtained from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator at 37°C/5% CO₂.

Septin-2 silencing with shRNA

shRNA for human HE4 (Santa Cruz Biotechnology, sc-40936-SH) or control shRNA (Santa Cruz Biotechnology, sc-108066) was transfected into SKOV3WT cells using Lipofectomine® 2000 (Invitrogen, 11668) following the manufacturer's instructions. Individual single cells were selected by culturing under the pressure of 5 ug/mL of puromycin (Research products International, 58-58-2), and clonal populations were allowed to expand. Phenotypes of the clones were evaluated by western blotting using anti Septin-2 antibody (Novus Biologicals, NBP1-85212)

Proliferation Assay

SKOV3WT, Plasmid C, KO#9, and KO#11 were plated at equal densities in 100x20mm plates. Cells were trypsinized at 72 and 96 hours, and replicates of three were counted using a hemocytometer to compare proliferation rates. The experiment was repeated three times and error bars represent standard deviation. Statistical significance was determined by an unpaired, two-tailed Student *t*-test, where $p < .05$ was considered significant.

Immunohistochemistry and confocal immunofluorescent microscopy

Formalin-fixed paraffin embedded human ovarian tissue slides were obtained from the Women and Infants Pathology Department. The human ovarian tissue microarray was obtained from US Tissue Biomax (OV802a). Slides obtained from Women and Infants were baked at 65 °C for two hours, and the microarray for 20 minutes. All slides were subsequently washed in xylene, 100% ethanol, 95% ethanol, 70% ethanol,

deoxygenated water, and FTA Hemagglutination Buffer. Antigen retrieval was then performed using DAKO antigen retrieval solution (10x) (Agilent, S1699), heated to 95 °C for 20 minutes. Slides were then blocked with 5% horse serum in FTA Hemagglutination Buffer and incubated overnight in primary Septin-2 antibody (Santa Cruz, sc-20408) at 4 °C. Secondary antibody, Alexa Fluora 488 (Thermo Fisher Scientific, A-11055) was then added to slides following incubation in the dark for one hour at room temperature. Slides were washed in between steps with FTA Hemagglutination Buffer and were cover-slipped with DAPI containing mounting medium (Vector Laboratories, H-1200). Images were acquired using a Nikon E800 microscope (Nikon Inc. Mellville, NY, USA) and an RT3 SPOT camera (Diagnostic Instruments, Sterling Heights, MI, USA). Random sampling of ten fields was based on DAPI staining. Mean intensity or integrated optical density (IOD), expressed as $\text{area} \times \text{mean} / 1\text{E}+07$, was acquired using a 40X objective. Statistical significance was determined by an unpaired, two-tailed Student *t*-test, where $p < .05$ was considered significant.

Western Blot

Protein was extracted from cell pellets in Cell Lysis Buffer (Cell Signaling, 9803) with 1 mM of PMSF, according to the manufacturer's protocol. The concentration of extracted proteins was determined by DC Protein Assay (Bio-Rad Laboratories, 5000116). Western blot analysis was performed by loading equal amounts of protein boiled at 70 °C with Novex Sample Reducing Agent (Life Technologies, NP009) and NuPAGE LDS sample buffer (ThermoFisher Scientific, NP0007) into a 4–12 %

gradient NuPAGE Novex Bis-Tris gel [Life Technologies, NP0321BOX (mini), WG1402BX10 (midi)]. The gel was then transferred using a semi-dry transfer to methanol-activated 0.2 µm PVDF membranes (Bio-Rad, 162-0177) at 0.12-0.24A for 1 h 20 m. Membranes were blocked in 5 % milk in phosphate-buffered saline with 0.05 % Tween 20 (PBS-T) for 30 m at room temperature. Finally, membranes were incubated in primary antibody diluted in 5 % milk in PBS-T overnight at 4 °C, and then in secondary antibody diluted in 5 % milk in PBS-T for 1 h at room temperature, with PBS-T washes in between. Amersham ECL Prime Western Blot Detection System (GE Healthcare, RPN2232) was employed for detection of the HRP-tagged secondary antibodies. The Biorad Chemidoc MP Imaging System was used to image all blots. GAPDH was used as a loading control. Antibodies and respective dilutions used are as follows:

GAPDH (cell signaling, 2118, [1:2000])

Septin-2(novus biologicals, NBP1-85212, [1:500])

LDHA(cell signaling, 3582S, [1:1000])

FASN (cell signaling, 3180S, [1:1000])

Enolase (santa cruz biotechnology, sc-100812 [1:500])

Transketolase (santa cruz biotechnology, sc-390179) [1:500])

Quantitative PCR

RNA was extracted from cells by Trizol /LiCl precipitation. Total RNA (1000 ng) was then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, 1708890), following the manufacturer's protocol. Quantitative PCR was performed in

triplicate by loading 1 μ l cDNA reaction, 1 μ M forward and reverse validated Septin-2 primers (Origene HP232247), 10 μ l SYBR Green (Applied Biosciences [ABI], 4367659) and 5 μ l RNase-free water to each well. Samples were run using the ABI 7500 Fast Real-Time PCR System. Data was then analyzed using the $\Delta\Delta$ Ct method. All gene expression levels were normalized to 18 s rRNA.

Densitometry

Densitometry analysis of western blots was performed using image J. Blot images were analyzed in 8-bit JPEG format, using the “analyze gel” function. Relative band densities were normalized to GAPDH loading control.

Sample preparation for LC-MS/MS analysis

Cell pellets were subjected in lysis buffer (8 M urea, 1 mM sodium orthovanadate, 20 mM HEPES, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, pH 8.0, 20 min, 4°C), sonicated and cleared by centrifugation (14 000 \times g, 15 min, 4°C). Protein concentration was measured (Pierce BCA Protein Assay, Thermo Fisher Scientific, IL, USA) and a total of 100 μ g of protein per sample was subjected for trypsin digestion. Typtic peptides were desalted using C18 Sep-Pak plus cartridges (Waters, Milford, MA) and were lyophilized for 48 hours to dryness. The dried eluted peptides were reconstituted in buffer A (0.1 M acetic acid) at a concentration of 1 μ g/ μ l and 5 μ l was injected for each analysis.

The LC-MS/MS was performed on a fully automated proteomic technology platform [16,17] that includes an Agilent 1200 Series Quaternary HPLC system (Agilent

Technologies, Santa Clara, CA) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The LC-MS/MS set up was used as described earlier [18]. Briefly, the peptides were separated through a linear reversed-phase 90 min gradient from 0% to 40% buffer B (0.1 M acetic acid in acetonitrile) at a flow rate of 3 μ l /min through a 3 μ m 20 cm C18 column. The electrospray voltage of 2.0 kV was applied in a split flow configuration, and spectra were collected using a top-9 data-dependent method. Survey full scan MS spectra (m/z 400-1800) were acquired at a resolution of 70,000 with an AGC target value of 3×10^6 ions or a maximum ion injection time of 200 ms. The peptide fragmentation was performed via higher-energy collision dissociation with the energy set at 28 NCE. The MS/MS spectra were acquired at a resolution of 17,500, with a targeted value of 2×10^4 ions or a maximum integration time of 200 ms. The ion selection abundance threshold was set at 8.0×10^2 with charge state exclusion of unassigned and $z = 1$, or 6-8 ions and dynamic exclusion time of 30 seconds.

Bioinformatics analysis

Peptide spectrum matching of MS/MS spectra of each file was searched against a species-specific databases (UniProt; downloaded 2/1/2015) using MASCOT v. 2.4 (Matrix Science, Ltd, London, UK). A concatenated database containing “target” and “decoy” sequences was employed to estimate the false discovery rate (FDR) [19]. Msconvert from ProteoWizard (v. 3.0.5047), using default parameters and with the MS2Deisotope filter on, was employed to create peak lists for Mascot. The Mascot database search was performed with the following parameters: trypsin enzyme cleavage specificity, 2 possible missed cleavages, 10 ppm mass tolerance for precursor

ions, 20 mmu mass tolerance for fragment ions. Search parameters permitted variable modification of methionine oxidation (+15.9949 Da) and static modification of carbamidomethylation (+57.0215 Da) on cysteine. The resulting peptide spectrum matches (PSMs) were reduced to sets of unique PSMs by eliminating lower scoring duplicates. To provide high confidence, the Mascot results were filtered for Mowse Score (>20). Peptide assignments from the database search were filtered down to a 1% FDR by a logistic spectral score as previously described [19,20].

Relative quantitation of the identified peptides

Relative quantification of peptide abundance was performed via calculation of selected ion chromatograms (SIC) peak areas. Retention time alignment of individual replicate analyses was performed as previously described [21]. Peak areas were calculated by inspection of SICs using in-house software programmed in R 3.0 based on the Scripps Center for Metabolomics' XCMS package (version 1.40.0). This approach performed multiple passes through XCMS' central wavelet transformation algorithm (implemented in the centWave function) over increasingly narrower ranges of peak widths and used the following parameters: mass window of 10 ppm, minimum peak widths ranging from 2 to 20 seconds, maximum peak width of 80 seconds, signal to noise threshold of 10 and detection of peak limits via descent on the non-transformed data enabled. SIC peak areas were determined for every peptide that was identified by MS/MS. In the case of a missing MS/MS for a particular peptide, in a particular replicate, the SIC peak area was calculated according to the peptide's isolated mass and the retention time calculated from retention time alignment. A minimum SIC peak area equivalent to the typical spectral noise level of 1000 was

required of all data reported for label-free quantitation. Individual SIC peak areas were normalized to the peak area of the standard synthetic peptide DRVYHPF that was exogenously spiked prior to reversed-phase elution into the mass spectrometer. Quantitative analysis was applied to replicate experiments. To select peptides that show a statistically significant change in abundance between control vs treatment cells, q-values for multiple hypothesis tests were calculated based on p-values from two-tailed unpaired Student's t tests using the R package QVALUE as previously described [22,23].

IV.4 Results

Septin-2 is overexpressed in EOC

A preliminary proteomic study determined interacting partners of the clinical EOC biomarker HE4. It was noted that septin-2 was the most upregulated HE4-interacting protein (13-fold) in SKOV3 ovarian cancer cells overexpressing HE4 compared to null vector cells (data not shown). This finding prompted us to begin to characterize septin-2's role in EOC, as it had not been previously documented in the literature. To establish the clinical relevance of septin-2 in EOC, we evaluated its levels in EOC samples of a variety of histopathologies and compared these to levels in benign controls. Immunohistochemical analysis of septin-2 levels in a human ovarian tissue microarray comprising normal, serous, mucinous, clear cell, and dysgerminoma histopathologies revealed that mean intensity of the septin-2 staining was statistically significantly greater in serous EOC (703.3889 pixels) than in adjacent normal tissue (539 pixels) ($p=0.0037$) (Fig.1a). While all other histopathologies exhibited higher

mean intensity levels of septin-2—mucinous (603 pixels), clear cell (821 pixels), and dysgerminoma (744 pixels)—compared to the normal adjacent tissue, none were considered statistically significant possibly due to low numbers of samples available. To further investigate expression levels of septin-2 in patient samples, immunohistochemistry of septin-2 was performed in EOC and benign tissue from our institution. Integrated optical density (IOD) was calculated for each sample, which revealed statistically significant higher levels in serous (721 area*mean/1E+06, $p=0.04$) (Fig 1b. and 1c.) and clear cell (31 area*mean/1E+06, $p=0.009$) histopathologies (Fig.1d. and 1e.) compared to respective benign controls (239 area*mean/1E+06) and (6 area*mean/1E+06).

Stable knockdown of septin-2 influences cell proliferation

In order to study septin-2's function in EOC, stable septin-2 knockout shRNA clones were generated in human serous ovarian SKOV3 wild type (WT) cells. Two clonal populations were employed for these studies—knockout 9 (KO9) and knockout 11 (KO11)—based on confirmation of successful septin-2 downregulation. A stable line was also generated by clonal expansion of cells transfected with control shRNA, designated Plasmid C. To confirm the efficacy of knockdowns at the genomic level, qPCR was employed. Septin-2 levels in KO9 were 1.93- and 4.16-fold lower than WT and Plasmid C cells, respectively. Septin-2 levels in KO11 were 1.67- and 3.88-fold lower than WT and Plasmid C cells, respectively (Fig 2a).

To further validate successful knockdown of septin-2, protein levels were detected by western blot. We observed substantial decreases septin-2 levels in KO9 and KO11 compared to the WT and Plasmid C controls (Fig 2b). Septin-2 levels in KO9 were decreased by 72% compared to WT and by 62.3% compared to Plasmid C. Septin-2 levels in KO11 were reduced by 76.4% and 67.7% compared to WT and Plasmid C, respectively (Fig.2c).

To begin to determine the consequence of septin-2 knockdown in SKOV3 cells, proliferation of the shRNA clones was evaluated. WT, Plasmid C, KO9, and KO11 cells were seeded at equal cell densities and allowed to expand. The cells were trypsinized at 72 and 96 hours, and numbers of live cells in each clonal population were quantified (Fig 2d). At 72 hours, KO9 clones exhibited a 67.5% decrease in cell proliferation compared to WT, and a 60.4% decrease compared to Plasmid C. KO11 clones demonstrated a 66.4% and 59.1% decrease in proliferation from respective WT and Plasmid C cell numbers. The 96-hour timepoint revealed a 51.1% reduction in KO9 cells compared to WT and a 39.3% reduction compared to Plasmid C. KO11 cells showed a 62.6% and 53.6% decrease compared to WT and Plasmid C cells, respectively. All decreases in cell counts displayed by KO9 and KO11 at both timepoints were determined to be statistically significant ($p < 0.02$). This finding strongly suggests that the downregulation of septin-2 has a profound impact on cell proliferation in EOC cells.

Proteomic analysis of septin-2 knockdown in EOC cells

A comparative label-free proteomic analysis was performed to examine global protein expression level differences resulting from the knockdown of septin-2. Interestingly,

significant differences in protein-peptide levels between control cells and septin-2 knockouts was observed only in KO11 populations, even though our proliferation results demonstrated that KO9's phenotype was similar to that of KO11. We concluded that it was possible that the knockdown resulted in less significant effects on protein levels, but still enough to affect proliferation, or that spontaneous loss of the knockdown had occurred during cell culture. Therefore, we proceeded with analysis using KO11 cells. As expected, a principal component analysis of three biological replicates of WT, Plasmid C, and KO11 revealed separate clusters when comparing principal component 1 and principal component 2 scores (Fig.3). In contrast, for KO9 sample, the 3 biological replicates were very scattered (Data not shown). Therefore, for any further analysis or validation process KO9 was not included.

Mass spectrometry of the control and knockdown cells identified 19976 unique peptides corresponding to 3565 unique proteins. Of those, only one peptide/protein in Plasmid C exhibited an absolute fold change greater than 1 with a q-value < 0.05 compared to WT (Fig 4a). This result allowed us to conclude that there was no significant difference between both control cell populations. Conversely, 5% of all peptides in KO11 cells revealed relative fold change greater than 1 ($q < 0.05$) compared to WT cells. In addition, 93.5% of those peptides identified as exhibiting substantial expression differences displayed a lower peak area in KO11 than WT, indicating a majority of peptides was downregulated (Fig 4b). Representative examples of peak-area of four peptide sequences from the proteins galectin-3 binding protein (LFALS3BP), transketolase (TKT), poly(A) binding protein (PABPC4), and enolase-

1(ENO1) show differential expression between control and knockdown cells. KO11/WT peak area ratios were calculated for LFALS3BP (0.051, $q=0.012$), TKT (0.081, $q=0.0012$), PABPC4 (0.50, $q=0.011$), and ENO1 (632.7, $q=0.30$) (Fig 4c). It is interesting to note that, all four of these proteins have previously been shown to play a role in tumorigenesis [24–27]. Heat maps were constructed to illustrate the clustering of the 231 differentially expressed proteins in each of the three replicates of WT, Plasmid C, and KO11 (Fig 5a) and representative peptides in the most differentially expressed proteins (Fig 5b). Comparison of both heat maps reveals an overall similar pattern of peak-area quantitation, with many of the proteins and peptide sequences within KO11 exhibiting downregulation compared to WT and Plasmid C controls.

Finally, gene ontology (GO) analysis with differentially expressed proteins showed enrichment of for, proteasomal/ubiquitin in the biological process category and RNA binding in the molecular function category (Fig 6). Enrichment was also noted for terms related to the ribonucleoprotein complex and cytosol in the cellular component category. KEGG pathway analysis revealed citric acid cycle (TCA cycle) and spliceosome enrichment among differentially expressed proteins (Fig 6).

Representative proteins related to these pathways were further validated by immunoblot analysis. Enolase, LDHA, Transketolase, and FASN expression in WT and KO11 was examined via western blot. (Fig 7a.) Band density normalized to GAPDH revealed a 7.8% increase in Enolase expression from WT to KO11. A corresponding 24.2%, 52.6%, and 64.9% decrease was observed comparing WT and KO11 in LDHA, Transketolase and FASN levels respectively. (Fig 7b.)

IV.5 Discussion

For the first time, we have characterized septin-2 function in EOC and examined its proteomic effects on a global level. Several biological pathways were found to be differentially regulated in septin-2 knockout ovarian cancer cells, exemplified by representative proteins from (Fig 4c.) Galectin-3 is a member of the β -galactoside binding protein family that is involved in diverse functions inherent to cancer, such as metastasis, immune surveillance, inflammation, apoptosis, molecular trafficking, and mRNA splicing [28]. Transketolase is a pentose phosphate pathway enzyme essential for cancer growth due to its ability to control NADPH production and counteract oxidative stress [26]. Poly(A) binding protein is a highly conserved protein that plays an important role in mRNA stabilization and translation [29], which controls cell growth, proliferation, and differentiation [30]. Enloase1, found to be differentially expressed in cancer, is a key glycolytic enzyme that catalyzes 2-phosphoglycerate to phosphoenolpyruvate in the last steps of the glycolytic catabolic pathway [31].

Of these pathways identified, it was most expected that autophosphorylation and proteasomal/ubiquitin protein functions were affected by septin-2 knockdown. It has been previously established that proper control of septins' phosphorylation status is required for the completion of cytokinesis [32]. In fungus, Meseroll et. al (2013) discovered that changes in specific phosphorylation sites on septins (Cdc3p and Cdc11p) leads to the disruption of higher order septin structures, indicating septin phosphorylation is also a vital regulator of their own structure formation [33].

Similar to phosphorylation, ubiquitination represents another important septin post-translational modification. Septins have an established role interacting with proteins

involved in degradation pathways, such as ubiquitin ligases and de-ubiquitylating enzymes, which modulates protein turnover [12,34,35]. Recently, it has also been reported that SUMOylation of human septins is a critical process contributing to proper septin filament bundling and cytokinesis [36]. Unlike ubiquitin, SUMO (small ubiquitin-like modifiers) modification does not always lead to protein degradation, as SUMOylation can also modulate localization, interaction, and activity of the target protein [37]. Ribet et.al (2017) reported that septin-7 is constitutively SUMOylated throughout the cell cycle, and septin variants that are unable to be SUMOylated halt septin bundle formation and lead to defects in cytokinesis, highlighting its crucial role in septin filament bundling and cell division [36].

GO analysis revealed that septin-2 is also involved in post-transcriptional modifications, as the spliceosome pathway was found to be enriched among septin-2 regulated proteins (Fig 6). This result suggests that septin-2 plays a major role in the editing of both precursor messenger RNA (pre-mRNA) and proteins. The spliceosome, a large molecular complex involved in the removal of non-coding introns from pre-mRNA, represents a potential oncogenic target as evidence has shown that tumors rely on normal spliceosome function for cell survival [38,39]. In addition, Poly(A) binding protein, which we reported as an example of a differentially expressed protein (Fig. 4c), is a translation initiation factor that binds to the mRNA 3'poly(A) tail [30] and also influences cell growth and survival. Since we have shown that the knockdown of septin-2 promotes irregular expression of a multitude of pathways related to mRNA and protein modifications, it seems reasonable that its downregulation would also affect tumor cell growth.

As the depletion of septins can lead to cytokinesis failures, it is logical that cellular proliferation would subsequently be affected. [36] In this study, we observed a reduction in proliferation with septin-2 knockdown. (Fig.2d) Corroborating results from our study in EOC, Zhang et. al (2016) treated breast cancer cells with the broad septin inhibitor forchlorfenuron(FCF) and also observed a decrease in cell proliferation [15], which they attributed to the suppression of MEK and ERK1/2 (extracellular signal-regulated kinase 1/2) signaling [15]. Another study showed that septin-8 interacts with MAPK5 (mitogen activated protein kinase 5), further suggesting that septins play a role in the MAPK/ERK pathway [40]. Septin-9 has also been implicated in cell proliferation, as a septin-9 variant SEPT9_i1 binds to c-Jun-N terminal kinase (JNK), preventing its degradation and therefore promoting tumor cell proliferation [4]. In addition, another septin-9 variant SEPT9_i3 has been found to be phosphorylated by cell-cycle-dependent kinase 1 (CDK1), controlling entry into mitosis and promoting cell survival and proliferation [41]. These investigations highlight that septin-2, and septins in general, play an important role in cellular proliferation and potentially promote tumor growth.

Interestingly, the most novel conclusion drawn from this investigation was the robust enrichment seen in cellular metabolism and energy dynamics in proteins affected by septin-2 downregulation. This novel finding regarding septin-2 is in agreement with previous studies reporting on septin functions related to energy metabolism. One study identified that fungal septins FaCdc3 and FaCdc12 are required for lipid metabolism [42]. In addition, septin-9 was found to induce lipid droplet growth through binding to phosphatidylinositol-5-phosphate(PtdIns5P), a phospholipid with a well-established

role in dynamics and intracellular membrane trafficking [43]. PtdIns5P binding in turn controls septin-9 filament formation and its interaction with microtubules [44].

Furthermore, septin-11 was found to be expressed in human adipocytes and upregulated in obese individuals. SEPT11 mRNA was positively correlated with markers of insulin resistance in adipose tissue, and silencing of septin-11 muted insulin signaling and insulin-induced lipid accumulation in adipocytes [45].

Our findings, however, represent the first time a septin family member has been implicated in cellular metabolism as it relates to tumorigenesis. Acetyl-CoA, one of the pathways most differentially expressed by septin-2, is a key metabolic player that links glycolysis, fatty acid oxidation, ketogenesis, amino acid metabolism, the TCA cycle, and lipid synthesis [46]. In normoxic conditions, acetyl-CoA is derived from glucose. However, under hypoxic conditions like in cancer, acetyl-CoA has been found to derive from acetate, suggesting that targeting the acetyl-CoA pathway in cancer could represent a viable treatment option [47]. The TCA cycle, another important metabolic pathway, was also deregulated in the septin-2 knockdown clones. While previous dogma stated that tumor cells do not utilize the TCA cycle for energy, it has now been found that some cancer cells with deregulated oncogenes and tumor suppressor genes actually do rely on the TCA cycle [48]. In addition, the metabolic proteins transketolase and enolase, which are involved in glycolysis and the pentose phosphate pathway, respectively, were found to be differentially expressed by septin-2 inhibition (Fig 4c), demonstrating that septin-2 is involved in various facets of cellular metabolism within EOC. Pathways related to metabolism and energy production have previously been found to contribute to EOC tumorigenesis, as it has been shown that

glycolysis drives chemoresistance in EOC and that high levels of fatty acid synthase (FASN) contribute to tumor cell growth through the promotion of human epidermal growth factor [49,50]. Therefore, it can be hypothesized that the inhibition of septin-2 would exhibit a therapeutic effect in EOC via suppression of tumor metabolic pathways.

Overall, our study demonstrates the novel finding that septin-2 is involved in EOC pathogenesis. This investigation represents a springboard for future studies to determine the efficacy of septin-2 inhibition, in addition to more clearly elucidating its diverse mechanistic pathways in EOC tumorigenesis. While our proteomics study was performed in a serous ovarian cancer cell line, it would be interesting to repeat the stable knockdown experiment in a clear cell EOC line, since septin-2 was also found to be overexpressed in this histopathology. Additionally, both in vitro and in vivo studies could be performed to confirm that inhibition of septin-2 affects cell viability and tumor growth in order to determine if targeting of septin-2 synergizes with platinum-based chemotherapeutics.

IV.6 References

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Figure IV. 1 Septin-2 is overexpressed in EOC

(a.) Tissue Microarray analysis reveals that septin-2 is overexpressed significantly ($p=.0037$) in serous EOC compared to adjacent normal control. Mucinous, clear cell, and dysgerminoma all exhibited a non-significant increase in septin-2 expression (mean intensity) (b.) Staining of human EOC tissue showed a statistically significant higher ($p=0.04$) septin-2 expression in serous compared to benign serous. (c.) Representative images of Serous EOC staining (left panel) vs benign (right panel). (b.) Staining of human EOC tissue showed a statistically significant higher ($p=0.009$) septin-2 expression in clear cell compared to benign tissue. (e.) Representative images of clear cell EOC staining (left panel) vs benign (right panel).

Fig.IV.1(a)

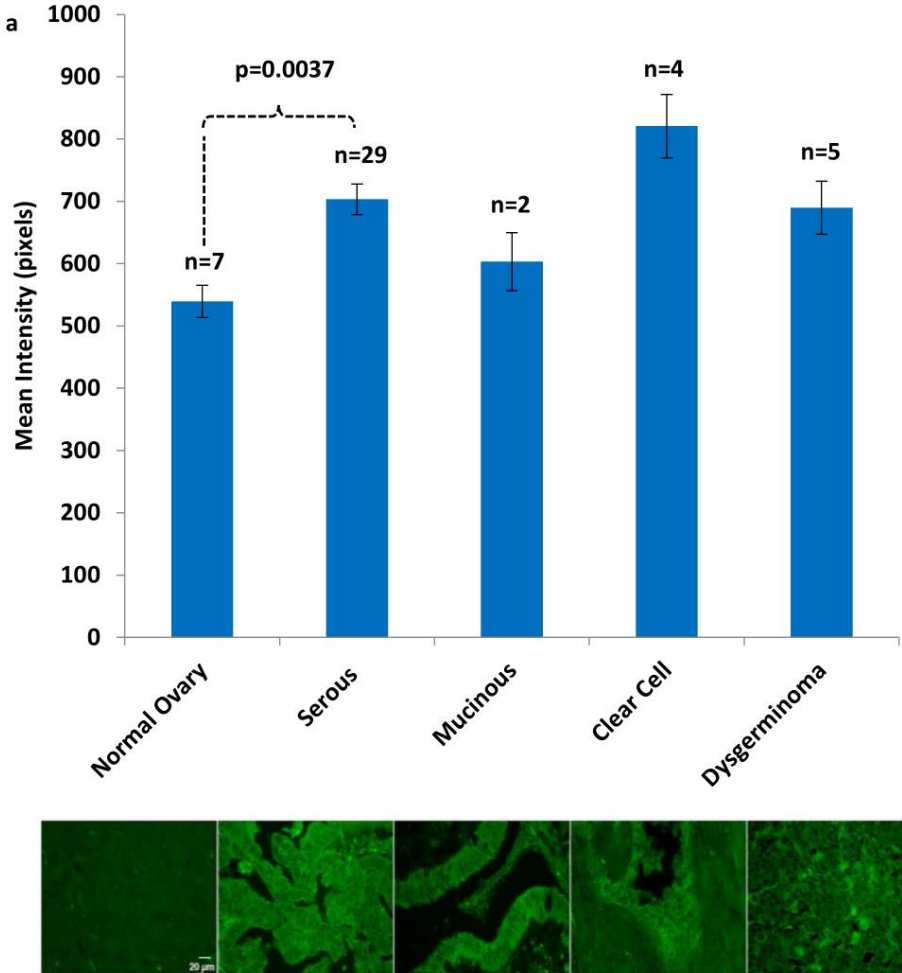


Fig IV.1(b-e)

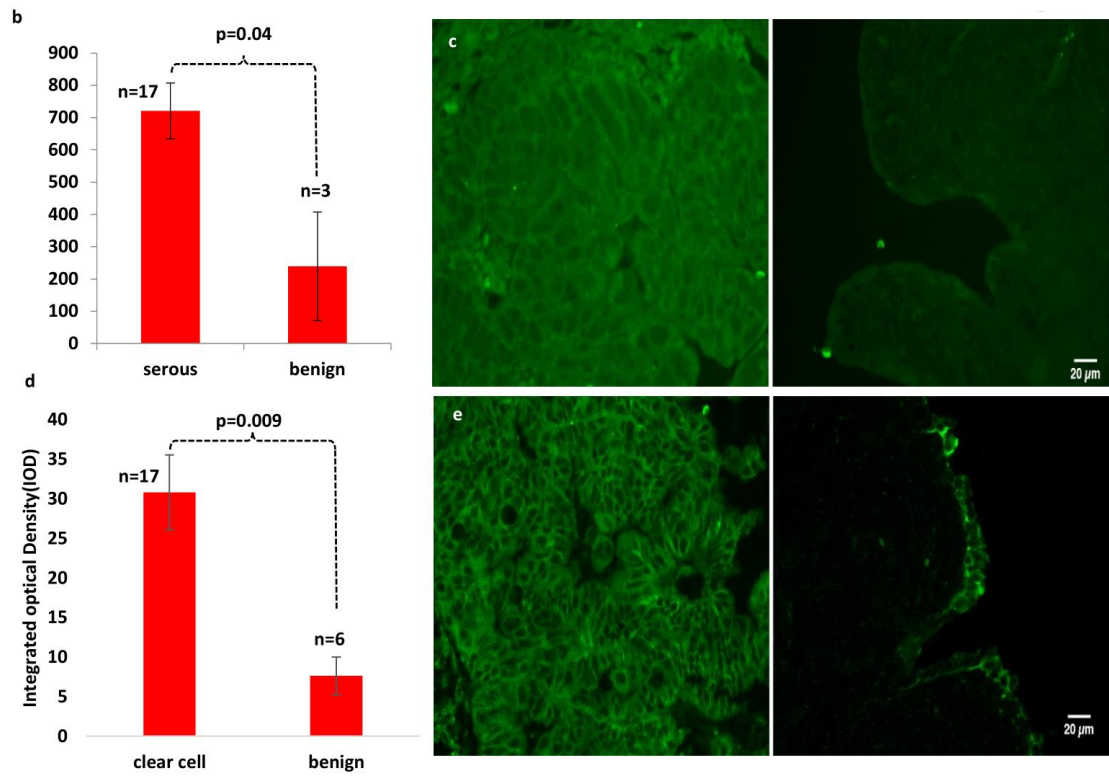


Figure IV.2 Stable septin-2 knockdown shows a decrease in proliferation

(A.) Gene expression levels of septin-2 in KO9 and KO11 were significantly decreased ($p < 0.01$) compared to WT and plasmid c control levels. (B.) Septin-2 is decreased in KO9 and KO11 at the protein level. (C.) Relative band density of (B.). (D.) Proliferation rates of KO9 and KO11 were significantly lower ($p < 0.02$) at both 72 and 96 hours compared to control WT and plasmid c.

Fig.IV.2

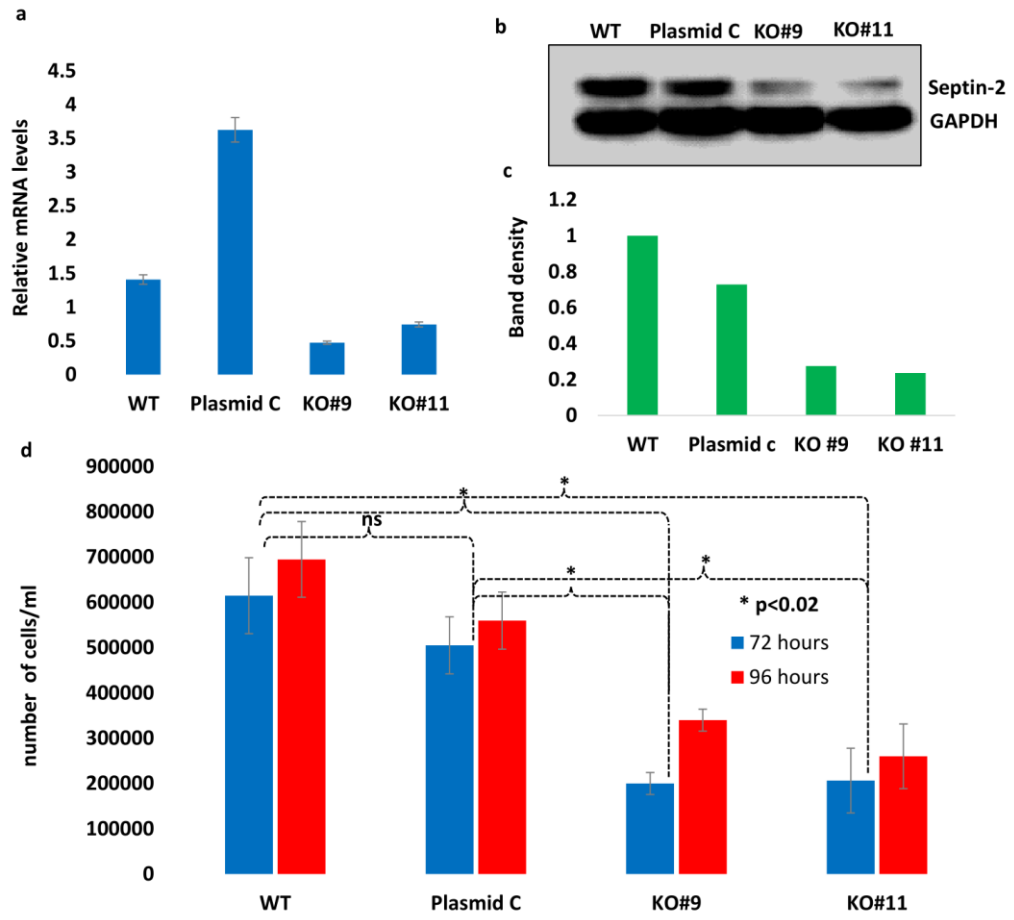


Figure IV.3 Principal Component Analysis of CO

WT, Plasmid C, and KO11 samples show clustering based on grouping. However, WT is more dispersed and shows overlap with Plasmid C. Visualization of principal component 1(PC1) versus principal component 2 (PC2).

Fig IV.3

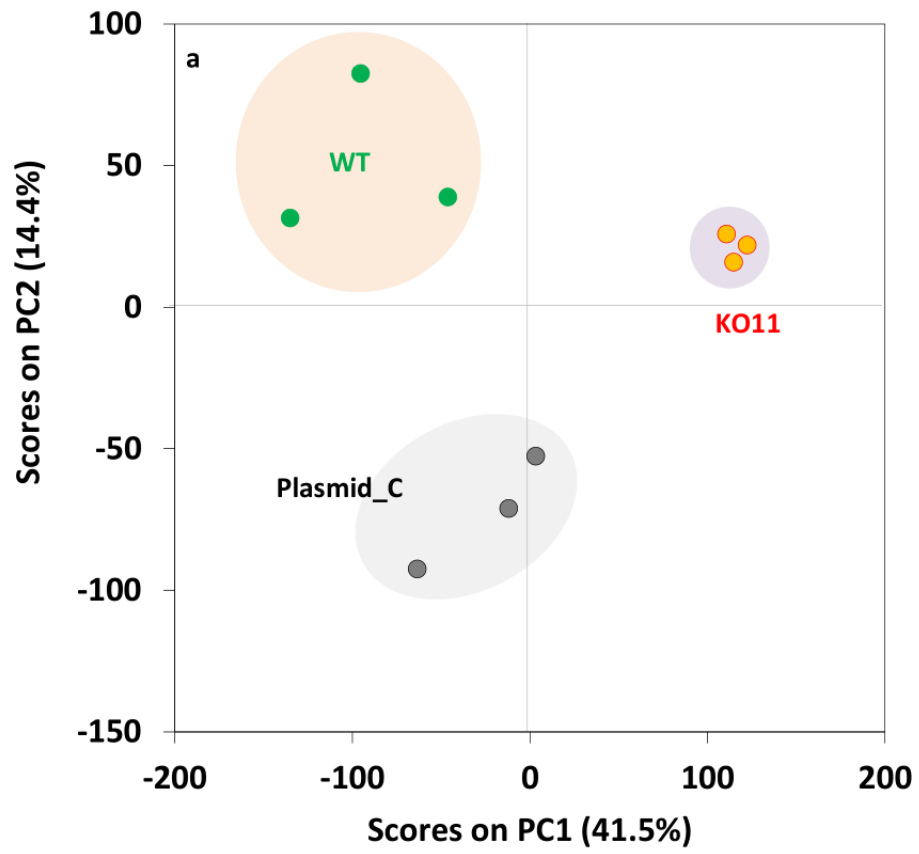


Figure IV.4 Volcano plot of fold change versus q-value of peak area for distinct peptides

Of the 19976 distinct peptides (3565 proteins) identified, (A) only one peptide/protein (0%, red in inset pie chart) in Plasmid C and (B) 5.0% peptides in KO11 showed large difference (absolute fold change more than 1, and $q < 0.05$) against WT. Nearly 93.5% peptides showed lower peak-area (down regulation) in KO11. (C) represents the examples of peak-area/expression levels in replicates for four peptides are shown: 1. Galectin-3-binding protein (LGALS3BP, K7EKQ5), 2. Transketolase (TKT, P29401), 3. Poly(A) binding protein 4 (PABPC4, Q4VC03), 4. Enolase 1 (ENO1, P06733). The peptide sequence, KO11/WT peak-area ratio and respective q-values are listed for each protein.

Fig.IV.4

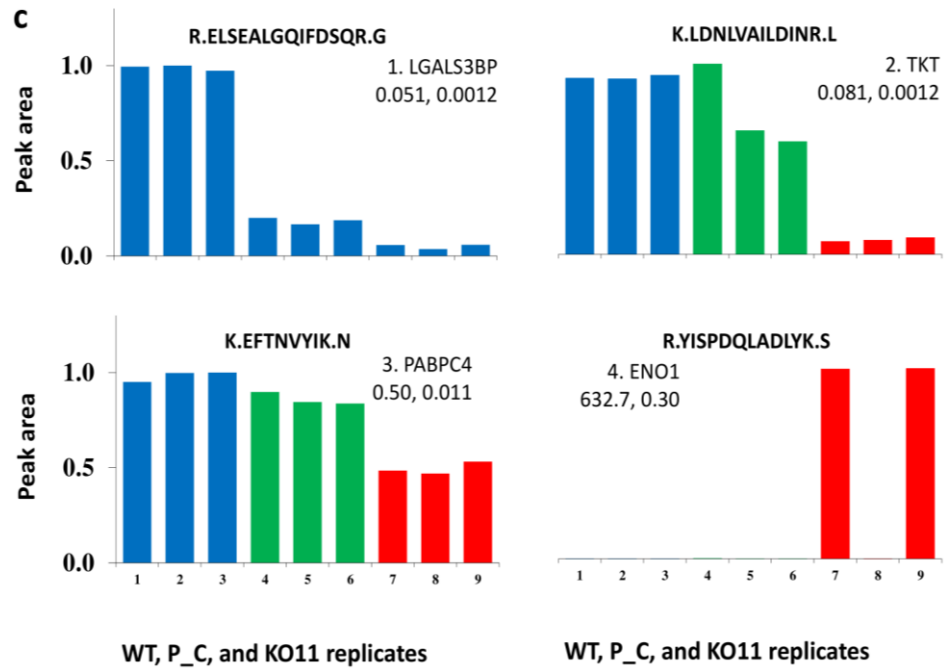
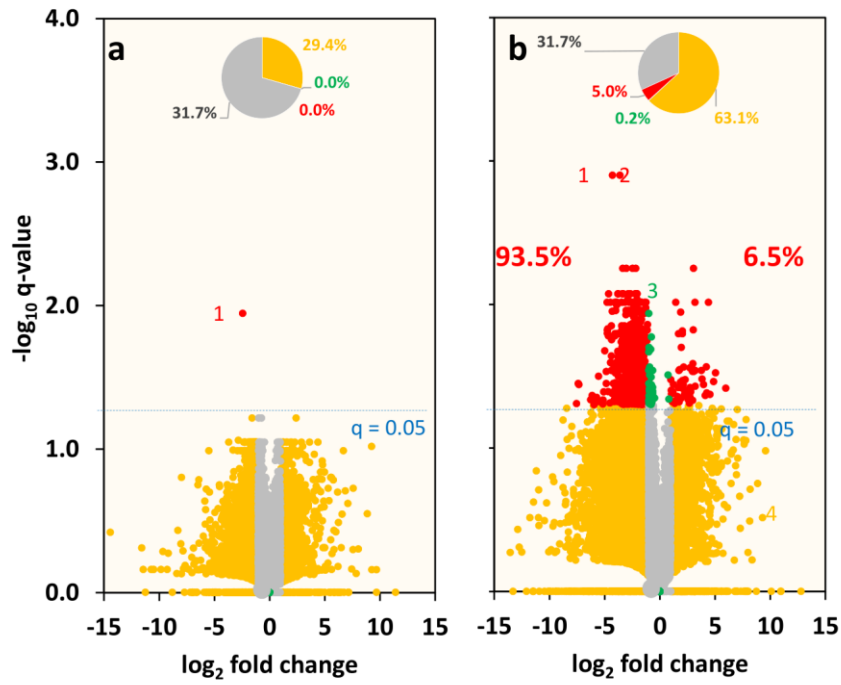


Figure IV.5 Hierarchical clustering and heat map of differentially expressed proteins and peptides

(A.) Clustering of the 231 differentially expressed proteins (B.) Peptides in most differentially expressed proteins, for example, Q9P2E9 (RRBP1, Ribosome binding protein 1) with 60 peptides, showing an overall similar pattern of peak-area quantitation.

Fig.IV.5

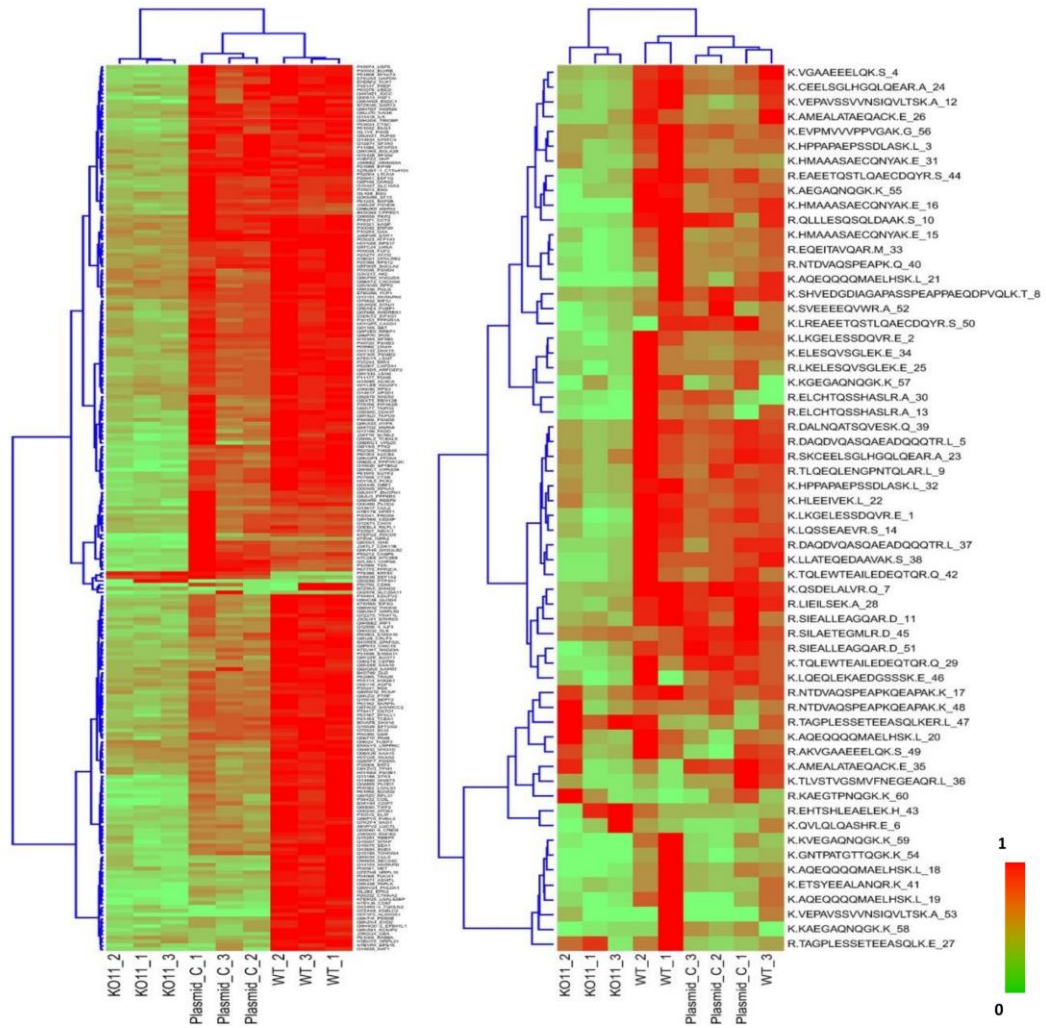


Figure IV.6 Gene ontology (GO) analysis

Gene ontology (GO) analysis using DAVID (<https://david.ncifcrf.gov/>). Proteins with differential expression (n = 231, $q < 0.05$, in KO11 versus WT) is compared with proteins (n = 3334) that showed no differential expression. Former showed enrichment for proteasomal/ubiquitin related GO terms ($q \ll 0.05$, Bonferroni) in biological process (BP) category. In cellular component (CC) and molecular function (MF) categories, differentially expressed proteins showed enrichment for ribonucleoprotein and RNA related terms. No enrichment was seen in molecular function category. Differentially expressed proteins showed enrichment for KEGG pathways relating to citrate cycle/energy and spliceosome.

Fig.IV.6

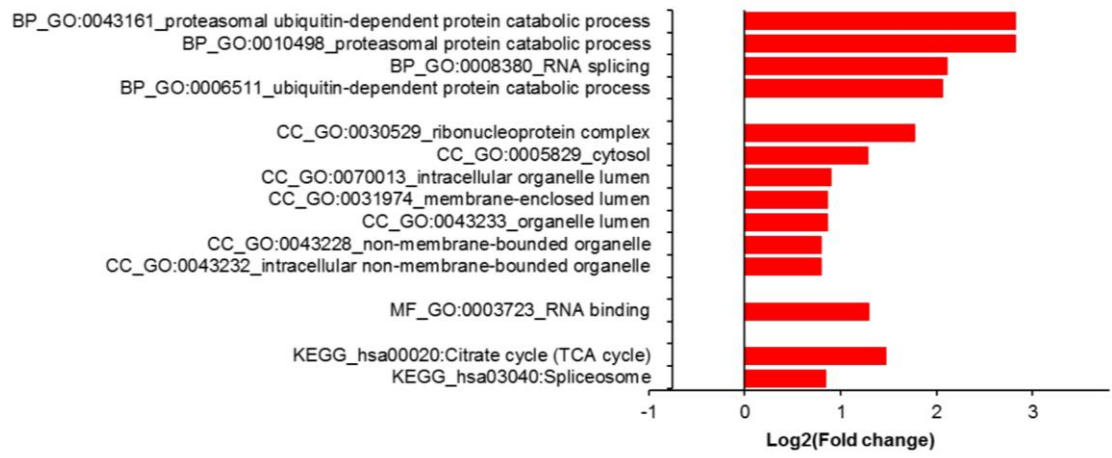
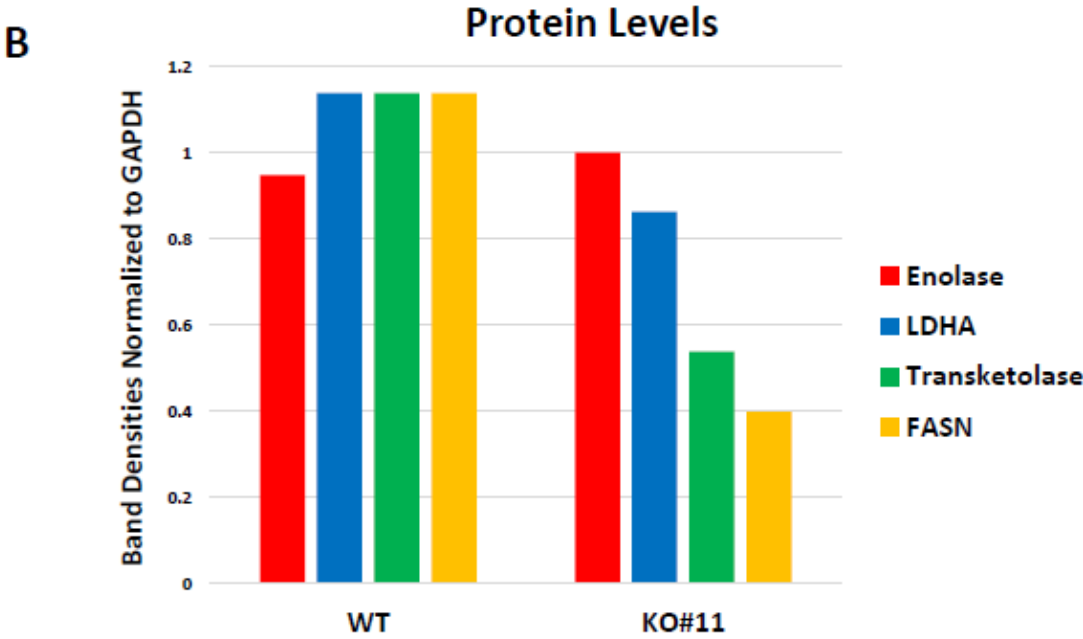
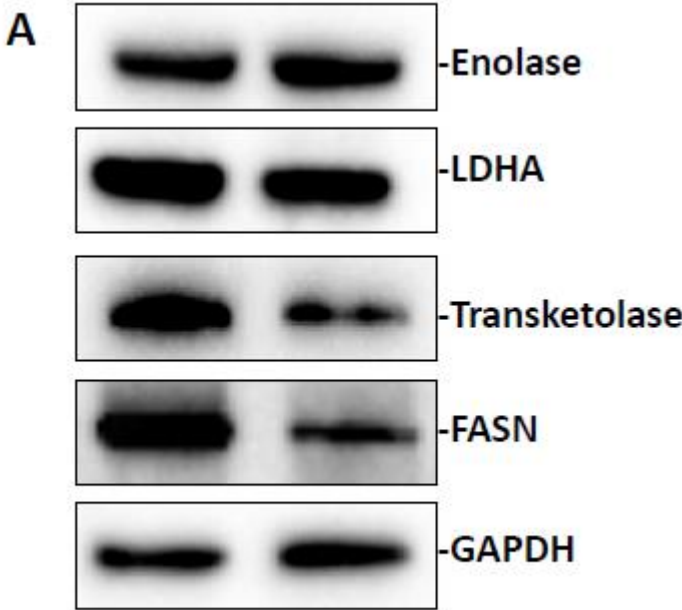


Figure IV.7 Verification of Enriched Proteins Identified by Proteome Analysis

(A.) Western Blot analysis of protein expression validated in both WT and KO11 (B.)

Relative Band Densities of proteins in (A.), normalized to GAPDH.

Fig.IV.7



CHAPTER 7

CONCLUSION

Epithelial ovarian cancer (EOC) is such a deadly disease largely owing to the two major challenges of diagnosis and treatment. Ovarian cancer is detected at a late stage when tumor cells have already detached and metastasized directly into the peritoneal cavity, making it challenging for all lesions to be removed surgically [1]. Therefore, extensive disease remains in the body even after surgery. While treatment has evolved to include PARP inhibitors and anti-angiogenic therapies, prognosis remains poor. Immunotherapies for the treatment of cancer have recently garnered much attention, as it has been observed that the number of intratumoral T-cell numbers correlate to a better clinical outcome [2]. However, establishing a breakthrough immune target for ovarian cancer has been met with challenges, as the response rate remains low [3]. Therefore, a critical need for novel therapies for EOC still exists.

HE4 plays a unique role in EOC as it has been implicated in both diagnosis and prognosis of the disease. As a clinical biomarker, HE4 represents a promising early detection method. Compared to the more established biomarker CA-125, it is less frequently elevated in benign disease and is potentially able to identify patients that are at high risk for primary platinum resistance [4]. While much is known about HE4 clinically, far less is known about its biological functions in EOC. The goal of this investigation was to determine the mechanisms in which HE4 drives ovarian pathogenesis, and to ultimately provide evidence as to whether HE4 should be recommended as a therapeutic target for EOC.

As HE4 was initially suggested to have a potential role in innate immunity, [5] these studies aimed to better understand HE4's function in tumor immunity. For the first time, this investigation has shown that HE4 is involved in promoting ovarian tumor immune evasion, through influencing expression of two proteins, osteopontin (OPN) and dual specificity phosphatase 6 (DUSP6). Subtractive hybridization revealed that when peripheral blood mononuclear cells (PBMCs) were treated with recombinant HE4, OPN was the most downregulated protein, and DUSP6 was the most upregulated.

OPN is a secreted glycoprotein that has been identified as having important T helper 1 (Th1) cytokine functions. [6] Specifically, it was discovered that HE4 suppresses OPN in CD3⁺ T cells, while also impairing the secretion of IL-12 and IFN- γ , two important cytokines downstream of OPN that promote T-cell survival [6,7]. Furthermore, when ovarian cancer cells were cultured with media from PBMCs cultured with recombinant HE4, those cells were less susceptible to cell death, which was reversed upon silencing of HE4. Also, in human EOC patient tissue, serum HE4 levels inversely correlated to the number of OPN positive T cells in patient tumors.

The second objective in defining HE4's role in tumor immunity was to delineate the effect of HE4's upregulation of DUSP6. DUSP6 is an extracellular signal-regulated kinase (ERK) phosphatase that has been found to regulate CD4⁺ T cell activation and differentiation through the inhibition of T-cell receptor (TCR) dependent ERK activation [8]. Interestingly, upon testing HE4's upregulation of DUSP6 in specific subsets of cells within PBMCs, the upregulation was found to be restricted to CD8⁺

T-cells and CD56+ natural killer (NK) cells, and not CD4+ T cells. It was also discovered that HE4 promotes ERK 1/2 phosphorylation in these cell populations. Upon co-culture of PMBCs with ovarian tumor cells it was found that adding recombinant HE4 enhanced cell proliferation. However, this effect was attenuated by the addition of an allosteric DUSP6 inhibitor (BCI). PBMCs devoid of CD8+/CD56+ cells did not produce the same result, proving that CD8+ and CD56+ populations were solely responsible for the observed effects. This result was particularly interesting in light of HE4's hypothesized role in innate immunity, since NK cells, as part of the innate immune response, have been found to play an important role in helping tumor cells escape immune surveillance [9].

These two studies indicate that through targeting of HE4, it may be possible to restore a normal tumor immune response. To confirm this, future directions include testing the inhibition of HE4 via a neutralizing antibody in an immune competent mouse model to see how this affects tumor burden. In addition, testing HE4 inhibition in vivo in combination with platinum-based chemotherapeutics and immune checkpoint inhibitors to determine synergistic effects is important. Results from these studies will be valuable, as many successful EOC regimens are combination therapies that produce higher response rates and lower resistance rates compared with monotherapies [10].

Before HE4 can truly be recommended as a novel therapy that can remedy tumor immune evasion, results from these in vivo experiments should be obtained.

The study of DUSP6 and HE4 in immune cells lead to an additional investigation that examined DUSP6's role in epithelial ovarian cells. This was of particular interest since DUSP6 has not been well defined in cancer, and it has been published that HE4

interacts with the ERK signaling pathway in EOC [11–13]. This study confirmed that DUSP6 functions similarly to HE4 in EOC pathogenesis, as the inhibition of both factors promotes apoptosis in EOC cells. Furthermore, DUSP6 is overexpressed in serous EOC patient tissue and intratumoral levels of HE4 and DUSP6 correlate. Since it has been published that HE4 promotes chemoresistance in EOC [14], the effect of DUSP6 on platinum response was also evaluated. When DUSP6 was inhibited with BCI in combination with carboplatin and paclitaxel, it produced a synergistic response over single-agent chemotherapeutic. To assess downstream effects of this inhibition, it was shown that BCI altered genomic levels of the ERK related response genes early growth response protein 1 (EGR1), a strong promoter of apoptosis and proto-oncogene c-Jun [15]. EGR1 was upregulated in cells co-treated with BCI and either paclitaxel or carboplatin, compared to a single-agent treatment, while c-Jun expression was decreased upon co-treatment. This study was able to define a new role for DUSP6 within EOC, indicating that targeting this factor is important both to restore proper tumor immune function and to overcome chemoresistance in EOC cells.

Moreover, as HE4 has the ability to be detected in patient serum, it would be interesting to determine if DUSP6 could also be detected in patient blood. Additional future directions include establishing stable DUSP6 knockdown and overexpressing clones to test cancer related phenotypes. Furthermore, as HE4 overexpressing and stable knockout cell lines have been previously established, global genomic arrays could then be performed to establish similarities and differences between the overexpression and knockout populations of each factor.

Finally, the last part of this thesis sought to characterize the novel protein septin-2 in EOC. Septin-2 is a member of the septins protein family, which comprises 13 GTP binding proteins that play important roles in various cellular processes including cytokinesis [16,17]. Septin-2 was identified in a small proteomics study as most enriched with HE4 immunoprecipitation in HE4 overexpressing cells versus null vector cells. For the first time, this study revealed that septin-2 is overexpressed in both serous and clear cell EOC patient tissue. Establishment of stable knockout clones in an ovarian cell lines showed that proliferation was drastically decreased in septin-2 knockout clones. Global proteome analysis was employed to determine the relevant pathways in which septin-2 is involved with in EOC, revealing that down regulation of septin-2 produced differential expression of major metabolic and cellular energy pathways.

As this was a pilot study with the simple goal of defining septin-2 in EOC, more research needs to be completed in order to understand its mechanistic role in ovarian tumorigenesis. Future directions involve an *in vivo* study to determine if septin-2 knockout lead to a decrease in tumor growth, alike to the reduction of cell proliferation observed *in vitro*. Furthermore, it will also be important to elucidate the mechanistic relationship between septin-2 and HE4, in addition to determining how septin-2 and HE4 interact with metabolic and cellular energy pathways. This is an especially original finding as both proteins have not been previously found to interact with cellular metabolism and may lead to new novel therapeutic targets for EOC.

As a reputable clinical biomarker, HE4 is valuable in the diagnosis and prognosis of EOC; however, knowledge of its role in treatment of EOC is deficient in comparison.

Overall, this thesis compilation improves the understanding of HE4's diverse biological function in EOC, through highlighting its role in the promotion of tumor immune dysfunction and characterizing novel interacting proteins. As there is a dire need for innovative targeted therapies for EOC patients, this thesis presents new evidence that inhibiting HE4 represents promise not only in downregulating molecular mechanisms that promote tumorigenesis, but also in restoration of normal tumor immune function. Furthermore, global genomic and proteomics analysis of differential HE4 levels revealed its relationship to novel factors that had not previously been characterized in EOC prior to this investigation. Taken as a whole, this dissertation offers original insights that emphasize the importance of HE4's role in the pathogenesis of EOC.

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