Angiostatic Regulators in Ovarian Cancer

by

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DEDICATION

This work is dedicated to the people in my life that have been diagnosed with cancer. Those that I have lost have given me the courage to live and those that have survived have given me the determination to fight.
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LIST OF ABBREVIATIONS

AS      Angiostatin
Bcl-2   B-cell lymphoma-2
bFGF    Basic Fibroblast Growth Factor
BRCA1   Breast Cancer Gene 1
BRCA2   Breast Cancer Gene 2
BSA     Bovine Serum Albumin
CA125   Cancer Antigen 125
CaCl₂   Calcium Chloride
cAMP    cyclic Adenosine Monophosphate
cDNA    complementary Deoxyribonucleic Acid
CpG     Cytosine-phosphate-Guanine
CREB    cAMP Response Element Binding Protein
CsCl₂   Cesium Chloride
CUB     Complement like
CuCl₂   Copper Chloride
Cy      Cystadenoma
DNA     Deoxyribonucleic Acid
EC      Endothelial Cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
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<tr>
<td>ES</td>
<td>Endostatin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FHIOSE</td>
<td>Familial History Immortalized Ovarian Surface Epithelium</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
<tr>
<td>FT</td>
<td>Fallopian Tube</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>Her2/neu</td>
<td>Human Epidermal Growth Factor 2/derived from Neuroblastoma</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia Inducible Factor 1</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>hTR</td>
<td>Human Telomerase RNA</td>
</tr>
<tr>
<td>IC</td>
<td>Inclusion Cyst</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IOSE</td>
<td>Immortalized Ovarian Surface Epithelium</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
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<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatidic Acid</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
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<tr>
<td>N</td>
<td>Normal</td>
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<td>NP</td>
<td>Neuropilin</td>
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<td>NP-1</td>
<td>Neuropilin-1</td>
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<tr>
<td>NP-2</td>
<td>Neuroplini-2</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian Surface Epithelium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Paraovarian Cyst</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Poorly Differentiated</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>PIGF-2</td>
<td>Placenta Growth Factor-2</td>
</tr>
<tr>
<td>PP</td>
<td>Primary Peritoneal</td>
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<tr>
<td>PSI</td>
<td>Plexin Semaphorin Integrin</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SEMA3</td>
<td>Class 3 Semaphorin</td>
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<tr>
<td>SEMA3F</td>
<td>Semaphorin 3F</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specific Protein 1</td>
</tr>
<tr>
<td>SV-40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline with Tween</td>
</tr>
<tr>
<td>TVS</td>
<td>Transvaginal Ultrasonography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>WD</td>
<td>Well Differentiated</td>
</tr>
<tr>
<td>ZEB-1</td>
<td>Zinc Finger E-box Binding Homeobox 1</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>Zinc Chloride</td>
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ABSTRACT

Angiogenesis by either normal or neoplastic cells involves a delicate balance of both angiogenic and angiostatic regulators. In the ovary, normal physiological angiogenesis occurs around the developing follicle and corpus luteum in response to hormonal shifts. Interestingly, carcinomas arising from the ovary are usually highly vascularized and are commonly clinically observed to produce cyst fluids or ascites which contain both angiostatic and/or angiogenic regulators. However, in contrast to normal angiogenesis, angiogenesis associated with epithelial ovarian cancer usually produces aberrant vasculature that may promote neoplastic progression. Therefore, the ovary and ovarian cancers provide models to study the mechanisms governing the strict balance of angioregulators in both normal and tumor angiogenesis. While most studies to date have focused on angiogenic regulators for normal and aberrant angiogenesis, we investigated the potential for dysregulation of angiostatic regulators to contribute to the etiology of epithelial ovarian cancer. Therefore, in this study, we examined two angiostatic regulators, angiostatin and semaphorin 3F, in epithelial ovarian cancer.

Angiostatin, a cleavage product of the circulating zymogen plasminogen, was isolated from serum and urine of mice bearing a Lewis lung carcinoma and
in vivo studies have demonstrated its potent angiostatic properties. Thus, we investigated the potential prognostic/diagnostic advantage of aberrant angiostatin expression with epithelial ovarian cancer. We found that urinary angiostatin, compared to other angioregulators in plasma or urine, could serve as an effective biomarker for early detection of epithelial ovarian cancer, especially when used in combination with cancer antigen 125. Additionally, urinary angiostatin correlated with both recurrent disease as well as successful tumor ablation further supporting its potential as a disease biomarker.

Alternative biological functions for the axon guidance molecule, semaphorin 3F, have been reported particularly in regard to angiogenesis, tumor progression and metastasis. However, the underlying mechanisms governing semaphorin 3F regulation and dysregulation remain unclear. Therefore, we first investigated the clinical relationship between semaphorin 3F expression and epithelial ovarian cancer progression. These immunohistological studies revealed that, similar to lung cancer, semaphorin 3F expression decreased with progression supporting a tumor suppressor-like role for semaphorin 3F. Additionally, we found that calcium, an essential cellular signaling molecule, could mediate transcriptional suppression of semaphorin 3F expression in a CREB-dependent manner.

Lastly, given the antagonistic relationship between semaphorin 3F and vascular endothelial growth factor, we sought to determine whether semaphorin 3F and vascular endothelial growth factor promoted opposing effects on a common downstream target. In the course of these studies we determined that
telomerase is a novel molecular target of semaphorin 3F in ovarian cancer cells such that semaphorin 3F suppresses telomerase activity while vascular endothelial growth factor promotes telomerase activity. In addition, we found that the inverse relationship between semaphorin 3F and telomerase was mediated through transcriptional inhibition of the hTERT promoter by semaphorin 3F.

In conclusion, this research shows that dysregulation of the angiostatic regulators, angiotatin and semaphorin 3F, may contribute to the etiology of epithelial ovarian cancer. In the future, dysregulation of these and other angiostatic regulators may be exploited for therapeutic intervention or as biomarkers for early detection which would allow women more treatment choices and hopefully, reduce the mortality associated with this insidious disease.
CHAPTER I

INTRODUCTION

Ovarian Cancer

Ovarian cancer is the most lethal gynecologic malignancy and is neither a common nor rare disease. The American Cancer Society estimates approximately 22,000 new cases of ovarian cancer in the United States in 2010 and over half, approximately 14,000, of these women will succumb to the disease (1). Over the past two decades advances in both cytoreductive surgery and combination chemotherapy have contributed to a modest increased overall 5-year survival. Furthermore, when cancer is confined to the ovaries, stage I, up to 90% of patients can be cured with currently available therapy. Unfortunately, due to a lack of early symptoms and no reliable screening method, ovarian cancer remains a “silent” killer, with 70% of women diagnosed at an advanced stage. Therefore, ovarian cancer represents a great clinical challenge in gynecologic oncology.
Epidemiology

The incidence of ovarian cancer is highest in the U.S. and Europe and lowest in developing countries (2). Although the gap is narrowing, in the U.S., there is a higher frequency of ovarian cancer among Caucasian women rather than African-American or Asian-American women (2). Ovarian cancer is more prevalent among perimenopausal and postmenopausal women, generally occurring after the age of 40 (3). The majority of ovarian cancers are sporadic, whereas the occurrence of hereditary ovarian cancer accounts for only a small proportion (5-10%) of total cases; however, family history of disease is the most significant risk factor for ovarian cancer. Advances in molecular genetics have identified specific germline mutations in the breast cancer 1 and 2 genes (BRCA1, BRCA2) and women who harbor these alterations carry an increased susceptibility to both ovarian and breast cancer (4). Additional risk factors include early menarche, late menopause, nulliparity, estrogen, infertility, fertility drugs, obesity, and use of talc as well as other environmental factors (3). Conversely, numerous studies have identified protective factors for risk of ovarian cancer. These include use of oral contraceptives, multi-parity, tubal ligation, and history of breastfeeding (5-7).

Histopathology

More than 90% of ovarian cancers have been traditionally thought to arise from the simple ovarian surface epithelium (OSE) which covers the ovary (Figure 1). This dynamic epithelium remains in a relatively uncommitted state with
mesenchymal features and a propensity to undergo epithelial-mesenchymal
transition (3, 8). As a result, in contrast to other epithelial malignancies, ovarian
cancers tend to be more differentiated than their tissue of origin and present as
morphological derivatives of coelomic epithelium of the fallopian tube, endocervix
and endometrium (8). The predominant form of epithelial ovarian cancer (EOC)
is denoted as serous which resembles tubal epithelium, followed by mucinous
and endometrioid which resemble endocervical and endometrial epithelium,
respectively (7). Less common epithelial histologic subtypes of ovarian cancer
include clear cell, Brenner, small cell, and undifferentiated carcinoma. Additional
non-epithelial types of ovarian tumors can develop from germ cells, responsible
for producing ova, and sex-cord stromal cells, which generate reproductive
hormones. Consequently, the variety of histological subtypes of ovarian cancer
contributes to the heterogeneity of this disease (9). Furthermore, specific
subtypes are associated with different degrees of aggressiveness, especially with
regards to clinical characteristics, survival, and genetic alterations. Interestingly,
a retrospective study by Hollingsworth et al. provided strong evidence of an
association between the degree of tumor vascularization and overall survival
(10).
**Figure 1. Ovarian surface epithelium.** The origin of ovarian cancer has been traditionally attributed to the OSE (arrow). OSE tends to have a simple cuboidal or low columnar morphology and is separated from the ovarian cortex by a distinct basement membrane. Hematoxylin and Eosin stain, Original Magnification, 166x with correction factor.
Pathogenesis

The etiology of ovarian cancer remains unclear, nevertheless, several hypotheses have been proposed for the pathogenesis of ovarian cancer. First, “incessant ovulation” postulated by Fathalla in 1971 suggests that repetitive OSE trauma and repair, in addition to exposure to an estrogen-rich follicular fluid, promotes the mitotic activity of OSE which increases the likelihood of genetic alterations that eventually lead to malignant transformation (11). Moreover, *in vitro* experiments by Nicosia *et al.* and Godwin *et al.* confirmed increased mitotic activity of rabbit OSE with chromosomal aberrations, proliferation or formation of preneoplastic lesions as a consequence of repetitive ovulation (12, 13). This hypothesis is further supported by the decreased risk for disease associated with multi-parity and use of oral contraceptives, emphasizing a correlation between the number of ovulatory cycles with ovarian cancer risk (14).

Second, the gonadotropin stimulation hypothesis, proposed by Stadel in 1975 and expanded by Cramer and Welch suggests that excessive exposure to the gonadotropins, particularly follicle stimulating hormone (FSH) and luteinizing hormone (LH), can directly and indirectly stimulate OSE to form inclusion cysts derived from crypts or invaginations in the epithelium (15, 16). FSH targets granulosa cells of the ovarian follicle, whereas LH targets theca, granulosa, and luteal cells (17). These hormones bind specific receptors on the surface of target cells and activate intracellular second messenger signaling (17). Likewise, *in vitro* studies demonstrate increased proliferation of OSE in response to both FSH and LH (18). Similar to the incessant ovulation hypothesis, this theory is further supported by the decreased risk for ovarian cancer associated with multi-parity
and use of oral contraceptives. In addition, this model also predicts increased risk associated with age, since levels of both gonadotropins, FSH and LH, are elevated in postmenopausal women (18).

Last, the Müllerian system hypothesis, which as an alternative to a coelomic cell of origin, attributes the source of ovarian cancers to tissues that are primary or secondary derivatives of the Müllerian system, such as the fimbriated end of the fallopian tube (7, 19, 20). Histological similarities among epithelia lining inclusion cysts, paraovarian cysts, and fallopian tube have been well documented, as have similarities among carcinomas arising from the ovary, fallopian tube, and peritoneum (19, 20). This hypothesis, then, would explain why epithelial ovarian neoplasms present as morphological derivatives of epithelia of the fallopian tube (serous adenocarcinoma), uterus (endometriod), and endocervix (mucinous adenocarcinoma) without requiring an intermediate metaplastic step (19).

**Molecular Alterations**

In general, different cell types are regulated by specific genes. Therefore, determining the impact of specific inherited or acquired genetic alterations may aid in the characterization of tumors and, thus, identification of their cell of origin. There are two classes of genes, oncogenes and tumor suppressor genes, which have been implicated in tumor pathogenesis. Oncogenes encode proteins that stimulate growth, whereas tumor suppressor genes encode proteins that inhibit proliferation of normal cells (21). In cancer, activation of proto-oncogenes occurs
in a single allele by mutation, over-expression, or translocation, so that oncogenes are referred to as dominant transforming genes. Conversely, tumor suppressor genes are inactivated in cancer and are considered recessive transforming genes because loss of both alleles or loss of heterozygosity (LOH) is required for neoplastic transformation (21).

Several tyrosine kinases have been identified as oncogenes, however overexpression and/or amplification of the epidermal growth factor (EGF) and human epidermal growth factor 2 derived from neuroblastoma (Her2/neu) receptors are most frequently observed in ovarian tumors and are associated with a very poor prognosis (22). These receptors target phosphatidylinositol-3-kinase (PI3K), an intracellular kinase, and result in aberrant autocrine/paracrine signaling (23).

Several lines of evidence suggest that, re-expression of homeobox (HOX) genes, which regulate differentiation of Müllerian derived cells during development, may also contribute to the differentiation of ovarian carcinomas (23). Furthermore, in vitro studies indicate differential expression of HOX 9, 10, and 11 in transformed mouse OSE results in serous, endometrioid and mucinous tumors, respectively (24).

The most frequent genetic aberration of a tumor suppressor gene occurs in the p53 gene. Normally, p53 acts as a transcriptional regulator and is often referred to as the guardian of the genome. Investigators have identified several p53 mutations, most of which occur in highly conserved regions of the gene and are of functional importance (25). Specifically in ovarian cancer, the presence of
putative precursor alterations in p53 staining or ‘p53 signatures’, accumulate in tubal epithelium of women at risk of developing ovarian carcinomas (women with BRCA mutations) and pre-invasive lesions (26). Moreover, the p53 signature characterizes high-grade serous histological subtypes (26). Although a preclinical stage has not been observed, identifying recurrent genetic mutations or ‘signatures’ warrants further investigation. Independent of its cell of origin the pathogenesis of EOC remains complicated and not well understood.

Detection

Eradication of this “silent” disease depends, in part, on an effective early detection method. A major prognostic factor is tumor stage at diagnosis and since most stage I ovarian cancers can be cured with conventional treatment, detection at a preclinical or early stage would have an impact on overall survival. An effective screening strategy requires a high sensitivity (true positive rate or probability that a subject with cancer will have a positive result), >75%, but even higher specificity (1-false positive rate or probability that a subject without cancer will show a negative result), >99.6%, in order to achieve a positive predictive value of 10% and avoid unnecessary surgery (27). Current screening strategies have centered on transvaginal ultrasonography (TVS) and tumor markers or a combination of these two approaches. Unfortunately, these methods alone or in combination do not satisfy the aforementioned criteria.

Although, TVS can provide precise imaging of the ovaries, in practice it is incapable of distinguishing small cancerous lesions from benign masses (28). In
addition, three major trials conducted in the US, UK, and Japan revealed limitations in both sensitivity and specificity (29-31) and have raised concerns about cost-effectiveness.

Research of potential biomarkers has largely focused on the serum tumor marker cancer antigen 125 (CA125), a large surface glycoprotein thought to play a role in epithelial cell attachment. At present, CA125 is the gold standard for ovarian cancer detection; however, it has limitations in sensitivity because only about 50% of women with stage I have an elevated CA125 level. Furthermore, CA125 lacks specificity, especially in premenopausal women, where many other conditions of the genital tract can produce an elevated CA125 level, for instance endometriosis. Specificity has been improved when CA125 is monitored over time or paired with TVS (27, 32) but still does meet the stringent criteria for an effective screening strategy.

Several emerging biomarkers currently under investigation for early detection of ovarian cancer appear promising. Of particular interest due to its role in regulation of the proangiogenic cytokine, vascular endothelial growth factor (VEGF), is the naturally occurring phospholipid, lysophosphatidic acid (LPA). Independent reports by Xu et al. and Sutphen et al. have found elevated LPA serum levels in 90% of stage I patients and elevated LPA levels in preoperative samples compared to healthy controls, respectively (33, 34). Furthermore, recent reports from Anderson et al. and Badgwell et al. demonstrate significantly elevated urinary levels of B-cell lymphoma-2 (Bcl-2) and mesothelin, respectively, in addition to complementarity with CA125 (35, 36).
Further validation of these emerging biomarkers in combination with other markers is required and could provide a convenient, non-invasive and cost-effective strategy for early detection of ovarian cancer.

**Angiogenesis**

Angiogenesis is the formation of new blood vessels from pre-existing vasculature (37). This process is strictly regulated by angioregulators, which are defined as endogenous factors that are angiogenic and promote angiogenesis, or angiostatic and inhibit angiogenesis.

Embryonic development, growth and maintenance of cells and tissues are dependent on a vascular supply (38). Although most vasculature in the adult is quiescent, physiological angiogenesis occurs during wound healing and prominently in the female reproductive system, pertinent to this work most notably taking place in the ovary (39). Dysfunctional or uncontrolled angiogenesis leads to several pathological conditions such as chronic inflammation (40), immunological diseases (41), and cancer (42). Elucidating which factors positively or negatively regulate this process has been a great challenge. However, ongoing research continues to unravel the intricate mechanisms involved in the tight regulation of pro- and anti-angiogenic signals.

Angiogenesis can be triggered by several factors including metabolic stress, especially under hypoxic conditions, as well as, mechanical forces like shear stress induced by blood flow, and genetic mutations such as, activation of
oncogenes, like K-ras. Regardless of the cause, angiogenesis involves a multi-step and orderly process and initiated by the expression of angiogenic growth factors by tissues (37, 43). The release of pro-angiogenic factors activates receptors of the endothelium so that and endothelial cells (ECs) begin to degrade their basement membrane (44, 45). Once the ECs penetrate the basement membrane they migrate towards the angiogenic stimulus (46) and endothelial sprouting is initiated. Sprout extension continues until individual sprouts join or anastomose and align with other sprouts or capillaries which results in tube formation (47). This process continues until the angiogenic factors are down-regulated or are counterbalanced by angiostatic factors.

Although, regulation of angiogenesis is reliant on a delicate balance of angioregulators, most research, to date, has focused on positive regulators. VEGF has emerged as the most prominent angiogenic regulator and the VEGF pathway appears highly conserved among different species (48, 49). Other pro-angiogenic factors include basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), angiopoietins, and the transcription factor hypoxia-inducible factor-1 (HIF-1). Conversely, studies aimed at identifying novel angiostatic molecules and elucidating the role of these endogenous inhibitors of angiogenesis are ongoing. However, in general angiostatic regulators associate with the extracellular matrix and suppress angiogenesis by exerting inhibitory effects on EC migration or by stimulating apoptosis (50).
Ovarian Angiogenesis

During the ovarian cycle angiogenesis occurs around the growing follicle and developing corpus luteum which is self-limited or transient, and ceases once the cycle is complete (37). In addition, studies have demonstrated hormonal shifts contribute to alterations of the ovarian vasculature (51). Consequently, several reproductive disorders are associated with dysregulated angiogenesis and vascular regression, for example, endometriosis, polycystic ovary syndrome, and cancer. Similar to embryonic angiogenesis, in the ovary angiogenesis is driven by growth factors and cytokines and mitigated by inhibitors of angiogenesis (52). Therefore, the ovary provides a physiological model to study both pro- and anti-angiogenic mechanisms.

During follicular maturation, primary and early secondary follicles are surrounded by a single layer of capillaries. Once changes in the oocyte occur and follicular cells proliferate and become more cuboidal, blood vessels begin to appear in late secondary follicles. Ferrara et al. have established a critical role for the pro-angiogenic factor, VEGF, and in situ hybridization reveals high levels of VEGF expression are maintained as follicles develop (53, 54). As a consequence, human follicular fluid is angiogenic (55). Furthermore, in vivo studies neutralizing VEGF directly or via receptor inhibition showed marked inhibition of follicular development and a decrease in both endothelial cell proliferation and vascular area (55-57).

The basement membrane keeps the blood vessels restricted to the theca layer and prevents invasion of the avascular granulose layer (58). Just prior to
ovulation there is a surge in LH, which results in vasodilation of capillaries in addition to an increase in vascular permeability, tissue edema, and ischemia (59). At ovulation, the breakdown of the basement membrane occurs in association with intense angiogenesis, concurrently the blood vessels invade the resulting corpus luteum. More than 75% of cells are of vascular origin in the mature corpus luteum (60) and it receives one of the highest blood supplies per gram of tissue than any other organ (61). Interestingly, the corpus luteum demonstrates angiogenic activity when transplanted into the hamster cheek pouch and rabbit cornea (39, 62, 63)

**Tumor Angiogenesis**

Folkman first hypothesized that tumor growth is dependent on angiogenesis (64). Indeed, tumors cannot grow more than 1-2 mm in size unless they recruit their own blood supply. Like normal physiological angiogenesis, to satisfy this requirement, neoplastic cells produce angiogenic factors, namely VEGF, which stimulate formation of new blood vessels from the endothelium of the pre-existing vasculature. However, in contrast to the angiogenesis that takes place in the ovary, once tumor angiogenesis is initiated it continues indefinitely and only ceases when the tumor is completely ablated or the host dies (37). Folkman and colleagues aptly designated this time point the “angiogenic switch” which indicates an imbalance of angioregulators in favor of angiogenic factors and, thus, initiation of tumor angiogenesis. Using in vivo model system Gullino et al. have demonstrated that angiogenic activity occurs prior to neoplastic
transformation (65), even in the absence of morphological changes (66, 67).
Therefore, the angiogenic switch, serves as a control point for most solid tumors,
including highly vascularized ovarian neoplasms.

Although, tumor angiogenesis parallels normal physiological
angiogenesis, distinct differences in the intrinsic vasculature morphology and
functionality are evident. For instance, certain tumors demonstrate structural
alterations in their capillary networks such as fenestrations in ECs and blind
ends, in addition to occasional interruptions in the basement membrane (68, 69)
and extensive tortuosity reflective of vascular compression. Furthermore, the so-called leaky vessels of tumor vasculature appear due to constant exposure to
VEGF which increases in vascular permeability. Distorted tumor vasculature is a
direct result of dysregulated angiogenesis and is compounded by a continual
outgrowth of the blood supply. Alternative to traditional angiogenesis,
vasculogenic mimicry suggests that in addition to ECs, pluripotent embryonic-like
and highly aggressive tumor cells contribute to neovascularization in tumors (70).

Ovarian cancers have a propensity to be highly vascularized and often
metastasize to the peritoneal lining. Additionally once these tiny implants
become vascularized, ascites accumulates in the abdomen, a clinical observation
of progression (71). Nicosia and colleagues have reported elevated levels of the
angiogenic regulators, VEGF and HGF, in patients with benign ovarian cysts or
functional cysts and patients with malignant tumors (72). Moreover, in contrast to
ascites, ovarian cyst fluid contains VEGF and demonstrates angiogenic
properties (72). Counter-intuitively, angiostatin (AS) (see below) and other anti-
angiogenic plasminogen cleavage products are also present in malignant ascites fluid and contribute to its net angiostatic properties (73). Therefore, since ovarian cancers are associated with the production of ascites and cyst fluids which contain positive and/or negative angioregulators, evaluation of angioregulators in bodily fluids may be clinically relevant for ovarian cancer.

**Angiostatin**

O'Reilly et al. in Folkman’s laboratory observed rapid angiogenesis and growth of residual tumors following surgical removal of primary tumors. Therefore, they postulated that although a primary tumor can stimulate angiogenesis locally, it is capable of inhibiting a secondary tumor at a distant site (74). Inhibition of the distant metastasis was hypothesized to be a consequence of an unbalanced production of both positive and negative angioregulators by the primary tumor, where angiogenic regulators could promote angiogenesis of the primary tumor and angiostatic regulators could suppress metastatic growth. In an effort to elucidate the phenomenon of inhibition of tumor growth by tumor mass, O'Reilly discovered the first naturally occurring angiostatic regulator, AS. Interestingly, AS was purified from the serum and urine of mice bearing a Lewis lung carcinoma and supports the concept of tumor dormancy, whereby, AS generated by the primary lung tumor diffused into circulation and inhibited a distant metastatic growth (74).

AS, is a 38 kDa internal cleavage product of the circulating zymogen, plasminogen (Figure 2) (74). Paradoxically, AS is generated via proteolytic
cleavage by proteinases which are activated in response to an angiogenic signaling cascade. Production of AS, then, illustrates the obligatory coupling of angiogenic and angiostatic regulators as a consequence of normal physiological angiogenesis. AS was originally described to contain the first four kringle domains of plasminogen. However, several studies have confirmed that a variety of proteases are able to cleave plasminogen creating different isoforms of AS with markedly different anti-angiogenic activity based on the presence of specific kringle domains (75). For instance, kringle 5 appears to possess more potent angiostatic activity than other kringle domains, so that AS isoforms containing kringle 5 are more effective inhibitors (76, 77). Functionally, AS inhibits migration and proliferation of ECs, most likely through its cell surface receptor ATP synthase (78). Consequently, delineating the molecular mechanisms involved in production, regulation, and dysregulation of AS may be clinically useful for therapeutic intervention.
Figure 2. Schematic of angiostatin structure and generation by proteolytic cleavage of plasmin and plasminogen. The asterisks indicate where plasminogen activators (urokinase and tissue plasminogen activator) cleave the zymogen to yield plasmin, an active fibrinolytic serine proteinase. Plasmin undergoes autoproteolysis in the presence of a free sulfhydryl donor to yield angiostatin as described by O’Reilly et al. (74).
Axon Guidance and Angiogenesis

The fundamental principles of blood vessel and nerve fiber growth involve sprouting, migration, and proliferation of ECs and axons, respectively in response to concentration gradients. These networks develop in response to common attractive and repulsive guidance cues, such as semaphorins which bind to cellular receptors to facilitate regulation. Consequently, specialized ECs and axons, identified as tip cells and growth cones, respectively, undergo cytoskeletal rearrangement and extend filopodia to become motile and invasive (79, 80). Intriguingly, many genes thought to be specific to neurons also play a role in angiogenesis, suggesting a developmental similarity between nervous tissue and vasculature. This is further supported by the observation that blood vessels and nerve fibers often align in parallel in order to provide oxygen and nutrients to the peripheral nervous system and arterial innervation (79, 81). Therefore, further investigation of these multifaceted cues may identify a novel angiostatic regulatory mechanism.

Semaphorins

Semaphorins are a large family of cell associated proteins. Although, initially identified to be involved in axon guidance and growth cone collapse, semaphorins have been found to be widely expressed outside the nervous system. There are eight classes of semaphorin genes and more than 30
members, which are implicated in several biological functions including cell adhesion, migration, and angiogenesis (82).

Semaphorins are divided based on structure: classes 1 and 2 consist of invertebrate semaphorins, whereas classes 3 to 7 comprise vertebrate semaphorins and class V consists of semaphorins encoded by viral genomes. All semaphorins are characterized by a conserved 500 amino acid, cysteine-rich, extracellular ‘sema’ domain, which mediates binding specificity and is necessary for signaling (82). The sema domain has a seven-blade β-propeller motif and structural similarity to α-integrins (83). Additionally, semaphorins contain a putative cysteine-rich protein binding domain known as a plexin-semaphorin-integrin (PSI) domain located adjacent to the sema domain (84). Class 3 semaphorins are unique in that they are the only secreted family members and are further distinguished by the presence of a basic C-terminal domain which is required for receptor binding (Figure 3). Class 4-7 semaphorins are anchored to the membrane and distinguished by structural features that include immunoglobulin-like domains, thrombospondin repeats (class 5), or a glycophasphatidylinositol (GPI) anchor (class 7).

**Receptors**

There are two classes of high-affinity receptors for semaphorins, plexins and neuropilins (NPs) (85-87). In humans, plexins are divided into four subfamilies (A,B, C, D) and expression is ubiquitous, whereas invertebrates only have two plexin genes which are more exclusively expressed in the nervous
tissue (86). Like semaphorins, plexins contain a ‘sema’ domain in the extracellular moiety in addition to 3-4 PSI domains (Figure 3). Although, the putative cytoplasmic domain of plexins lacks endogenous tyrosine kinase activity, this segment has demonstrated a weak intrinsic GTPase-activating protein (GAP) which facilitates R-Ras inactivation (88). Most class 4-7 semaphorins directly bind to plexins and activate plexin-mediated signal transduction. In contrast to other types of semaphorins, class 3 semaphorins (SEMA3s) are unable to directly bind plexins and, therefore, utilize the NPs, NP-1 and NP-2, as co-receptors, with the exception of SEMA3E.

NPs are only expressed in vertebrates and are single-span transmembrane glycoproteins characterized by two extracellular complement-like (CUB) domains (designated a1/a2 domains) as well as, two FV/FVIII coagulation factor-like domain (designated b1/b2 domains) and a meprin-like MAM domain (designated as c domain) (Figure 3) (85, 86, 89). NPs have a relatively short cytoplasmic domain and no signaling consensus sequence has been identified. Interestingly, although the NPs do not interact with membrane-bound semaphorins, they serve as co-receptors for VEGF family members where signal transduction is facilitated via activation of tyrosine kinase receptors, vascular endothelial growth factor receptor 2 (VEGFR2) and vascular endothelial growth factor receptor 3 (VEGFR3) (89-92). Additionally, NPs also interact with other heparin-dependent ligands such as, bFGF, placenta growth factor-2 (PIGF-2) and hepatocyte growth factor (HGF) (93). However, the interaction between
VEGF family members and NPs suggests a potential role in vascular and tumor biology for NPs in addition to their SEMA3 ligands.

In contrast to most biological signaling pathways which are unidirectional, semaphorin signaling is bidirectional, occurring in an autocrine or paracrine manner. These guidance cues operate in a mode similar to that of a traffic sign, harboring the ability to provide two alternative signals, specifically inhibition or induction of cell motility. The underlying mechanisms driving semaphorin signaling are unclear, studies suggest that this dynamic signaling is dependent upon the oligomerization of specific receptors and distinct downstream molecular pathways (94). Although counter-intuitive, this bi-directional semaphorin signaling is similar to the action of angiogenic and angiostatic regulators governing angiogenesis. Consequently, bidirectional semaphorin signaling and/or its dysregulation have implications for cell motility and invasion, and especially as that pertain to tumor angiogenesis and may promote tumor progression.

**Class 3 semaphorins**

In recent years, the putative role of SEMA3 signaling has expanded beyond the nervous system. SEMA3s are approximately 100 kDa and consist of seven soluble proteins designated SEMA3A-G (95). As secreted proteins, SEMA3s specifically target cells expressing NPs, most notably neurons, ECs, epithelial cells (like OSE) and tumor cells. As a result, SEMA3s involved in axon guidance and angiogenic VEGF family members share NP receptors. There is a
high degree of specificity in binding between the NPs and their ligands. NP-1 has a higher affinity for SEMA3A, in addition to VEGF$_{165}$, PIGF-2, HGF, whereas NP-2 has a higher affinity for SEMA3F, SEMA3G, and VEGF$_{145}$. However, SEMA3B, SEMA3C, VEGF$_{165}$, and VEGF$_{121}$ can bind either NP receptor (85, 86, 89, 91, 93, 96-101). SEMA3E is the only SEMA3 that does not bind NP receptors, however, it does directly bind to Plexin D1 (102).

Consequently, inter-relationships between axon guidance SEMA3s and angiogenic VEGF with NP, VEGFR, and plexin receptors potentially regulate a wide range of signaling pathways involved in cell adhesion, migration, tube formation, sprouting, permeability, angiogenesis, and metastasis. For instance, SEMA3A initially identified to repel axon movement also inhibits EC and tumor cell motility (103, 104). Clearly, investigating the mechanisms governing the molecular cross-talk between the variety of cellular mechanisms influenced by SEMA3 signaling has potential prognostic and therapeutic implications.
Figure 3. Schematic representation of class 3 semaphorins and receptors, neuropilins and plexins, structure. SEMA3s function in a paracrine manner which is mediated through specific binding of SEMA3s to NP receptors on the surface of target cells followed by complexing with another transmembrane receptor family, known as Plexins, and subsequent activation of intracellular signaling pathways.
Semaphorin 3F

Semaphorins have been studied extensively in vertebrates, and was initially identified to play an important role in brain development as a potent chemo repellant to axonal extensions and neuronal migration (105). Current research has established additional biological functions for SEMA3F outside the nervous system, most notably in regard to angiogenesis, as well as tumor progression and metastasis. In fact, the SEMA3F gene was originally isolated from 3p21.3, a region known to be deleted in lung, breast, and ovarian cancers (106, 107) (remove 106 and replace with 1996 Roche Oncogene #158). Exogenous SEMA3F expression in tumor cells resulted in reduced tumor formation in nude mice thereby implicating SEMA3F as a tumor suppressor (108).

Since SEMA3F and VEGF share a common receptor, several studies have investigated the antagonistic relationship between SEMA3F and VEGF which is attributed, in part, to overlapping ligand-binding regions in the b1/b2 extracellular domains of both NPs (Figure 3). In a lung cancer cell line, Roche et al. have suggested, in addition to competition for binding, that an alternative mechanism driving the angiostatic activity of SEMA3F is down regulation of VEGF mRNA via inhibition of HIF-1α expression (109). Likewise, immunohistological studies indicate a loss of SEMA3F expression with advanced stage of disease and while VEGF expression is increased (110). Furthermore, in ovarian cancer the ratio of VEGF to SEMA3 may have potential prognostic implications, such that patients with a higher VEGF/SEMA3 ratio are associated with poorer survival compared to patients with a lower VEGF/SEMA3 ratio (111).
Although several signaling pathways are affected by SEMA3F, including inactivation of the mitogen-activated protein kinase (MAPK) and Akt pathways, regulation of SEMA3F remains unclear (109). Interestingly, there is some evidence suggesting that p53 and/or the transcription factor zinc finger E-box binding homeobox 1 (ZEB-1) may be involved (112, 113). In summary, considering the inter-relationships and parallels during axon guidance and angiogenesis, further studies are warranted to elucidate the molecular mechanisms regulating SEMA3F function and dysregulation, especially as they impact ovarian tumor formation and progression.

Central Hypothesis

Dysregulation of angiostatic regulators plays a role in ovarian cancer.

Specific Aims

Ovarian cancers develop into morphologically complex, highly vascularized structures. Although, the role of angiogenic regulators has been well documented the role of angiostatic regulators has been understudied. Therefore, this study examined two angiostatic regulators, angiostatin and semaphorin3F, in ovarian cancer. Three specific aims were proposed to address this hypothesis:
1) Evaluate the sensitivity and specificity of urinary angiostatin as a potential biomarker for ovarian cancer.

2) Establish the clinical relationship between semaphorin3F expression and ovarian cancer progression.

3) Expand upon the antagonistic relationship between VEGF and semaphorin3F, by examining semaphorin3F regulation and semaphorin3F-mediated telomerase activity in ovarian cancer cells.
CHAPTER II

URINARY ANGIOSTATIN LEVELS ARE ELEVATED IN PATIENTS WITH EPITHELIAL OVARIAN CANCER

Abstract

The poor prognosis associated with EOC is due to the lack of overt early symptoms and the absence of reliable diagnostic screening methods. Since many tumors over express angiogenic regulators, the purpose of this study was to determine whether elevated levels of the angiogenic or angiostatic molecules VEGF, HGF, endostatin (ES), and AS were elevated in plasma and urine from patients with EOC.

VEGF, HGF, ES and AS were assayed by ELISA in samples from pilot cohort consisting of healthy women (N=48; pre-menopausal N=23, post-menopausal N=25), women with benign gynecological disease (N=54), patients with primary peritoneal cancer (PP) (N=2) and EOC (N=35). Wherever possible, parallel serum samples were measured for CA125 levels by ELISA.

AS was the angioregulator that independently discriminated EOC patients from healthy individuals. Levels of urinary AS (uAS) from healthy individuals or
women with benign gynecological disease averaged 21.4 ng/mL±3.7 and 41.5 ng/mL±8.8, respectively. In contrast, uAS averaged 115 ng/mL±39.2 and 276 ng/mL±45.8 from women with Stage I (N=6) and late stage (N=31) EOC, respectively. Further, uAS was elevated in EOC patients regardless of tumor grade, stage, size, histological subtype, creatinine levels, menopausal status, or patient age, but appeared to complement CA125 measurements.

Levels of AS are elevated in the urine of patients with EOC and may be of diagnostic and/or prognostic clinical importance. Further studies of uAS as a biomarker for EOC alone or in combination with other markers are warranted.

Introduction

EOC is the most lethal gynecologic neoplasm. In 2009, it will strike over 21,000 women, seventy percent of whom will be first diagnosed at advanced stage (114). As a result, less than 50 percent of patients are alive five years after initial diagnosis.

In order to detect early stages of EOC and avoid unnecessary surgery screening strategies require a sensitivity >75% and a specificity of 99.6% (27, 115). Currently, three screening procedures are in use for EOC detection: bimanual pelvic examination, serum CA125 and transvaginal ultrasonography (TVS) (27, 115). Pelvic examination is an important part of routine gynecologic examination but lacks sensitivity and specificity. Although CA125 is elevated in 80 percent of patients with EOC, its use as an early predictor of malignancy is
limited because only half the patients with stage I disease have elevated CA125 levels. In addition, CA125 lacks specificity as a screening procedure being elevated in a significant number of healthy women or in patients with benign ovarian lesions. The pairing of TVS with CA125 improves specificity, although the former procedure is not practical for cancer screening because of its potential for false positive results and unnecessary surgery. Unfortunately, these strategies alone or in combination do not satisfy the aforementioned criteria.

Pairing of multiple markers and clinical symptoms is also being explored with promising, but yet unresolved clear advantages over CA125 toward early diagnosis (61, 116, 117). Molecular alterations that occur in the early or recurrent cancer may serve as biomarkers of tumor growth and progression and may provide new approaches to detect EOC.

During the early tumor development, cells acquire the capacity to stimulate angiogenesis (53). For instance, a primitive blood capillary with its surrounding fibrocollagenous stroma is found at the base of incipient papillae of ovarian serous neoplasms (118). Experimental tumors cannot grow more than 2 to 3 mm in size unless they are vascularized. To satisfy this requirement, neoplastic cells produce angiogenic factors which stimulate formation of new vessels from the endothelium of the preexisting host vasculature (119). The switch to an angiogenic phenotype during the early stages of tumor progression is modulated by both angiogenic and angiostatic molecules in a “Ying and Yang” fashion (119). Thus, evaluation of angioregulators’ levels in body fluids may contribute to the early detection of EOC. The angiogenesis-dependent nature of
tumor growth is particularly relevant for this cancer which can reach large proportions and correlations between microvascular density and tumor aggressiveness have been established (120). Thus, analysis of angiogenic factors that regulate EOC growth and progression may have important implications for the diagnostic and prognostic evaluation of this disease.

Our lab previously reported that the cyst fluid of EOC contains large amounts of VEGF (72). VEGF and basic fibroblast growth factor (bFGF) levels were evaluated in patients with benign ovarian cysts, functional cysts, borderline tumors, and patients with malignant tumors. There was a marked difference in VEGF levels between malignant cysts and benign, borderline or functional cysts. Malignant neoplasms had an average 26-fold increase in VEGF over benign lesions and a 6-fold increase over borderline tumors. Unlike VEGF, bFGF was generally very low or undetectable in malignant cysts and did not correlate with malignancy. We also found that VEGF levels in ovarian cyst fluid were 3-fold higher in 6 patients with evidence of disease 1-2 years after surgery (~50 ng/mL) as compared to 7 patients with no evidence of disease (~18 ng/mL) (72).

Consequently, evaluation of circulating or excreted angiogenic and/or angiostatic markers may be clinically relevant for EOC.

Other studies have also shown that high intratumoral concentrations of VEGF and other angiogenic cytokines, such as HGF, may be reflected by elevated levels in peripheral blood, in urine and in effusions of patients with a wide spectrum of cancers, including EOC (121-124). Malignant tumors also generate small inhibitors of angiogenesis such as ES, AS, and thrombospondin (73, 125-128).
Additionally, ES and AS have been reported at detectable levels in urine of patients with malignant disease and could, therefore, provide biomarkers for cancer (129-131). Given these reports along with our earlier finding of elevated VEGF in the cyst fluid of EOC (72), we sought to determine if plasma and urinary levels of the angioregulators VEGF, HGF, ES, and AS correlate with EOC disease status.

**Methods and Materials**

**Patient cohort**

With prior institutional approval, urine and blood samples were collected from a cohort of healthy pre-menopausal (N=23) and post-menopausal (N=25) individuals, women with benign gynecologic disorders (N=54) and patients with EOC (N=35) or primary peritoneal (PP) cancer (N=2) at the H. Lee Moffitt Cancer Center and Tampa General Hospital in collaboration with the University of South Florida. Two cases designated as low malignancy potential (LMP) tumors were also evaluated. All except 8 specimens were collected prior to initial surgical cytoreduction while the latter 8 specimens presented with recurrent disease at the time of enrollment in this study. In addition to EOC, the cancer category consisted of women diagnosed PP cancer, which is often related to EOC. The samples collected from women with benign disease consisted of a broad range of ovarian and non-ovarian genital tract (GT) lesions (Table 1).

Tissue blocks were identified and reviewed by SVN to confirm the histopathology of benign and malignant lesions, the latter according to FIGO
criteria. The clinical databases of these women were also reviewed and information regarding patient age, tumor type, stage, grade, size, CA125 values and surgical and adjuvant treatment abstracted whenever available. This information and tumor pathology were correlated with plasma and urinary levels of angioregulators, which are summarized in Tables 1 and 2, in a total of 141 women.

Sample preparation

Urine and blood samples were collected from patients, anonymized and decoded to protect patient identity, and released from the tissue banks for this research project. All samples were kept on ice following collection. Urine samples were treated with a standard protease inhibitor cocktail (80 μg/ml 4-(2 aminoethyl)-benzene sulfonyl fluoride, 200 μg/ml EDTA, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin, Sigma Scientific, St. Louis, MI) within minutes to a few hours of collection and centrifuged at 3000×g. Urinary supernates and plasma samples were then aliquoted and stored at −20 °C for up to 4 years without change in activity.

Enzyme-linked immunosorbant assay

Angiogenic molecules were assayed using quantitative sandwich enzyme-linked immunosorbant assay (ELISA) kits for VEGF, HGF, and ES (all kits from R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. uAS was assayed using lysine-ELISA as described previously by Cao et al (129).
In accordance with previous studies (129), uAS threshold levels were set to include 95% of AS values of urine samples from healthy women.

When not derived from clinical data, CA125 levels in subjects’ plasma were assayed by individual ELISA tests (Bio-Quant, San Diego, CA) according to the manufacturer’s instructions. The enzymatic reactions were detected at 450 nm or 492 nm using a Dynex MRX plate reader (Dynex Technologies, Chantilly, VA) and results were expressed as the mean absorbance of triplicate samples ± S.E. for VEGF, HGF, ES, while CA125 and AS results were expressed as the mean of duplicate samples.
Table 1. Histological diagnoses and clinical characteristics of the study cohort.

<table>
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<tr>
<th>Sample</th>
<th>Cancer Pathological Parameters</th>
<th>Age mean ± SE</th>
<th>AS mean ng/ml ± SE</th>
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<tr>
<td><strong>Healthy</strong> (48)</td>
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<td>53.5 ± 1.7</td>
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<td>Pre-Menopausal (23)</td>
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<td>Genital Tract Lesions (16)</td>
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<td><strong>Cancer</strong> (37)</td>
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Western blot analysis

For Western blot analysis, plasma samples were solubilized in SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), separated via 10% SDS-PAGE and electroblotted onto PVDF membranes by wet transfer. Immunoblotting was performed using antibodies directed against the kringle 1-3 regions of human plasminogen (1:1000, R & D Systems, Minneapolis, MN). β-actin (1:10,000, Sigma-Aldrich, St. Louis, MO) was used as a loading control. Blots were visualized using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Blots were analyzed and scanned with ImageQuant image analysis software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Values reported for AS were normalized to the blots’ respective β-actin levels.

Statistical analysis

Samples for VEGF, HGF, and ES were run in triplicate, whereas samples for CA125 and AS were run in duplicate and the data subject to descriptive, one-way Kruskal Wallis, Spearman correlation, and/or receiver operator curve and area under the curve (ROC-AUC) analyses. P-values <0.05 were considered statistically significant.
Results

**uAS levels are elevated in EOC patients**

We evaluated the levels of VEGF, HGF, and ES in the plasma and urine of healthy controls and of patients with benign gynecologic disorders or EOC; uAS was also evaluated. Kruskal Wallis global test indicated seriousness of clinical status (EOC > benign lesions > healthy status) correlated with plasmatic (p ≤ 0.0016) and urinary (p ≤ 0.01) VEGF, plasmatic HGF (p ≤ 0.0021), plasmatic ES (p ≤ 0.01) and uAS (p < 0.0001) (Figures 4, 5A, Table 2). uAS was the variable that independently discriminated EOC patients from healthy controls. Although there have been some studies reporting the detection of non-ovarian cancers by measuring various proteins in urine, only few reports have been recently published relative to EOC detection in this biological fluid (35, 123, 129) and to our knowledge this is the first report of AS as a biomarker of EOC. Since plasminogen interfered with the measurement of plasmatic AS by ELISA, plasmatic AS was detected in representative samples (6/group) by Western immunoblotting and did also appear to correlate with disease status (Figure 5B). We believe uAS is better because renal filtration allows smaller, positively charged fragments of plasminogen, like AS, but not its higher molecular weight precursor, to be excreted in urine. In addition, creatinine levels measured at the time of urine collection indicated that elevated uAS in cancer patients was not related to renal dysfunction (data not shown).
## Table 2. VEGF, HGF, ES, and AS in the Study Cohort as Descriptive Statistical Information

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P, plasma; U, urine; ND, not determined
Figure 4. Plasma and urinary levels of angiogenic regulators are elevated with EOC progression. ELISA was utilized to measure (A.) VEGF (mean pg/ml ± S.E.), (B.) HGF (mean pg/ml ± S.E.) and (C.) ES (mean ng/mL ± S.E.) in the plasma and urine of healthy volunteers (N=24) and of patients with benign gynecologic disorders (N= 54) or EOC (N= 39). Samples were examined in triplicate and the data expressed as mean ± S.E/category (normal-purple, benign-pink, cancer-green). Lined bars–plasma; Solid bars–urine; * p≤0.01
Figure 5. **AS levels are elevated in EOC patients.** (A.) uAS was evaluated using lysine-ELISA. Samples were examined in duplicate and the data expressed as mean ng/mL ± S.E. per category. Healthy individuals were further divided based on menopausal status (inset). (B.) Plasmatic AS was detected in representative samples (6/category) by Western immunoblotting using a monoclonal antibody against the kringle 1-3 regions of human plasminogen. Densitometric analyses are expressed as relative intensity of plasmatic AS levels normalized to β-actin protein levels. Normal-purple; Benign-pink; Cancer-green; Lined bars–plasma; Solid bars–urine; ** p≤0.0001
Clinical status

Based on the initial findings of a better discriminating effectiveness of AS, we expanded our analysis with regards to clinical parameters in this cohort of healthy controls, women with benign gynecologic disorders and patients with EOC. Though this cohort comprises a small pilot study, it is representative of our institutional clinical practice in regard to EOC histology, grade and stage distribution.

The amount of uAS was generally negligible (average of 21.4 ng/mL±3.7; 95% confidence interval = 13.9-28.9) in healthy controls regardless of menopausal status (Figure 5A inset) with only 8% of samples above a previously established cutoff threshold of 16 ng/mL for uAS in normal individuals (129, 131) (Figure 5A, Table 1). In contrast, uAS associated with EOC and PP cancer was generally >10x than found in healthy controls with an overall mean value of 249 ng/mL±39.7 and 216 ng/mL, respectively (95% confidence interval of all cancers = 171-323.5) (Figure 6A, Table 1). Interestingly, 5/37 and 3/37 of EOC patients had uAS ≤16 and ≤50 ng/mL, respectively, but it is noteworthy that two samples were derived from patients with mucinous ovarian cancer and one was derived from endometrioid ovarian cancer (Figure 6A).
Figure 6. uAS levels are elevated in EOC patients. Urinary samples were analyzed in duplicate by lysine-ELISA and data expressed as mean ng/mL per patient in (A.) healthy controls and cancer histological types (muc, mucinous; serous, serous adenocarcinoma; PP, primary peritoneal) as well as serous tumor grade; (B.) according to tumor stage including recurrent EOC, grade and size and (C.) among women with benign ovarian and non-ovarian genital tract (GT) lesions; 2 cases of LMP were also included. Mean uAS (ng/mL) indicated for each category. ROC-AUC analyses of uAS in EOC vs (D.) healthy controls and (E.) women with benign gynecologic diseases.
The distribution of uAS was evaluated by histological EOC subtype, including serous carcinomas representing over 75% of the pilot cohort. There was a trend for elevated uAS with increasing tumor grade (189 ng/mL±42.3 for Grade 2 neoplasms to 269 ng/mL±53.2 for Grade 3 carcinomas) and especially stage (115 ng/mL±39.2 for Stage I to 208, 246 ng/mL±49.2, and 333 ng/mL±90.5 for Stages 2, 3, and recurrent respectively) although these were not statistically different (Figures 5A, 6A-B). Of interest, on average, uAS levels of Stage I EOCs were 5 and 3 fold higher than in healthy controls or women with benign disease, respectively; likewise, although not statistically different (p ≤ 0.1232), uAS Stage I was 2 fold lower than late stage EOC (Figures 5A, 6B). Similarly, there was a non-statistical tendency for elevated uAS with tumor size (108.7 ng/mL±34.9, 393.3 ng/mL±112.2, 247.6 ng/mL±42.3, for microscopic, <2 cm, and >2 cm, respectively) (Figure 6B). In contrast, patient age did not appear to be related to elevated uAS (Table 1).

uAS was also analyzed in women with benign ovarian lesions or non-ovarian GT lesions (Figure 6C, Table 1). uAS in patients with benign ovarian lesions displayed a mean level ≤ 50 ng/mL with nearly 82% of samples <16 ng/mL and 7/54 samples with levels ranging from 64 to 176 ng/mL (95% confidence interval = 24.9-73.9) (Figure 6C). Interestingly, the 3 highest uAS values were noted in 1 case of serous surface papillomatosis and 2 serous cystadenomas; unfortunately, these were blinded samples and follow-up information on these patients was not available.
ROC analyses indicated sensitivity and specificity of uAS for distinguishing healthy controls from cancer patients as 88% and 92.3%, respectively (Figure 6D). Additionally, the set cutoff threshold (16 ng/mL) was >90% accurate with an AUC of 0.953 (Figure 6D). Sensitivity and specificity for distinguishing benign samples from cancer patients were 84.1% and 84.4%, respectively (Figure 6E), while the set cutoff threshold of 80 ng/mL as determined by a 95% confidence interval was 83% accurate with an AUC of 0.88 (Figure 6E).

Levels of uAS were also compared in 11 patients immediately prior to and within 3 weeks following initial cytoreductive surgery (Figure 7A); no chemotherapy was administered during this interval. uAS decreased to control levels in those patients (# 17, 21, 22 and 42) in which chart review indicated successful tumor ablation and to a lesser extent in suboptimally debulked patient # 40. A postoperative increase was observed in patient # 20 who developed ascites. Urinary samples were also collected in patients # 5, 27, 43, 49 and 51 at 7 and/or 12 months after initial surgery (Figure 7A). In these 5 patients, uAS was indicative of resistant (# 5, 43 and 51) or sensitive (# 27 and 49) disease. These data suggest, then, that uAS levels may correlate with surgical debulking and/or recurrent EOC and warrant further investigation.
Figure 4. Elevated uAS correlates with recurrent EOC and complements CA125 measurements. (A.) uAS was evaluated by lysine-ELISA before and after surgery in 11 patients. Samples were examined in duplicate and data expressed as mean ng/mL per patient. A- 3 weeks after surgery, B- 7 months after surgery, and C- 12 months after surgery. (B.) uAS and blood levels of CA125 were measured from the same healthy controls and EOC patients (muc, mucinous; serous, serous adenocarcinoma; PP, primary peritoneal) prior to initial cytoreductive surgery. uAS data expressed as mean ng/mL per patient and CA125 data expressed as mean U/mL per patient. * False-positive or False-negative
**Elevated uAS levels complement CA125 values**

While studies continue to identify EOC biomarkers (132, 133), CA125 remains the current "gold standard" for EOC detection followed by TVS and pelvic examination. CA125 is useful in the follow-up of EOC patients after surgical and chemotherapeutic management but its value in early detection and overall management is not ideal due to the test’s limited sensitivity and specificity (27) and to the fact that this malignancy is neither rare nor frequent (115). While nearly 2/3 of patients with clinical disease will have elevated CA125 levels, less than 50% of early stage EOC will be detected by CA125. Therefore, in this initial evaluation, we compared uAS to CA125 levels in 12 healthy controls and 23 cancer patients to address the potential for uAS to serve as a biomarker for EOC (Figure 7B). Elevated uAS (>16ng/mL) was associated with 88% EOC detection, correctly identifying 15/17 serous, 3/4 mucinous and 2/2 PP carcinomas in cancer patients (Figure 7B). In contrast, CA125 levels (>35 U/mL) from matched samples was associated with only 74% EOC detection, correctly identifying 13/17, 3/4, and 1/2 of patients with serous, mucinous and PP cancer, respectively, as cancer positive (Figure 7B). All healthy controls were correctly classified as cancer-negative by uAS (<16 ng/mL) whereas 2/12 healthy controls were incorrectly identified as cancer-positive by CA125 (Figure 7B). In addition, PPV and NPV were 0.923 and 0.836, respectively; and 95% confidence intervals were 133.5-316.6 ng/mL for uAS and 97.6-285.8 U/mL for CA125. Lastly, statistical analyses revealed a positive Spearman correlation of 0.5431, p ≤ 0.0007 between uAS and CA125. Therefore, there is evidence of a statistically
significant positive correlation indicative of complementarity between uAS and CA125 in the ability to detect ovarian cancer. Further, EOC could be detected in 91.3% (21/23) of samples when using the criteria that one or both of these biomarkers were elevated.

**Discussion**

While angiogenesis is an essential biological process for embryonic development and normal physiological processes, it is also involved in a number of pathologic conditions including chronic inflammation, immunological diseases, and cancer (134). Angiogenesis is regulated by several factors that can either promote or inhibit the development of new blood vessels and since EOCs are generally highly vascularized tumors our study aimed at the evaluation of angioregulators in bodily fluids as potential biomarkers for EOC.

Investigated angioregulators included VEGF, HGF, ES and AS. VEGF, a 30-42 kDa homodimer produced by a variety of cell types including cancer cells, has emerged as a critical regulator of the angiogenic process by promoting endothelial migration, proliferation, protease activity and capillary tube formation (53). VEGF levels in various body fluids are increased during cancer progression (72, 121). HGF is a pleiotropic growth factor that is implicated in the growth and spread of some epithelial tumors (124) and is present in benign and malignant ovarian tissues, cysts and ascites (122). A proportion of ovarian tumors also express high levels of the HGF receptor, c-Met (135), and this expression may
add a selective growth advantage to a narrow subset of differentiated EOCs. ES is a 20-kDa C-terminal fragment of collagen XVIII originally isolated from a murine hemangioendothelioma that has been shown to specifically inhibit endothelial cell proliferation, angiogenesis and tumor growth (128). Blood and urinary levels of ES have been reported as elevated in vulvar and other malignant disease (129-131, 136), but the role of ES in EOC has not yet been explored.

AS is a specific 38kDa internal fragment of plasminogen that inhibits angiogenesis by blocking endothelial cell growth via its kringle 1-3 regions (129, 131, 137). AS synergizes with ES in inhibiting angiogenesis and EOC growth (138) and the two angiostatic molecules may thus be valid targets for anti-angiogenic therapy in cancer via recombinant viral strategies (134, 139). AS and other plasminogen cleaved products are present in malignant ascites and may contribute to the net anti-angiogenic properties of this fluid (73). In a single immunohistochemical study of AS expression in EOC, survival time was longer in patients with AS-positive and VEGF-negative tumors than in patients with AS-negative and VEGF-positive tumors (127). AS has also been sparingly reported as elevated in the urine of leukemic and some solid cancer patients suggesting that urinary detection of this angiogenesis inhibitor may provide new diagnostic, prognostic and potentially therapeutic tools (129, 131).

Our study suggests that uAS provides a more sensitive marker than other angioregulators. Specifically, our data indicates significantly elevated uAS discriminates EOC from healthy controls and women with benign gynecologic
disease. Further, the most apparent clinical features related to uAS are detection of early stage EOC and complementarity with CA125. While the former represents an important target group associated with high survival (>95%), the latter suggests potentially important diagnostic and prognostic roles for uAS; especially when both biomarkers were taken into consideration over 91% of ovarian cancer was detected and all normal individuals were identified as healthy.

In future, ELISA or spot assays of combined or dominant urinary proteins may be used for diagnostic and prognostic applications. These assays may be used in combination with tests currently utilized to detect EOC at an earlier stage, thereby decreasing patient mortality. After surgery, patients could also be evaluated for recurrence by easily monitoring the urine for such proteins. Measuring these angiogenic regulators may also be pursued in other readily accessible body fluids such as saliva as done for other diseases (140). Finally, the identification of an EOC-related angiogenic profile may lead to the formulation of adjuvant therapies utilizing target-specific anti-angiogenic drugs.

Validation of AS as a urinary biomarker for the clinical detection may offer a non-invasive, convenient, and cost-effective screening and diagnostic tool for detection of this most lethal gynecologic malignancy. This would allow women to make better decisions about their health options and potentially reduce the mortality associated with this insidious disease.
CHAPTER III

EXPRESSION OF SEMAPHORIN 3F AND ITS RECEPTEORS IN EPITHELIAL OVARIAN CANCER, FALLOPIAN TUBES AND SECONDARY MÜLLERIAN TISSUES

Abstract

While semaphorins and their receptors appear to play a role in tumor carcinogenesis, little is known about the role of SEMA3F in EOC development. Therefore, we sought to determine the clinical relationship between S3F and its receptors, NP-2 and NP-1 with EOC progression. We analyzed the immunohistological expression of SEMA3F, NP-2 and NP-1 in clinical specimens of normal ovaries (N), benign cyst adenomas (Cy), well-differentiated adenocarcinomas (WD), poorly-differentiated adenocarcinomas (PD), inclusion cysts (IC), paraovarian cysts (PC), and fallopian tubes (FT). Tissue sections were evaluated for staining intensity and percentage of immunoreactive epithelia. We found that expression of SEMA3F and NP-2 decreased while NP-1 expression increased with EOC progression. Interestingly, we also found elevated expression of SEMA3F, NP-2, and NP-1 in epithelia of ICs, PCs, and
Our findings indicate that loss or deregulation of semaphorin signaling may play an important role in EOC development.

**Introduction**

EOC is the most lethal and the second most commonly diagnosed gynecological malignancy. It is estimated that in 2009, it will strike over 21,000 women seventy percent of whom will be first diagnosed at advanced stages and will die within five years (141). In general, EOC is characterized by poor prognosis due to lack of early symptoms, which contributes to advanced stage of disease at presentation, and by the absence of accurate screening methods to detect early stages of the disease. The origin of this malignancy has been traditionally attributed to the OSE. However, alternative theories to a coelomic origin attribute the source of EOC to primary or secondary Müllerian system derivatives such as the fimbriated end of the fallopian tube and paraovarian vestigial structures, respectively (7, 19, 20). The Müllerian system theory would explain why epithelial ovarian neoplasms present as morphological variants of fallopian tube (serous adenocarcinoma), uterus (endometrioid), or endocervix (mucinous adenocarcinoma) epithelia without requiring an intermediate metaplastic step (19). Independently of its cell of origin, the pathogenesis of this most lethal gynecologic malignancy is, however, not well understood.

Semaphorins are a large family of transmembrane, secreted, or GPI-anchored, proteins involved in axon guidance and growth cone collapse through
interaction with their receptors, the neuropilins and plexins (82). There are eight classes of semaphorin genes all of which are characterized by a conserved 500 amino acid, cysteine-rich Sema domain, which mediates binding specificity and is necessary for signaling (82). Plexins are transmembrane receptors that form complexes with NP transmembrane receptors, which only directly interact with SEMA3 members, and mediate signal transduction following binding to a semaphorin (87). Additional biological functions for semaphorins and their receptors include regulation of angiogenesis as well as tumor progression and metastasis (142, 143).

With regard to angiogenesis, SEMA3s are of interest since members of this class have demonstrated either pro- or anti-tumorigenic functions. SEMA3s are unique in that they directly bind NP homo- or hetero-dimeric receptors and are unable to bind directly to plexins with the exception of SEMA3E (85, 86, 144, 145). However, signaling is regulated through an oligomeric complex involving a NP dimer and one of four type-A plexins (87, 146-148). Interestingly, NPs also function as co-receptors with VEGFRs for VEGF whose over expression contributes to tumor growth and metastasis (89). In addition to VEGF family members, NPs also interact with other heparin-dependent growth factors like bFGF and HGF (93). Of interest is SEMA3F, a class 3 secreted protein which plays a critical role during neural development in both the peripheral and central nervous systems through interaction with its high affinity receptor NP-2 and low affinity receptor NP-1 (148). SEMA3F has also been shown to inhibit angiogenesis by decreasing blood vessel density and through competition with
VEGF for a shared receptor complex (108, 149). Specifically, SEMA3F induces a poorly vascularized, encapsulated, non-metastatic phenotype through chemorepulsion of endothelial cells in melanoma (150). In breast cancer, SEMA3F disrupts intercellular contacts of MCF7 breast cancer cells through delocalization of E-cadherin and β-catenin (142). Further, SEMA3F and VEGF demonstrate opposing effects for cell attachment and spreading (151), as well as migration (152).

SEMA3F loss or delocalization has been shown to correlate with advanced tumor stage in a number of cancers including lung (110); however, a correlation between SEMA3F and tumor stage, grade, and histological subtype remains to be demonstrated in ovarian cancer. In order to begin to better understand epithelial ovarian carcinogenesis, we sought to determine the clinical relationship between SEMA3F and EOC progression. Therefore, we analyzed the immunohistochemical expression of SEMA3F and its two receptors NP-1 and NP-2 in clinical specimens.

**Materials and Methods**

**Tissue Specimens**

With institutional approval, 44 specimens were retrieved from the tissue bank at H. Lee Moffitt Cancer Center and Research Institute. Serial 4-5μm sections were hematoxylin and eosin stained and classified according to FIGO criteria (International Federation of Gynecology and Obstetrics) as normal.
ovaries (N, n = 12), benign serous cyst adenomas (Cy, n = 10), well differentiated serous cystadenocarcinomas (WD, n = 4), poorly differentiated serous cystadenocarcinomas (PD, n = 6) and fallopian tubes (FT, n = 4). Three of 4 WD carcinomas were late stage (III-IV) whereas all PD specimens were of late stage. We also evaluated epithelia of inclusion cysts (IC, n = 6) and paraovarian cysts (PC, n = 2) from patients with otherwise normal ovaries and fallopian tubes.

**Immunohistochemistry**

Immunohistochemical staining was performed on serial paraffin-embedded sections by the horseradish peroxidase (HRP) conjugated system using a Dako Autostainer Plus (Dako North America, Inc., Carpinteria, CA). Microwave antigen retrieval was achieved using 10x Antigen Retrieval AR-10 (Tris) (BioGenex, San Ramon, CA) or 10mM citrate buffer for 17 minutes. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide. Sections were rinsed twice with deionized water, washed in Tris buffered saline (TBS)/Tween for 5 minutes and immunostained on the Dako Autostainer with the following: rabbit anti-SEMA3F polyclonal antibody (Chemicon, Billerica, MA) at 1:50 for 1 hour at room temperature, rabbit anti-neuropilin-1 polyclonal antibody (ECM Biosciences, Versailles, KY) at 1:200 overnight at 4°C, and the mouse anti-NP-2 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:75 for 1 hour at room temperature. Secondary antibodies for SEMA3F and NP-2 were Vector Elite ABC Peroxidase, using rabbit IgG and mouse IgG, respectively; DAB was the chromogen. The secondary antibody for
NP-1 was EnVision+ Peroxidase polymer. Sections were counterstained with modified Mayer’s hematoxylin.

Immunostaining of SEMA3F, NP-1, and NP-2 was evaluated by two independent observers (SVN and CD) and scored based on staining intensity from 1 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong) and percent of positive epithelial cells (1, 1-10%; 2, 10-50%; and 3, >50%). Cellular localization of SEMA3F, NP-1, and NP-2 was also assessed. To confirm the specificity of the antibodies, non-immune rabbit IgG and goat IgG were used as negative controls in place of primary antibodies for tissue specimens. Specificity was further confirmed by Western blot analyses of cell lysates and visualization of the corresponding protein bands at the appropriate molecular weights for the respective antibodies (data not shown).

**Statistical analyses**

Statistical analysis of staining for SEMA3F, NP-1, and NP-2 among clinical samples was analyzed by Spearman rank correlation and Fisher exact test for differences in staining intensity and histological type. ANOVA analyses were performed to determine significant differences in percentage of positively stained epithelia between N, N combined with FT, Cy, and cancer (WD combined with PD) groups. Spearman and Fisher exact tests were performed with SAS version 9.2 (SAS Institute, Cary, NC) and ANOVA tests were performed with Microsoft Excel (Microsoft, Redmond, WA). P-values < 0.5 were considered statistically significant.
Results

**SEMA3F expression decreases with epithelial ovarian cancer progression.**

When all histological subtypes were considered, the expression level of SEMA3F in epithelial cells was relatively weak and decreased with tumor progression. We found a significant inverse correlation between SEMA3F staining intensity and histology where 83.3% (10/12) of N, 80% (8/10) of Cy, and 75% (3/4) of WD specimens expressed weak SEMA3F staining, whereas the majority of PD specimens, 67% (4/6), completely lacked SEMA3F expression (Figures 8-9) (p<0.0001). No differences were observed as function of stage. Interestingly, when we evaluated the percentage of positive epithelia in the sections expressing SEMA3F a significantly higher percentage of normal OSE was immunoreactive compared to Cy (p<0.001) and cancer (p<0.001) (Table 3). The staining pattern throughout the tissue sections was predominantly cytoplasmic though a small portion (<20%) of epithelial cells demonstrated basal membranous staining pattern in normal, benign, and well differentiated carcinomas (Figure 8).

Stromal cells of all histological groups did not express SEMA3F with the exception of endothelial cells that, together with positive control placental tissues, expressed SEMA3F in a cytoplasmic and membranous localization (Figure 8, arrow), thus providing in our cohort an internal positive control for SEMA3F expression. Immunostaining was not observed in negative control samples (Figure 8, inset).
Table 3. Epithelial expression of SEMA3F and NP-2 decreases while NP-1 increases with ovarian epithelial tumor progression.

<table>
<thead>
<tr>
<th></th>
<th>SEMA3F</th>
<th>NP-2</th>
<th>NP-1</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>67.5 ± 1.6</td>
<td>71.4 ± 3.0</td>
<td>80.5 ± 1.4</td>
</tr>
<tr>
<td>Cy</td>
<td>42.9 ± 2.3</td>
<td>48.8 ± 2.1</td>
<td>86.5 ± 1.8</td>
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<tr>
<td></td>
<td>* p ≤ 0.001 • p ≤ 0.05</td>
<td>* p ≤ 0.001 • p ≤ 0.001</td>
<td>* p ≤ 0.001 • p ≤ 0.001</td>
</tr>
<tr>
<td>WD</td>
<td>21.6 ± 3.9</td>
<td>19.2 ± 4.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>** p ≤ 0.001 ○ p ≤ 0.05</td>
<td>** p ≤ 0.001 ○ p ≤ 0.001</td>
<td>** p ≤ 0.001 ○ p ≤ 0.001</td>
</tr>
<tr>
<td>PD</td>
<td>29.5 ± 1.5</td>
<td>17.1 ± 2.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>** p ≤ 0.001 ○ p ≤ 0.05</td>
<td>** p ≤ 0.001 ○ p ≤ 0.001</td>
<td>** p ≤ 0.001 ○ p ≤ 0.001</td>
</tr>
<tr>
<td>IC</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PC</td>
<td>100</td>
<td>85.7 ± 4.5</td>
<td>82.5 ± 2</td>
</tr>
<tr>
<td>FT</td>
<td>100</td>
<td>72.2 ± 3.6</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: * - N vs Cy  ** - N vs WD+PD  • - N + FT vs Cy  ○ - N + FT vs WD+PD
Abbreviations: normal (N), serous cystadenoma (Cy), well-differentiated serous adenocarcinoma (WD), poorly differentiated serous adenocarcinoma (PD), inclusion cyst (IC), paraovarian cyst (PC) and fallopian tube (FT)
Data represent the average percent of positive epithelium expressing SEMA3F, NP-2, and NP-1 ± SE.
Figure 8. **SEMA3F expression decreases while NP-1 increases with epithelial ovarian cancer progression.** Representative illustrations of immunohistochemical staining of normal (N), serous cystadenoma (Cy), well-differentiated (WD) and poorly differentiated (PD) serous adenocarcinomas) for SEMA3F, NP-2, and NP-1. Placental tissue was used for positive control (C) and arrow indicates expression of SEMA3F by endothelial cells. Primary antibodies were replaced with non-immune serum in negative control sections (inset). Original magnification: 400x.
Figure 9. Graphical depiction of SEMA3F, NP-2, and NP-1 expression with epithelial ovarian cancer progression. Immunohistochemically stained sections of normal (N), serous cyst adenomas (Cy), well-differentiated (WD) and poorly differentiated (PD) serous adenocarcinomas were evaluated for expression of SEMA3F, NP-2 and NP-1 and scored as negative (Neg), weak (W), moderate (M), or strong (S) as described in Materials and Methods.
NP-2 expression decreases with epithelial ovarian cancer progression.

NP-2 was generally weakly expressed in all histological groups but the proportion of positive epithelial cells significantly decreased with tumor progression. The expression of NP-2 in 33% (4/12), 20% (2/10), 50% (2/4), 33% (2/6) of N, Cy, WD, and PD was generally weak (Figures 8-9) and with no significant statistical difference. In contrast to normal ovaries where 71.4% of OSE positively expressed NP-2, the percentage of positive epithelia was significantly lower in Cy, WD and PD where only 48.8%, 19.2%, and 17.1% were positive, respectively (p<0.001) (Table 3). The overall staining pattern was cytoplasmic and membranous in all histological groups (Figure 8). Interestingly, most cells expressing NP-2 in the examined WD carcinomas were localized in highly distinctive clusters within the tissue specimens of early stage compared to late stage (Figure 10).

In contrast to epithelial cells, over 90% of stromal cells in normal ovaries strongly expressed NP-2 (Figure 8). Similar to normal tissue, stromal cells in Cy, WD, and PD tissues expressed NP-2, however, the level of expression was moderate (Figure 8). Like SEMA3F, all endothelial cells within the stroma and positive control placental tissues expressed NP-2 immunostaining.
Figure 10. NP-2 expression occurs in distinct clusters of tumor cells. Representative illustration of NP-2 expression in well-differentiated serous adenocarcinoma (WD). Original magnification 100x and inset 200x.

NP-1 expression increases with epithelial ovarian cancer progression.

In contrast to SEMA3F and NP-2, the overall expression of NP-1 increased significantly with tumor progression. Most (93.8%, 30/32) of the tissues examined expressed NP-1 (Figure 9). The overall staining intensity of NP-1 in N and Cy sections ranged from weak, 58% (7/12) and 60% (6/10), to moderate, 25% (3/12) and 30% (3/10), respectively (Figures 8-9, Table 3). In contrast, the vast majority of cancerous tissues, 75% (3/4) of WD and 83% (5/6) of PD samples, strongly expressed NP-1 (Figures 8-9); however, no differences were observed as function of stage. The percentage of positive epithelial cells also significantly increased as 80.5%, 86.5%, and 100% of epithelia were positive for NP-1 in N, Cy and cancer tissues, respectively, (Table 3).
Most stromal cells in N and Cy tissues expressed NP-1, although the staining intensity was less than for NP-2. Stromal staining was less in cancerous than in N and Cy tissues (not shown).

**SEMA3F, NP-2, and NP-1 expression is elevated in inclusion cysts, paraovarian cysts, and fallopian tube epithelium.**

Given the uncertain cellular origin of EOC, coelomic versus extrauterine Müllerian, we also evaluated the immunohistochemical expression of SEMA3F, NP-2, and NP-1 in ICs, PCs, and FT tissues. We found an elevated staining intensity and percentage of epithelial cells expressing SEMA3F and its receptors in ICs, PCs, and FT sections when compared to normal ovarian and cancerous tissues (Figures 11-12, Table 3). In contrast to WD and PD tissues where only 21.6% and 29.5% of the epithelium were positive, 100% of the epithelium lining the ICs expressed S3F (Table 3). All PCs and FT epithelia expressed SEMA3F either moderately or strongly (Figures 11-12, Table 3). Similar to the normal ovary, only endothelial cells, but no other surrounding stromal cells expressed SEMA3F.
Figure 11. SEMA3F expression is elevated in inclusion cysts, paraovarian cysts, and fallopian tubes. Representative illustrations of immunohistochemical staining of normal ovary (N), inclusion cyst (IC), paraovarian cyst (PC), and fallopian tube (FT) for SEMA3F, NP-2, and NP-1. Original magnification 400x.
Figure 12. Graphical depiction of SEMA3F, NP-2, and NP-1 expression in inclusion cysts, paraovarian cysts, and fallopian tubes compared to normal ovaries. Immunohistochemically stained sections of normal ovary (N), inclusion cysts (IC), paraovarian cysts (PC) and fallopian tubes (FT) were evaluated for staining intensity and designated as negative, weak, moderate, or strong following staining with antibodies directed against SEMA3F, NP-2, and NP-1.
NP-2 expression but not intensity was comparable to SEMA3F in epithelial cells of ICs and PCs (Figures 11-12, Table 3). In contrast to WD and PD where only 19.2% and 17.1% of the epithelial cells were positive for NP-2, respectively, 100%, 85.7%, and 72.2% of the epithelia lining ICs, PCs, and FT, respectively, were positive (Table 3). In contrast to the strongly staining stromal cells of the normal ovary, weak NP-2 stromal staining was found in FT and PCs. All endothelial cells were strongly immunoreactive for NP-2.

Epithelial expression of NP-1 in ICs, PCs, and FT was universal (Table 3) and similar to cancerous tissues; in contrast to normal ovaries, 50%, 50%, and 25%, respectively of IC, PC, and FT epithelia exhibited strong NP-1 staining (Figures 11-12). Similar to NP-2, stromal cells displayed negative to weak NP-1 expression while all endothelial cells were positive.

**Discussion**

Loss or delocalization of SEMA3F has been shown to correlate with advanced tumor stage in lung cancer (110, 153). In this study, we sought to determine the clinical relationship between SEMA3F and epithelial ovarian cancer progression. Overall, we observed a significant decrease in both intensity and frequency of SEMA3F staining with EOC progression. Although, tumors of high grade and advanced stage expressed the least amount of SEMA3F, tumor grade was the only parameter that indicated a significant relationship between SEMA3F expression and EOC progression in this initial cohort of patients.
Levels of SEMA3A have also been reported to be significantly reduced in advanced EOC and metastases (111). Taken together, these findings suggest that the loss or deregulation of semaphorin signaling may play an important role in E OC progression and support a tumor suppressor function for this molecule (108).

In contrast, the SEMA3F receptors NP-2 and NP-1 have been reported to be over-expressed in some cancers, including EOC (111, 153). In agreement with previous reports, we found that the staining intensity and percentage of epithelium expressing NP-1 significantly increased with EOC progression and was predominantly cytoplasmic. However, we found that NP-2 expression decreased with EOC progression. Differences in these results compared to other reported findings may reflect methodological differences in sample preparation, scoring of immunostaining, and case distribution. Interestingly, we observed prominent staining of NP-2 in isolated, but highly distinct clusters of tumor cells in early stage and low grade (WD) ovarian cancer tissues similar to that described by Brambilla et al. (110) in non-small cell lung cancer. These observations, in addition to the cytoplasmic localization of receptors we observed and previously reported in both lung and ovarian cancers (111, 153), may further support a role for a SEMA3F-NP pathway in epithelial cell adhesion and/or migration.

Carcinomas arising from the ovary, FT, and peritoneum have histological and clinical similarities (20). Histological similarities with epithelia lining ICs, PCs, and FT have also been documented and explained on the basis of common coelomic or Müllerian system origin (19, 20). In the present study, while there
was only weak expression of SEMA3F and NP-2 in EOC, OSE, and IC there was strong expression of NP-1 in FT, PC, and EOC. This shared phenotype indirectly supports a common Müllerian origin for epithelial ovarian cancer. Given the slightly younger pre-menopausal age of normal individuals compared to the peri-to post-menopausal age of benign and ovarian cancer patients, a potential contribution of menopausal status on SEMA3F expression cannot be ruled out. Although in this initial series, there was no noticeable difference in SEMA3F expression among normal specimens, additional studies are needed to further evaluate independency from hormonal status. Interestingly, Joseph et al. recently reported on regulation of SEMA3B and SEMA3F by gonadotropins (FSH and LH) and estradiol in ovarian cancer cell lines (154). SEMA3F expression was enhanced by estradiol only indicating that SEMA3F was less sensitive to hormone treatment compared to SEMA3B, which was stimulated by FSH and LH in addition to estradiol (154). Therefore, these studies suggest that hormonal regulation of SEMA3F may play a role in the ovary and EOC, certainly, additional studies are necessary to further elucidate the mechanism(s) involved in hormonal regulation of SEMA3F.

In conclusion, our data suggests that the SEMA3F-NP pathway may be deregulated in EOC pathogenesis. Further investigation of SEMA3F and its receptors in epithelial ovarian cancer is warranted to delineate the molecular pathway(s) by which such deregulation may promote tumor progression and, if so, provide novel molecular targets for therapeutic intervention.
CHAPTER IV

SEMAPHORIN 3F DYSFUNCTION INDUCES TELOMERASE ACTIVITY IN OVARIAN CANCER CELLS

Abstract

SEMA3F is a secreted with potent angiostatic activity and although studies have indicated that loss expression of SEMA3F correlates with cancer progression, including EOC, less is known about SEMA3F regulation and/or dysregulation. Since studies of the nervous system suggest that calcium influences SEMA3 signaling and since cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is activated by calcium, we investigated the potential for calcium and CREB to regulate SEMA3F in OSE and ovarian cancer cells. In the present study, we demonstrated that both calcium and CREB suppress SEMA3F expression and CREB could specifically target the -4810 to -4418 region of the SEMA3F promoter. Since we have previously demonstrated that VEGF can target specific Sp1 sites within the hTERT promoter to stimulate telomerase activity and given the antagonistic relationship between SEMA3F and VEGF, we also evaluated the relationship between SEMA3F and telomerase using semi-quantitative RT-PCR, Western blot
analyses, and PCR-ELISA. We found a significant inverse relationship, in addition SEMA3F could regulate telomerase activity by targeting regions of the hTERT promoter, alternative to the VEGF responsive regions. These results demonstrate that calcium and CREB negatively regulate SEMA3F expression in OSE and ovarian cancer cells. SEMA3F loss is associated with an increase in telomerase activity. Moreover, ectopic expression of SEMA3F could mediate suppression of telomerase activity. Together, these data provide evidence that calcium and CREB can negatively regulate SEMA3F, in addition telomerase appears to be a novel molecular target of SEMA3F.

Introduction

Semaphorins are a large family of secreted, transmembrane, or GPI-anchored proteins that play a critical role as axon guidance molecules in the developing nervous system. However, they are also widely expressed outside the nervous system and influence a variety of cellular mechanisms including migration, proliferation, cytoskeleton rearrangement, angiogenesis and cancer progression (80, 103, 155-157). Secreted SEMA3s (158), which are distinguished by the presence of a C-terminal basic domain and function in a paracrine manner through a NP/plexin holoreceptor complex (82). Unlike other semaphorin family members, SEMA3s directly bind NP receptors with the exception of SEMA3E, however similar to other semaphorin family members the plexin receptor initiates signal transduction (82).
SEMA3F was originally isolated from a recurrent homozygous deletion in the 3p21.3 chromosomal region in small-cell lung cancer (159-161), a region also frequently lost in ovarian cancer (107). Normally SEMA3F functions to suppress tumor formation and/or progression. More specifically, exogenous SEMA3F inhibits tumor formation in several xenograft models (108, 149, 150). In addition, SEMA3F expression is associated with reduced blood vessel density and a nonmetastatic tumor phenotype, suggestive of angiostatic activity (150). The angiostatic activity of SEMA3F is due, in part, to competition of overlapping NP binding sites with the angiogenic factor VEGF (104). Alternatively, re-expression of SEMA3F in H157 lung cancer cells negatively affects VEGF mRNA expression due to decreased signal transducer and activator of transcription 3 (STAT3) phosphorylation and loss of Akt-dependent hypoxia-induced factor 1α (HIF-1α) protein (109, 112).

As seen in lung cancer (153), we and others have previously demonstrated decreased SEMA3F expression with EOC progression (154, 162), however, the positive and negative mechanisms regulating SEMA3F expression remain unclear. To date, DNA methylation correlates with suppression of SEMA3F expression (106). Similarly, Clarhaut et al. reported that the zinc finger transcription factor and key regulator in epithelial-mesenchymal transition, ZEB-1, down-regulates SEMA3F by targeting a specific E-box sites located in the CpG island of the SEMA3F promoter (112). Alternatively, chromatin remodeling by histone deacetylase inhibition contributes to SEMA3F activation (106). Recently, Joseph et al. demonstrated reproductive hormonal regulation of SEMA3F and
SEMA3B in ovarian cancer cells, such that, FSH, LH and estradiol stimulated SEMA3B, whereas SEMA3F could only be stimulated by estradiol (154).

The modulation of growth cone turning by guidance cues, like SEMA3F, is facilitated by calcium, an essential signaling molecule (163). Interestingly, levels of calcium fluctuate in preparation for rupture of the ovarian follicle and changes in calcium can stimulate damaged OSE to proliferate (164). Moreover, inhibition of calcium influx reportedly results in inhibition of ovarian cancer cell proliferation, invasion, and angiogenesis (165). Conversely, hormonal stimulation of SEMA3B and SEMA3F blocks invasion and angiogenesis in ovarian cancer cells (154).

Since loss of SEMA3F correlates with EOC progression and given the effects of calcium on SEMA3F in the nervous system and OSE in the ovary, calcium could potentially regulate SEMA3F in OSE and ovarian cancer cells.

In the present study, we investigated mechanisms that positively and negatively regulate SEMA3F and how loss may be related to tumor progression. We found that calcium suppressed SEMA3F expression in both OSE and ovarian cancer cell lines. We also identified the basic/leucine zipper (bZIP) transcription factor, CREB, as a novel SEMA3F transcriptional repressor, while two GC boxes are important for transcriptional activation of SEMA3F. Additionally we found, an inverse relationship between SEMA3F expression and telomerase activity such that, SEMA3F appears to suppress telomerase in a transcription-dependent manner. Consequences of decreased SEMA3F thus, involve mechanisms that promote cancer progression through induction of telomerase activity.
Methods and Materials

Cell lines and culture

A panel of ovarian carcinoma cell lines, including A2780s, A2780cp, C-13, CaOV3, ES-2, OV90, OV432, OV433, OV2008, OVCAR3, OVCAR5, PA-I, SW626, TOV21G and TOV112D cell lines, and SV40 large-T-antigen-immortalized ovarian surface epithelial (IOSE) cell lines, including familial history human immortalized ovarian surface epithelial 114 (FHIOSE 114), FHIOSE 117, FHIOSE 118, immortalized Moffitt Cancer Center 3 (IMCC 3), and IMCC 5 were used in this study (166). Cells were maintained in Medium 199/MDCB 105 (1:1) (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10µg/mL gentamicin (GIBCO BRL, Grand Island, NY) in a humidified 5% CO₂/95% air atmosphere.

Treatment with SEMA3F, VEGF, CBO-P11, calcium, BAPTA and metal ions

Two million IOSE and ovarian cancer cells were treated with recombinant SEMA3F (0.212µg/mL or 0.424µg/mL) (a generous gift from Dr. Klagsbrun), VEGF (50ng/mL) dissolved in BSA (Biosource, Camarillo, CA) and/or 1.3µM VEGF receptor inhibitor, CBO-P11, dissolved in Milli-Q water (Calbiochem, La Jolla, CA). Cultures were harvested at 24 hours and assayed for telomerase activity.

In order to determine the role of calcium in SEMA3F regulation, three million IOSE and ovarian cancer cells were treated ± 10 mM CaCl₂ or ± 1,2-bis(2-
aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, Sigma-Aldrich, St. Louis, MO), a calcium chelator (167). Cells were also treated with additional divalent cations in the form of chlorine salts. The salts used were ZnCl$_2$ (100µM, 1mM), MgCl$_2$ (10mM), CuCl$_2$ (10µM, 100µM), and CsCl$_2$ (1mM) (Sigma-Aldrich, St. Louis, MO). Following suspension of metal salts into medium, pH was adjusted to 7.4 then the solution was filtered using a 0.2-um syringe filter and added to cells in culture. Cells were harvested at 24 hours and assessed for SEMA3F expression.

**Transient transfection and small interfering RNA transfection**

Two million IOSE and ovarian cancer cell lines were transiently transfected using Program X-005 and Kit V on the Nucleofector device (Amaxa/Lonza, Walkersville, MD) with pSecTag (Invitrogen, Carlsbad, CA), pSecTag-S3F (generous gift from Dr. Tessier-Levigne) which encodes for the long splice form of SEMA3F, GFP (Amaxa/Lonza, Walkersville, MD), or pSG3-CREB (kindly provided by Dr. Cheng) plasmids. To inhibit expression of SEMA3F, two million OSE and ovarian cancer cells were transfected with SEMA3F siRNA or non-targeting control siRNA (Qiagen, Valencia, CA). Cells were harvested at 24 or 48 hours post-transfection. Each transfection was performed in three independent experiments.
To verify expression of SEMA3F and to determine the contribution of SEMA3F for transcriptional regulation of human telomerase reverse transcriptase (hTERT), semi-quantitative RT-PCR studies were performed, with each experiment repeated a minimum of three separate times. Total RNA was collected using TRizol reagent (GIBCO BRL, Grand Island, NY). One µg total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA for each sample. To ensure there was no DNA contamination, each sample for reverse transcription was prepared in duplicate, with one sample lacking reverse transcriptase. The cDNA samples were amplified using Applied Biosystems GeneAmp kit (Foster City, CA). The SEMA3F primers used were SEMA3F-Sense (AGCAGACCCAGGACGTGAG) and SEMA3F-Antisense (AAGACCATGCGAATATCAGCC) oligonucleotides (Sigma Genosys, The Woodlands, TX) and hTERT primers used were hTERT-Sense (CGGAAGAGTGTCTGGAGCAA) and hTERT-Antisense (GGATGAAGCGGAGTCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX). For an internal control β-actin primers were used; actin-Sense (GGGAATTCAAAACTGGAACGGTGAAGG) and actin-Antisense (GGAAGCTTATCAAAGTCCTCGGCCACA). PCR for S3F was performed for 35 cycles of 95°C for 90 s, 55°C for 90 s, and 72°C for 90 s. β-actin primers were added at cycle 18. PCR for hTERT was performed for 33 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. β-actin primers were added at cycle 17. Amplified products were then separated by gel electrophoresis, stained with 1x
SybrGreen (Cambrex Bioscience Rockland, Inc., Rockland, ME) and analyzed with the Kodak EDAS 120 Digital Analysis System.

**Telomerase assay**

To quantitatively detect changes in telomerase activity levels, cell lysates were assayed using the telomerase polymerase chain reaction enzyme-linked immunosorbant assay (PCR-ELISA) (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, cells were washed in PBS, trypsinized, and spun at 500 x g for 10 min. Pellets were washed twice in PBS, then resuspended in lysis buffer and kept on ice for 30 min, after which the lysates were centrifuged at 100,000 x g for 1 hour at 4°C. Lysates were then assayed using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) for the determination of protein concentration, according to the manufacturer’s instructions. In order to perform the telomerase PCR-ELISA within a linear range, cell extracts equivalent to 3μg of protein were used. Following PCR-ELISA, telomerase activity was detected using a ELx800 microplate reader (Bio-Tek Instruments, Winooski, VT) and recorded as absorbance units.

**Western blot analysis**

For Western blot analysis, cell lysates were solubilized in SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% b-mercaptoethanol), separated via 10% SDS-PAGE and electroblotted onto (0.45µm) PVDF membranes (Millipore, Billerica, MA) by wet transfer. Immunoblotting was
performed using antibodies directed against SEMA3F (1:5000, Millipore, Billerica, MA), phospho-CREB (1:2500, Cell Signaling, Danvers, MA), total-CREB (1:2500, Cell Signaling, Danvers, MA), phospho-Akt (1:2500, Cell Signaling, Danvers, MA), and total-Akt (1:2500, Cell Signaling, Danvers, MA). β-actin (1:10,000, Sigma-Aldrich, St. Louis, MO) was used as a loading control. Blots were visualized using the ECL Western Blotting Analysis System (Millipore, Billerica, MA) according to the manufacturer’s instructions. Blots were analyzed and scanned with ImageQuant image analysis software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Data represent mean relative intensities for S3F, phospho-CREB, total-CREB, phospho-Akt, and total-Akt, from three independent experiments normalized to the corresponding β-actin levels and expressed as mean net intensity.

**Luciferase reporter assay**

To measure promoter activity, 1.5µg SEMA3F luciferase reporter promoter constructs pGL3-6310-4013, -5131-3765, -5836-4013; previously described (106, 112) and pGL3 -4810-4418 and -4810-4013 or hTERT full-length and deletion constructs pGL3-1375, -1175, -976, -776, -578, -378, -181; hTERT constructs previously described (168), were transfected into IOSE and/or ovarian cancer cells using Program X-005 and Kit V on the Nucleofector device (Amaxa/Lonza, Walkersville, MD). In each experiment, the pRL-TK plasmid (100ng), encoding Renilla luciferase (Promega, Madison, WI), was co-transfected for normalization purposes. Luminescence was measured 48 hours after transfection using the
Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The pGL3-basic (promoterless) plasmid was used in each experiment to determine basal levels of luciferase. Reporter activity was normalized by calculating the ratio of Firefly/Renilla values and transcriptional activity was expressed as relative luciferase activity from triplicate ± S.E. from three independent experiments.

**Statistical analysis**

Samples for telomerase PCR-ELISA were run in triplicate from three independent experiments and the data subjected to Student t test analysis for determination of statistical significance for S3F suppression of telomerase. To determine the relationship between SEMA3F and telomerase activity we used a Spearman correlation coefficient (a nonparametric analog to the Pearson correlation coefficient) for statistical analysis.

**Results**

**Calcium suppresses SEMA3F expression in IOSE and ovarian cancer cells**

In agreement with earlier studies (154, 162), SEMA3F levels were lower in ovarian cancer versus normal cell lines (>45%). To ascertain whether calcium could mediate SEMA3F expression, we treated IOSE and ovarian cancer cells with calcium for 24 hours. SEMA3F mRNA and protein expression were evaluated using semi-quantitative RT-PCR and Western blotting, respectively (Figures 13A-B). Calcium suppressed SEMA3F mRNA expression in IOSE in
ovarian cancer cell lines (Figure 13A, top panel). Likewise, SEMA3F protein expression was suppressed in both IOSE and ovarian cancer cells following treatment with calcium (Figure 13B, top panel). Densitometric analyses indicated calcium suppressed SEMA3F protein expression in IOSE in ovarian cancer cell lines by 83% and 67%, respectively (Figure 13B, top panel).

To verify a role for calcium in SEMA3F inhibition, IOSE and ovarian cancer cells were treated with a calcium chelator, BAPTA, for 24 hours and assessed for SEMA3F RNA and protein expression. In agreement with above, calcium chelation with BAPTA induced SEMA3F expression by two-fold (Figures 13A-B, bottom panels). Cell viability following BAPTA treatment was not compromised as determined by trypan blue exclusion (data not shown). Additionally, to confirm specificity for calcium to suppress SEMA3F expression, FHIOSE 118 cells were treated with various divalent cations at varying concentrations for 24 hours (Figure 13C). Following treatment, samples were collected and assessed for SEMA3F protein expression. Additional metal salts examined resulted in SEMA3F expression similar to that of the control samples.
Figure 13. Calcium mediates SEMA3F Suppression in IOSE and Ovarian Cancer Cells.
Cells were treated with ± 10mM CaCl2 or ± BAPTA and were harvested at 24 hours to measure SEMA3F mRNA (A) by semi-quantitative and protein (B) expression by Western blot analyses. B-actin was used as a loading control and values are expressed as relative intensity of SEMA3F/Actin. (C) To confirm specificity of CaCl2, cells were treated with additional divalent cations and measured for SEMA3F protein expression at 24 hours after treatment.
Calcium-mediated suppression of SEMA3F is CREB-dependent

The bZIP transcription factor, CREB, has been implicated in regulating OSE survival and proliferation in response to gonadotropins during ovulation (169, 170). Furthermore in vitro and in vivo studies indicate CREB is frequently over-expressed in a number of human tumors, including EOC (170). Since CREB activation is dependent on calcium and cAMP (171, 172), we examined whether CREB contributes to negative regulation of SEMA3F expression. IOSE and ovarian cancer cells were transiently transfected with a CREB expression construct. CREB expression was verified by Western blot analysis (Figure 14A, left panel). Compared to control cells, ectopic expression of CREB inhibited SEMA3F mRNA and protein expression in IOSE and ovarian cancer cells by 75% and 68%, respectively (Figure 14A).

To determine whether CREB represents a novel transcriptional repressor of the SEMA3F gene, we performed luciferase reporter assays using [6310-4013] SEMA3F promoter and deletion constructs (previously described 16005989, 19177200) cotransfected ± CREB expression construct, to identify the promoter region(s) responsive to CREB. As shown in Figure 14B, greatest endogenous SEMA3F promoter activity occurred in the -4810 to -4418 region. CREB decreased luciferase reporter activity in IOSE cells by 54.2%, targeting the -4810 to -4418 SEMA3F promoter region (Figure 14B).
A. CREB - + - + IOSE118 OV2008

- + - + p-CREB

CREB

SEMA3F

Actin

0.06 0.65
0.08 0.54
0.08 0.72
0.42 0.65
0.63 0.16
0.34 0.11

B. Relative Luciferase Activity

Control CReB

- Con 6310-4013 5836-4013 5131-3765 4810-4418 4810-4013

* **
Figure 14. CREB down-regulates SEMA3F transcription in OSE and ovarian cancer cells.

(A.) FHIOSE 118 and OV2008 cells were transiently transfected with control or CREB expression vector and were harvested at 24 hours to measure SEMA3F mRNA by semi-quantitative RT-PCR and protein expression by Western blot analyses. B-actin was used as a loading control and values are expressed as relative intensity of SEMA3F/Actin. (B.) The SEMA3F luciferase reporter constructs were transfected ± CREB expression vector into FHIOSE 118 Firefly luciferase activity was measured and normalized to Renilla luciferase activity of the cotransfected plasmid pRL-TK for three independent experiments done in triplicate. (C.) The luciferase reporter constructs, with the [-6310-4013] SEMA3F promoter fragment, mutated or not (WT) for each or both 3 and 4 of the GC boxes present in the CpG island of this fragment, were transfected into FHIOSE 118 cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity of the cotransfected plasmid pRL-TK for three independent experiments done in triplicate. Bars, SE. Statistical analysis was performed with Student’s t-test: *p<0.05, **p<0.01.
SEMA3F promoter region -4810 to -4418 is required for expression in IOSE

MCF7 breast cancer cells, which express high levels of SEMA3F, have
promoter activity in a region surrounding the CpG island (106), whereas IOSE
and ovarian cancer cells that express SEMA3F demonstrate the highest
luciferase induction within a portion of the CpG island located at -4810 to -4418.
This region excludes two E-box sites but contains four putative GC-box sites
(Figure 14B-C). Therefore, our results suggest that the GC box sites are
important for positive regulation of SEMA3F in IOSE and ovarian cancer cells.
To determine the contribution of each GC box site for SEMA3F promoter activity
we performed additional luciferase reporter assays with [-6310-4013] SEMA3F
promoter reporter constructs containing all the GC box sites and with promoter
constructs with mutations in each GC box (GC mut1, GC mut2, GC mut3, GC
mut4) or combined GC box mutations (GC mut3-4). Mutations in GC box 2 or 4,
significantly decreased luciferase activity (54%, 58.6%) in FHIOSE 118 cells
(Figure 14C, right panel) compared to the wild type sequence -4810-4418 which
had the highest promoter activity. Taken together, these results suggest that GC
box sites 2 and 4 are necessary to induce SEMA3F expression in IOSE and
ovarian cancer cells, while, a nearby CREB binding site negatively regulates
SEMA3F.
SEMA3F expression is inversely correlated with telomerase in IOSE and ovarian cancer cell lines

In the ovary, telomerase is absent in normal OSE and pre-malignant lesions, while tumor cells from both ascites fluid and ovarian carcinomas express telomerase activity (173, 174). Previously we reported that VEGF can induce telomerase activity in an ERK1/2-dependent manner in ovarian cancer cells by targeting Sp1 binding sites within the proximal 976- to 378- regions of the hTERT promoter (175). Additionally, we have demonstrated that calcium promotes de novo telomerase activation in telomerase-negative IOSE cells and elevates endogenous activity in telomerase–positive ovarian cancer cell lines (167).

Using semi-quantitative RT-PCR, we surveyed normal and ovarian cancer cell lines for expression of SEMA3F and hTERT, the reverse transcriptase and rate limiting component of telomerase. SEMA3F RNA was strongly expressed in IOSE cell lines, while there was a marked decrease in SEMA3F expression in ovarian cancer cell lines (Figure 15A). As expected telomerase-negative IOSE cells demonstrated no hTERT expression, whereas all ovarian cancer cell lines expressed strongly hTERT mRNA (Figure 15A). SEMA3F protein expression was significantly inversely correlated with telomerase activity, as determined by Western blot and PCR-ELISA, respectively (Spearman correlation coefficient, r = -0.47, p = 0.035; Figure 15).

Interestingly, supporting a role for loss of SEMA3F with increasing tumor progression and EOC progression (i.e. tumor aggressiveness), we observed that parental chemo-sensitive cell lines A2780s, OV2008, and OV432 demonstrated
higher SEMA3F expression and lower telomerase activity compared to the chemo-resistant daughter cell lines A2780cp, C-13, and OV433 (Figure 15B).

Figure 15. SEMA3F expression is inversely correlated with telomerase in OSE and ovarian cancer cells. (A.) Endogenous hTERT mRNA and SEMA3F protein expression were measured in IOSE and ovarian cancer cell lines by semi-quantitative RT-PCR and Western blot, respectively. β-actin was used as a loading control. (B.) Graphical depiction of denistometric analyses of SEMA3F/β-actin protein expression and telomerase activity, which was measured by PCR-ELISA and values are expressed as relative telomerase activity from triplicate samples. (Inset) Western blot indicating higher SEMA3F expression in cancer cells correlates with chemo-sensitive cell lines versus their respective chemo-resistant daughter cell lines.
SEMA3F mediates suppression of telomerase activity in ovarian cancer cells

Given the inverse relationship we found between SEMA3F and telomerase, we examined whether SEMA3F could regulate telomerase activity in ovarian cancer cells. Ectopic expression of SEMA3F in OV2008 and C-13 cell lines was confirmed by semi-quantitative RT-PCR and Western blot and resulted in a marked decrease in hTERT mRNA expression in ovarian cancer cells compared to empty vector (Figure 16A). Similarly, telomerase activity was suppressed on average by 35% as determined by PCR-ELISA (Figure 16B). To further validate the specificity of SEMA3F for telomerase suppression we used siRNA to inhibit SEMA3F in FHIOSE 118 and OV2008 cell lines (Figure 16C). Interestingly, suppression of SEMA3F in OSE resulted in de novo telomerase activity (42.6%). Likewise, telomerase activity increased in ovarian cancer cells by 43.5% (Figure 16C).

SEMA3F targets the 378-region and 181-region of the hTERT promoter

Since, ectopic expression of SEMA3F decreased hTERT mRNA (Figure 16A, right panel), using full-length and deletion reporter constructs of the hTERT promoter, we performed a luciferase reporter assay to identify the promoter region(s) responsive to negative regulation by SEMA3F (Figure 16D). Compared to endogenous promoter activity, SEMA3F suppressed luciferase activity in the 378-bp and 181-bp hTERT promoter regions in OV2008 cells (Figure 16D). In contrast, SEMA3F failed to suppress activity of the full-length, -1175-bp, -976-bp,
and -776-bp hTERT promoter regions. The highest luciferase inhibition was achieved using the 181-bp or core hTERT promoter, where we observed a 1.5-fold decrease (Figure 16D). These results demonstrate that SEMA3F can negatively regulate telomerase by targeting the 378-bp and 181-bp regions of the hTERT promoter, in ovarian cancer cells.
Figure 16. SEMA3F suppresses telomerase activity in ovarian cancer cells. OV2008, C-13, and other ovarian cancer cells were transiently transfected with control or SEMA3F cDNA and were harvested at 24 hours after transfection. Over-expression of SEMA3F was confirmed by (A, left panel) semi-quantitative RT-PCR and (A, right panel) Western blot analysis. Values are expressed as relative intensity of SEMA3F normalized to β-actin. (B) Transfectants were assessed for telomerase activity by PCR-ELISA. Values are expressed as relative telomerase activity (Absorbance450nm-Absorbance690nm) of triplicate samples. (C.) FHIOSE 118 and OV2008 cells were transiently transfected with control or SEMA3F siRNA and were harvested at 48 hours after transfection. Silencing of SEMA3F was confirmed by Western blot analysis. Values are expressed as relative intensity of SEMA3F normalized to β-actin. Transfectants were assessed for telomerase activity by PCR-ELISA. Values are expressed as relative telomerase activity (Absorbance450nm-Absorbance690nm) of triplicate samples. (D.) OV2008 cells were transfected with full-length and deleted hTERT luciferase reporter constructs ± SEMA3F expression vector. Firefly luciferase activity was measured and normalized to Renilla luciferase activity of the cotransfected plasmid pRL-TK for three independent experiments done in triplicate. Bars, SE. Statistical analysis was performed with Student's t-test: *p<0.05, **p<0.01.
SEMA3F and VEGF have opposing effects on telomerase activity

SEMA3F and VEGF are secreted proteins that share common co-receptors and activate signaling pathways but with opposing effects (151), therefore, SEMA3F may compete with VEGF to suppress telomerase activity. To validate a role for negative regulation of telomerase by SEMA3F and to expand upon the antagonistic relationship between SEMA3F and VEGF, we treated FHIOSE 118, PA-I and SW626 cells with recombinant SEMA3F ± VEGF or ± CBO-P11, a known VEGFR-2 inhibitor. As expected, treatment with VEGF induced telomerase activity in ovarian cancer cells, whereas SEMA3F suppressed telomerase activity in a dose-dependent manner (Figure 17). When VEGF was administered with a lower concentration of SEMA3F telomerase activity was similar to that of the control; however with a higher concentration, SEMA3F was able to overcome competition with VEGF and suppress telomerase activity (Figure 17). Additionally, SEMA3F inhibition of telomerase appears to occur, in part, through the VEGFR-2 signaling pathway as indicated by the recovery of telomerase activity following treatment with both SEMA3F and CBO-P11 (Figure 17).
Figure 17. SEMA3F and VEGF have opposing effects on telomerase activity. FHIOSE 118, SW626, and PA-I cells were treated with ± VEGF, ± recombinant SEMA3F, ± CBO-P11. Cells were harvested at 24 hours after treatment and assessed for telomerase activity by PCR-ELISA. Transfectants were assessed for telomerase activity by PCR-ELISA. Values are expressed as percent of relative telomerase activity of triplicate samples.

Discussion

Given the correlation between SEMA3F dysregulation and EOC progression, it is imperative to elucidate the cellular mechanisms involved in SEMA3F regulation in normal physiological conditions and during neoplastic transformation in order to exploit the therapeutic potential of SEMA3F. The present study attempted to identify positive and negative regulators of SEMA3F in IOSE and ovarian cancer cells. We found that calcium specifically suppressed
both SEMA3F mRNA and protein expression, since treatment with additional metal cations had no effect on SEMA3F expression. Of interest are ongoing clinical trials investigating the potential of therapeutic strategies targeting inhibition of calcium influx via carboxyamidotriazole (CAI) treatment in ovarian cancers (176). CAI inhibits the influx of calcium into non-excitable cells, like endothelial or epithelial cells, and treatment with CAI results in inhibition of angiogenesis and signaling pathways that promote adhesion (165, 176). Taken together, these findings suggest SEMA3F may be a clinically useful downstream target of CAI therapy in the treatment of EOC.

Because calcium activates CREB (62, 171, 172), we investigated the potential involvement of CREB in calcium-mediated SEMA3F suppression. We identified two possible CREB binding sites in the SEMA3F promoter, one located near four putative GC box sites within the CpG island and the second site just upstream flanking the CpG island. We also determined that the -4810 to -4418 SEMA3F promoter region, which contains a portion of the CpG island, had the highest endogenous luciferase activity in IOSE and ovarian cancer cells and was most responsive to CREB.

Differences in effectiveness for CREB to repress SEMA3F promoter activity at CREB binding sites within and outside the CpG island may be attributed to the presence of multiple transcriptional start sites within the CpG island, direct interaction with other transcription factors/basal transcription machinery or interference due to methylation outside the CpG island (106). Preferential CREB-mediated SEMA3F promoter activity in the CpG island may
involve the Sp/KLF family of transcription factors which recognize elements within this region and which have been shown to interact with CREB (177). Conversely, Sp1 demonstrates a central role in TATA-less promoters and has been suggested to be an activator of SEMA3F (106, 178). Furthermore, our results indicate that GC boxes 2 and 4 were necessary and positive regulators for SEMA3F promoter activity in IOSE and ovarian cancer cells, so that discrete regions of the SEMA3F promoter are important for its expression. Future studies should address the interactions between CREB and other transcription factors or a component of the basal transcriptional machinery which may be involved is this indirect negative regulation of the SEMA3F gene. In addition, studies are warranted to further elucidate which specific Sp1/KLF family members are involved in positive regulation of the SEMA3F promoter via GC box binding sites 2 and 4.

Telomerase is a multimeric ribonucleoprotein that adds telomeric repeats to chromosomal ends, thereby stabilizing chromosome ends and conferring immortality to cells (173, 179). Since telomerase is expressed in more than 90% of human tumors and absent from most normal somatic cells and its expression correlates with tumor aggressiveness, telomerase is an attractive target for therapeutic intervention. Epithelial ovarian cancers have a propensity for therapeutic failure due to development of drug resistance and changes in telomerase may predict therapeutic outcome. We have previously reported that inhibition of telomerase improves chemosensitivity to cisplatin in cisplatin-resistant ovarian cancer cell lines (180). In the present study, we found that
chemo-resistant cell lines (A2780cp, C-13, OV433) were associated with increased telomerase activity compared to their respective parental cell lines (A2780s, OV2008, OV432), and subsequently compared to non-tumorigenic IOSE cells. Moreover telomerase activity was not only inversely related to SEMA3F expression but was transcriptionally regulated by SEMA3F. Interestingly, while we have previously shown VEGF and LPA activate telomerase activity by targeting Sp1 sites in the -978 to -378 region of the hTERT promoter (175), here we show that SEMA3F suppresses telomerase activity by targeting the -378 region and hTERT core promoter region. These results are consistent with earlier studies showing that expression of SEMA3F confers a poorly vascularized and non-metastatic phenotype (150) so that together, these results suggest that as an alternative to inhibiting angiogenesis, SEMA3F may also inhibit tumor progression and survival by negatively targeting telomerase.

Several transcription factors have been reported as positive and negative regulators of hTERT transcription. Sp1 protein has been shown to specifically target the hTERT core promoter and studies indicate that Sp1 sites may contribute to basal promoter activity (181-183). Additionally, mechanisms involving E-box binding proteins which are known to heterodimerize with a variety of transcription factors with helix-loop-helix domains, such as c-Myc or Mad-related family members, are implicated in both activation and repression of hTERT (184, 185). Several lines of evidence indicate transcriptional repression is associated with the function of a putative telomerase/hTERT repressor gene located on the short arm of chromosome 3 (186, 187). Furthermore, loss of this
region as a consequence of deletion or inactivating mutation may occur during 
neoplastic transformation (181, 187). Interestingly, this 3p region corresponds to 
the known 3p21.3 loci of the SEMA3F gene. These studies taken together with 
our findings indicating a significant inverse relationship between SEMA3F 
expression and telomerase activity, further implicates SEMA3F may function as a 
repressor of telomerase activity in telomerase-negative normal cells and this 
mechanism becomes defective during the carcinogenic process.

Lastly, the angiostatic activity of SEMA3F may be due to competition with 
VEGF for common co-receptors. In the present study, SEMA3F abrogated the 
ability of VEGF to enhance telomerase activity. SEMA3F-mediated telomerase 
occurred, in part, through VEGFR-2, supporting an antagonistic relationship 
between SEMA3F and VEGF due to competition for shared receptors as well as 
promoting opposing effects on a common downstream target.

SEMA3F acts like a tumor suppressor and dysregulation results in loss of 
expression which can promote tumor progression, angiogenesis and survival. 
To our knowledge, this is the first report of differential SEMA3F regulation in OSE 
and ovarian cancer cells. We found that the SEMA3F promoter was negatively 
regulated at -4810 to -4418 in a calcium and/or CREB-dependent manner, 
whereas positive regulation was mediated at GC box 2 and 4 within this same 
region. Additionally, we identified telomerase as a novel molecular target of 
SEMA3F. Since reactivation of telomerase is critical for cellular immortalization 
and malignant transformation, inhibition of telomerase by SEMA3F demonstrates 
an additional tumor suppressor function and may have clinical utility as adjuvant
therapy for enhanced chemosensitization. Clearly, further studies are warranted to exploit the therapeutic potential of these emerging and dynamic cancer regulators.
CHAPTER V

CONCLUDING REMARKS

Although most studies have focused on the role of angiogenic regulators for cancer development (37, 42, 53, 65), angiogenesis involves a delicate balance between angiostatic and angiogenic regulators and dysregulation of angiostatic regulators also contributes to neoplastic transformation, promotes tumor progression, and supports chemoresistance. Therefore, given the overall poor outcome of patients with ovarian cancer, filling fundamental gaps in knowledge regarding dysregulation of angiostatic regulators is both positively and clinically relevant for therapeutic implications. Novel findings herein define new roles for the angiostatic regulators, AS and SEMA3F, in ovarian cancer.

We report that AS, a potent inhibitor of angiogenesis, may be a useful urinary biomarker of high-grade and early stage ovarian carcinomas. uAS was shown to effectively discriminate normal healthy individuals from women with a broad range of benign gynecological pathologies, as well as ovarian cancer patients. However, given that several genetic insults are required for malignant transformation of OSE into neoplastic cells and since so many environmental variables influence the effectiveness of a screening method for ovarian cancer,
the possibility of a single biomarker to detect ovarian cancer seems unlikely. Recall, a recently proposed pathway which postulates that components of the Müllерian system, including the fallopian tube, may be the source of high-grade serous epithelial ovarian cancer rather than, or in addition to, the more traditionally accepted OSE (19, 20). This alternative cell of origin has been linked to the ‘p53 signature’, which characterizes serous tubal intraepithelial carcinoma (STIC) and high-grade serous histological subtypes (26). Similarly, the over-expression of high-mobility group AT-hook 2 (HMGA2) occurs in 70-80% of high-grade serous ovarian carcinomas and STIC and may represent an additional biomarker candidate (188). Therefore, establishing an association of high-grade serous carcinoma growth with increased production of p53, HMGA2 and/or other HMG family members, may identify additional ovarian cancer biomarkers in urine as well as other readily available bodily fluids such as saliva as done for other diseases (140, 188, 189).

Consequently, the future of early ovarian cancer detection is likely reliant upon the development of a panel of biomarkers, possibly including uAS, that collectively complement one another and meet the requirements for sensitivity, without compromising specificity. Such a sophisticated panel of biomarkers in conjunction with CA125, could then be used to augment imaging technologies and provide a viable two-step approach to screening. Certainly, improvements in early detection of ovarian cancer will reduce the mortality associated with this insidious disease.
This work also established a clinical and molecular relationship between loss of SEMAF expression with EOC progression and in agreement with previous studies suggesting a tumor suppressor-like role for SEMA3F (108, 149, 150, 159). In agreement with studies indicating calcium regulates SEMA3s in the nervous system, we found that SEMA3F was negatively regulated in a calcium/CREB-dependent manner in ovarian cancer cells while specific GC regions in the SEMA3F promoter are essential for SEMA3F expression in our ovarian model system.

In an attempt to expand upon the antagonistic relationship between SEMA3F and VEGF, we found an inverse relationship between SEMA3F expression and telomerase activity in IOSE and ovarian cancer cell lines so that, this is the first report to identify telomerase as a novel molecular target of SEMA3F. Consequently, loss of SEMA3F not only alleviates competition with VEGF which promotes angiogenesis, but also induces telomerase activity which confers immortality, tumor aggressiveness, and chemosensitivity. These studies, then, further support the antagonistic relationship between SEMA3F and VEGF such that activation of common co-receptors may activate different signaling pathways which results in opposing effects on a common downstream molecular target (Figure 18). Future studies are warranted to delineate the mechanisms contributing to SEMA3F function and dysregulation in ovarian cancer especially as it may pertain to tumor angiogenesis but also with regards to normal physiological ovarian processes.
Figure 18. Schematic of proposed SEMA3F signaling pathway. In this schematic representation of the antagonistic relationship between SEMA3F and VEGF, we illustrate our findings of an opposing effect on a common downstream molecular target, telomerase. We found that SEMA3F uses contrasting signaling pathways compared to VEGF. Additionally, SEMA3F could transcriptionally target specific regions of the hTERT promoter alternative to regions targeted by VEGF.
Lastly, AS is generated by cancer-mediated proteolysis of plasminogen and this is supported by reports identifying additional plasminogen cleavage products which demonstrate different degrees of angiostatic potency (75-77). Similar to AS, several lines of evidence implicate that regulation of SEMA3s, including SEMA3F, also involve proteolytic processing by pro-protein convertases, which are a family of calcium-dependent serine endopeptidases (190-192). These enzymes cleave substrates like hormones, growth factors, or neuropeptides, at specific consensus sequences to facilitate maturation from an inactive precursor form to biologically active peptides (190). Interestingly, all SEMA3s contain a conserved RXRR sequence in the C-terminal region, which is the major recognition site of the best studied pro-protein convertase, furin (190). Most recently it has been observed that furin processing of SEMA3F can affect its angiostatic potency (191). Likewise, furin-like pro-protein convertases reportedly target SEMA3B and render it inactivate (Varshavsky 18757406).

Furin has been associated with enhanced tumor invasiveness and metastasis since many substrates it activates are cancer-associated proteins (193). Overall, proteolytic processing by furin and other pro-protein convertases can either activate or inactivate SEMA3 signaling (191). Taken our findings that calcium suppressed SEMA3F and given that furin is calcium dependent, in our ovarian model system proteolytic processing may be an important mechanism involved in SEMA3F regulation. Since elevated levels of proteases have been linked to the malignant phenotype in a wide variety of tumors, including serous epithelial carcinoma of the ovary (125), future studies investigating dysregulation
of angioregulators, including the interrelationships between AS and SEMA3F, may provide clinically important information about the consequences of aberrant expression of enzymes and proteins involved in proteolytic cleavage.

In summary, research focused on angiostatic regulators, such as AS and SEMA3F, may not only benefit ovarian cancer patients, but may also have implications for other cancers. By elucidating the mechanism(s) responsible for dysregulation of SEMA3F, it may be possible to develop a therapeutic intervention that would not only disturb the vasculature by acting as a VEGF competitor but, by targeting telomerase positive cells, could act specifically on tumor cells. Likewise, AS not only has therapeutic potential but has promise as a biomarker for early detection of ovarian cancer. Therefore, a broader understanding of the wider roles of angiostatic regulators, as well as delineating the molecular mechanisms contributing to dysregulation of angiostatic regulators can provide insight into the etiology, clinical presentation and treatment of ovarian cancer. This may eventually allow women more choices for treatment and hopefully, reduce the mortality associated with this insidious disease.
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Appendix I List of Publications

Chapter II of this thesis has been published as:


Chapter III of this thesis has been published as:


Chapter IV of this thesis has been submitted for publication as:

ABOUT THE AUTHOR

Christina D. Drenberg received a Bachelor of Science degree in Biomedical Science with a minor in History, from the University of South Florida (USF). She entered the Ph.D. program in Fall of 2006 at USF’s College of Medicine, joined the Department of Pathology and Cell Biology in January 2007 and received a Master’s Degree in Medical Science in 2008.

While in the Ph.D. program, Ms. Drenberg was a Captain for a Relay for Life Team for the Association of Medical Science Graduate Students (AMSGS) and raised money for the American Cancer Society. In addition, she has served as Department Representative for AMSGS and represented the Graduate students by serving as Honor Council Representative. Ms. Drenberg has received several awards including Outstanding Presentation at the USF Health Research Day, the Dr. K Graduate Student Award for Leadership and Service, and Student Government Conference Presentation Program Grant Award. She has also been selected as a Department of Defense Teal Pre-doctoral Scholar in accordance with a New Idea Grant. Most recently, she was invited to present her research at the 2010 National Institute of Health National Graduate Student Research Festival and Ms. Drenberg will continue her training at St. Jude Children’s Research Hospital as a Post-doctoral Fellow.