Proteomic Identification of Mediators Implicated in the Metastatic Progression of Ovarian Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

Department of Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Ovarian cancer (OvCa) is the leading cause of death among gynecological malignancies, and is characterized by peritoneal metastasis and increased resistance to chemotherapy. Acquired drug resistance is often attributed to the formation of multicellular aggregates (MCAs) in the peritoneal cavity, which seed abdominal surfaces, particularly, the mesothelial lining of the peritoneum. Given that the presence of metastatic implants is a predictor of poor survival, a better understanding of the underlying biology surrounding OvCa metastasis may lead to the identification of key molecules that are integral to the progression of the disease, which therefore, may serve as practicable therapeutic targets. To that end, in vitro cell line models of cancer-peritoneal interaction and aggregate formation were used to identify proteins that are differentially expressed during cancer progression, using mass spectrometry-based approaches. First, we performed a proteomics analysis of a co-culture model of ovarian cancer and mesothelial cells, in which we identified numerous proteins that were differentially regulated during cancer-peritoneal interaction. We further validated one protein, MUC5AC, and confirmed its expression at the cancer-peritoneal interface. Next, we conducted a quantitative proteomics analysis of a cell line grown as a monolayer and as MCAs. After identifying a subset of overexpressed proteins, we determined that CLCA1 plays a role in MCA formation.
Additional studies using a CLCA1 blocker, as well as siRNA knockdown of CLCA1 in OvCa cells, resulted in a decreased ability of cancer cells to aggregate, a reduced ability to adhere to extracellular matrix components, and decreased cell viability. Moreover, we demonstrated that CLCA1 is able to regulate MUC5AC expression, as CLCA1-knockdown cells exhibited reduced expression of MUC5AC. In summary, the research presented in this thesis adds to our current understanding about ovarian cancer progression, and identifies two proteins that play a role in OvCa progression, which may serve as novel therapeutic targets.
Acknowledgements

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**List of Abbreviations**

α-SMA, alpha smooth muscle actin  
ACTION, adjuvant chemotherapy in ovarian neoplasm  
AD, anchorage-dependent  
AI, anchorage-independent  
ARID1A, AT rich interactive domain 1A  
BMI, body mass index  
BMP, bone morphogenetic protein  
BRAF, v-raf murine sarcoma viral oncogene homolog B1  
BRCA1, breast cancer 1, early onset  
BRCA2, breast cancer 2, early onset  
CA-125, cancer antigen 125  
CAF, cancer-associated fibroblast  
CCL2, chemokine (C-C motif) ligand 2  
CCL18, chemokine (C-C motif) ligand 18  
CCR2, chemokine (C-C motif) receptor 2  
CD4+, cluster of differentiation 4+  
CD163, cluster of differentiation 163  
CD20, cluster of differentiation 20  
CD44, cluster of differentiation 44  
CLCA1, calcium-activated chloride channel regulator 1  
CLIC4, chloride intracellular channel 4  
CM, conditioned media  
CSF-1, colony stimulating factor 1  
CTNNB1, catenin (cadherin-associated protein), beta 1  
CXCL12, chemokine (C-X-C motif) ligand 12  
CXCR2, chemokine (C-X-C motif) receptor 2  
DAB, 3,3’-diaminobenzidine tetrahydrochloride  
ECM, extracellular matrix  
ELISA, enzyme-linked immunosorbent assay  
EMT, epithelial-to-mesenchymal transition
ER, estrogen receptor
ERK2, extracellular signal-regulated kinase 2
ESR1, estrogen receptor 1
FABP4, fatty acid binding protein 4
FAP, fibroblast activation protein
FBS, fetal bovine serum
FDA, Food and Drug Administration
FDR, false discovery rate
FIGO, International Federation of Gynecology and Obstetrics
FoxP3, forkhead box P3
FXII, coagulation factor XII
GO, gene ontology
HA, hyaluronic acid
hADSC, human adipose-derived stem cell
HGF, hepatocyte growth factor
HPLC, high performance liquid chromatography
ICAT, isotope-coded affinity tag
ICON1, international collaborative ovarian neoplasm 1
IFN-γ, interferon gamma
IL, interleukin
IPA, ingenuity pathway analysis
iTRAQ, isobaric tag for relative and absolute quantification
KRAS, Kirsten rat sarcoma viral oncogene homolog
LC, liquid chromatography
LIF, leukemia inhibitory factor
LL-37, leucine, leucine-37
LPA, lysophosphatidic acid
MCA, multicellular aggregate
MCP-1, monocyte chemoattractant protein 1
MMP, matrix metalloproteinase
MRI, magnetic resonance imaging
MS/MS, tandem mass spectrometry
MSC, mesenchymal stem cell
MUC5AC, mucin 5AC
NF, normal fibroblast
NFA, niflumic acid
OvCa, ovarian cancer
PARP, poly (ADP-ribose) polymerase
PFS, progression-free survival
PI3K, phosphoinositide 3-kinase
PI3KCA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PLD, pegylated lysosomal doxorubicin
PMSF, phenylmethanesulfonyl fluoride
PTEN, phosphatase and tensin homolog
PVDF, polyvinylidene fluoride
qPCR, quantitative polymerase chain reaction
ROS, reactive oxygen species
SCX, strong cation exchange
SILAC, stable isotope labeling by amino acids in cell culture
STAT-3, signal transducer and activator of transcription 3
TAM, tumor-associated macrophage
TG2, transglutaminase 2
TGF-β1, transforming growth factor β1
TGFβ1-Ip, transforming growth factor β1 induced protein
TIA-1, T cell intracellular antigen-1
TIL, tumor-infiltrating leukocyte
TIMP-1, tissue inhibitor of metalloproteinase 1
TNF, tumor necrosis factor
TP53, tumor protein p53
uPA, urokinase-type plasminogen activator
VCAM1, vascular cell adhesion molecule 1
VEGF, vascular endothelial growth factor A
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Chapter 1 | Introduction

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1.1 Ovarian Cancer

1.1.1 Ovarian Cancer Statistics and Epidemiology

Epithelial ovarian cancer is the most lethal gynecological malignancy and accounts for 5% of all cancer-related deaths among women in North America. In the United States alone, the number of new cases of ovarian cancer this year is estimated to be 21,290 (1), and approximately 220,000 women are expected to develop it worldwide (2). The median age at time of diagnosis is 63 years; however, women who are genetically predisposed to the disease are often diagnosed at least ten years earlier on average (2). Currently, the overall 5-year survival rate is approximately 45%; however, this rate is contingent on the stage at diagnosis, as the 5-year survival rate can range from 95% for patients with localized disease, to a rate of 27% in advanced-stage cases (1). Unfortunately, the majority of patient cases (61%) present with a distant form of the disease (1).

Although the majority of ovarian cancer cases are sporadic (90-95%), there are several factors that influence the risk of ovarian cancer, with family history being the most important. Approximately 10-12% of ovarian cancer patients carry \textit{BRCA1} and \textit{BRCA2} germline mutations, while 2-3% have a family history of hereditary nonpolyposis colorectal cancer (or Lynch syndrome) (3-4). Moreover, polycystic ovaries and endometriosis have been connected with certain subtypes of ovarian cancer (2), including endometrioid and clear cell carcinomas (5-6). The risk of developing ovarian cancer increases with age, obesity (BMI $\geq 30$), fertility drug use, estrogen hormone replacement therapy, nulliparity, and exposure to talcum powder (4). On the other hand, a decreased risk has been observed with the use of oral contraceptives, a higher number of full-term pregnancies, fewer ovulatory events, a low-fat diet, tubal ligation and hysterectomy (2, 4, 7-8). Given that ovarian cancer is more common in industrialized
nations, it is believed that environmental factors, such as smoking, may also have an impact on the development of the disease (3, 9).

1.1.2 Classification and Histological Subtypes

The classification system used to define epithelial ovarian cancer is probably more complex than any other disease, as a number of biological aspects are taken into account, including tissue of origin, genetic mutations, tumor grade, and histopathological features. For this reason, treating ovarian cancer patients presents many challenges, as it is not a single disease, but is rather a collection of heterogeneous tumors that have distinct molecular and histological classifications (10). In general, ovarian carcinomas are classified as germ cell, sex cord-stromal, or epithelial tumors, depending on their cell of origin (3). Of these, epithelial ovarian cancers are the most common as they represent approximately 90% of all ovarian malignancies (3). As a result, most studies focus on this particular type of ovarian cancer, which is further categorized into different histological subtypes: high-grade and low-grade serous, clear cell, endometrioid, and mucinous tumors (2, 10).

High-grade serous carcinomas account for over 60% of epithelial ovarian cancers and are characterized by TP53 and BRCA1/BRCA2 mutations (2). The precursor lesion for these tumors has not been well defined. Although originally believed to develop from the ovarian surface epithelium, several observations have led to the more recent theory of the fallopian tube as the origin, which is based on the finding that the tissue morphology of high-grade serous tumors are similar to that of the fallopian tube (3). Furthermore, the identification of tubal carcinomas or serous tubal intraepithelial carcinoma in resected tissues from prophylactic salpingo-oophorectomies of women with familial risk has also supported the hypothesis of the tubal fimbriae as the site of origin (11-13). It is believed that malignant cells from the distal
fallopian tube implant onto the ovary, and serve as the source for most high-grade and low-grade serous ovarian cancers (14). Meanwhile, a few may arise from the malignant transformation of the ovarian surface epithelium (14). In general, low-grade serous carcinomas are considered to be indolent and harbor mutations in \textit{PI3KCA}, \textit{BRAF}, and \textit{KRAS} genes (2, 15). Endometrioid and clear cell carcinomas each account for 15-20% of epithelial ovarian cancer cases, and are strongly associated with endometriosis (10). Mucinous carcinomas are rare (3% of epithelial OvCas) (16), resemble the endocervical epithelium, and are often metastases to the ovary from tumors originating in the gastrointestinal tract (10).

In addition to the above guidelines, another classification system was created ten years ago that divides epithelial ovarian carcinomas into type I and type II tumours. This system was established using a combination of clinical, histological, and molecular genetic findings (16-17). Under this model, type I tumors are comprised of low-grade and slow-growing cancers that stem from borderline tumors. They are characteristic of low-grade serous, low-grade endometrioid, clear cell, transitional (Brenner), and mucinous carcinomas (5). These tumors display specific mutations that are uncommon in type II tumors, including \textit{BRAF}, \textit{KRAS}, \textit{PTEN}, \textit{ARID1A}, \textit{CTNNB1} and \textit{PIK3CA} mutations, and are usually indolent and genetically stable (16). In contrast, type II tumors include high-grade serous, high-grade endometrioid, malignant mixed mesodermal, and undifferentiated tumors, which are highly proliferative and are associated with frequent \textit{TP53} mutations (17-18). Since they are genetically unstable, they are often highly aggressive, and present themselves as advanced diseases (16).

Given that ovarian cancer subtypes are molecularly distinct, and derive from various non-ovarian tissues, many experts suggest that these subtypes should be treated as distinct
diseases (10). As a result, treatments need to be personalized for each patient in order to achieve the most favorable clinical outcome.

1.1.3 Ovarian Cancer Diagnosis and Treatment

1.1.3.1 Diagnosis

The lack of early symptoms often results in the late detection of ovarian cancer, which has important clinical implications, given that patient outcome is heavily dependent on disease stage at diagnosis (19). In addition, it is not uncommon for disease symptoms, such as abdominal pain, to be mistaken for disorders of the gastrointestinal tract (19). Other symptoms include urinary frequency, early satiety, and bloating (3). When a mass is suspected through physical pelvic examination, transvaginal ultrasound and serum concentrations of cancer antigen-125 (CA-125) are the main methods used during preoperative evaluation (2). However, poor sensitivity and specificity limitations are major caveats of CA-125, as increased levels have been reported in other conditions, such as lung disease, liver cirrhosis, endometriosis, and pelvic inflammatory diseases to name a few (20). Moreover, CA-125 displays poor sensitivity for early stage diseases (3). Once a suspicious adnexal mass has been confirmed, a biopsy or laparotomy is performed to determine its malignant potential, establish a histopathological diagnosis, as well as determine the stage of the disease (2). The International Federation of Gynecology and Obstetrics (FIGO) has established a classification system for staging ovarian cancers (2, 21). In general, stage I disease is limited to the ovaries, stage II cancers extend to the pelvis, stage III disease involves cancer spread to abdominal surfaces and/or lymph nodes, and stage IV cancers contain distant metastases to the liver, lung, and extra-abdominal sites (3). Serum levels of CA-125 are also useful for characterizing adnexal masses, as borderline and
invasive stage I tumors display low CA-125 values, compared to stage II-IV cancers (20). Depending on how far the disease has disseminated, total abdominal hysterectomy and bilateral salpingo-oophorectomy would be performed with the goal of achieving maximal cytoreduction (3).

1.1.3.2 Treatment

Standard Therapy

Several clinical features are examined to determine which available therapies would be most effective in yielding a favorable outcome. For instance, assessing histological subtype, tumor grade, stage, age, and the size of the tumor after primary cytoreduction allows physicians to make informed decisions regarding the implementation of anti-cancer strategies (22). Two randomized clinical trials, the International Collaborative Ovarian Neoplasm trial 1 (ICON1) and Adjuvant Chemotherapy in Ovarian Neoplasm (ACTION), have led to the use of adjuvant chemotherapy for the treatment of early stage disease (stages I and II) (23). The benefits of chemotherapy in early disease has been mostly observed in poorly differentiated stage I diseases and stage II diseases; in contrast, well- or moderately differentiated stage I patients are not treated further after surgery (3). Optimal surgical debulking followed by either platinum/taxane-based chemotherapy (carboplatin and paclitaxel) also continues to be the first-line treatment for advanced-staged patients with ovarian cancer (stages III-IV) (22, 24-25). The current recommendation is three cycles of paclitaxel and carboplatin for patients with early stage disease, and six cycles for those with advanced disease. Unfortunately, one of the major challenges faced by patients is cancer recurrence, as tumor cells acquire a chemoresistant phenotype making them insusceptible to additional anticancer agents (22). In such cases,
patients with recurrent OvCa are further subgrouped into platinum-sensitive or platinum-resistant, which is used to decide on subsequent treatment approaches (3).

**Targeting Angiogenesis**

To sustain growth and development, tumors need a constant supply of oxygen and nutrients, often achieved through the formation of new blood vessels. These angiogenic mechanisms are induced by several pro-angiogenic factors in the microenvironment, the most notable being vascular endothelial growth factor A (VEGF-A). Since the contribution of angiogenesis to tumor spread is essential, anti-angiogenic therapies targeting tumor vasculature have proven to be a practical approach when used in combination with other conventional treatments in targeting ovarian cancer (26). Phase III clinical trials evaluating the efficacy of the VEGF-A inhibitor, bevacizumab, have shown that when administered during and after carboplatin/paclitaxel treatment, there were improvements in progression-free survival (PFS) in patients with advanced OvCa, and for those with high risk of disease progression (27-28). Similar improvements in PFS resulted when bevacizumab was administered during and after chemotherapy in platinum-sensitive recurrent OvCa (29). Unfortunately, no benefits to overall survival were achieved; however, these findings have led to the approval of bevacizumab in Europe and the United States to be used in combination with standard therapy for treating platinum-resistant recurrent OvCa.

**Poly(ADP-ribose) polymerase (PARP) inhibitors**

Given that a subset of patients harbors BRCA1/2 mutations, which are essential for homologous recombination repair, targeting DNA repair machinery using PARP inhibitors has also proven to be a successful alternative for cancer therapy (30). Essentially, PARP inhibitors work to prevent single-stranded DNA break repair by blocking PARP enzymatic activity or by
PARP trapping, which leads to an accumulation of double-stranded breaks. As a result, BRCA-mutated cells are not able to repair damaged DNA, thereby inducing synthetic lethality, as BRCA-mutated cells will undergo apoptosis (31-32). In the United States, the Food and Drug Administration (FDA) has recently approved olaparib as a treatment for women with advanced OvCa with germline BRCA1/2 mutations that have undergone at least three lines of therapy (32-33). In Europe, it has also been approved for use on recurrent disease following platinum-based chemotherapy (33). Current trials are underway that are assessing the combinational performance of olaparib with the angiogenic inhibitor, cediranib, in recurrent disease (33).

**Targeting Other Molecular Pathways**

It is becoming increasingly clear that cancer progression is a multi-step process that requires the recruitment of host cells in order to grow and metastasize. For this reason, the targeting the tumor microenvironment and molecular pathways have become attractive avenues for the development of therapies that will complement and improve current standard treatments. This has been exemplified by the success of angiogenic and PARP inhibitors. Meanwhile, ongoing studies are evaluating therapies that target receptor tyrosine kinases, folate receptor alpha, epidermal growth factor receptor and PI3K pathways (3, 25, 34). Further development of these strategies will transform the way OvCa patients are treated in the future.
1.2 The Role of the Tumor Microenvironment during Ovarian Cancer Progression and its Clinical Implications

Despite several routes of investigation over the past three decades, epithelial ovarian cancer remains the most deadly of all gynaecological malignancies among women, with the serous subtype being predominant to clear cell, endometrioid, and mucinous histological subtypes. Inherent to a subset of ovarian cancers is the local dissemination of tumor cells to the omentum and peritoneal surfaces, often accompanied by an accumulation of ascites fluid. Functionally, the role of ascites during cancer progression is to facilitate migration and spread of cancer cells to the omentum by providing a milieu rich in soluble growth factors secreted by neighbouring cells. During cancer progression (Figure 1.1), malignant cells derived from ovarian surface epithelium or extra-ovarian sites undergo rapid proliferation, leading to the establishment of a primary tumor. Cancer cells from the tumor may either invade underlying stroma or shed into the peritoneal cavity, where they form multicellular aggregates (MCAs) that remain floating in ascites fluid. These MCAs may then form adhesions and implant on the mesothelial lining of the peritoneum, followed by subsequent invasion and further metastasis.

For several years, it was believed that cancer progression was driven solely by cell-autonomous processes, however, there is now abundant evidence suggesting that tumor cells are vulnerable to contributing components present within their surrounding microenvironment (35). Thus far, many attempts to alter the behaviour of individual cancer cells have been developed as key chemotherapeutic strategies, such as disrupting cell cycle processes and other vital molecular pathways. Although not entirely successful, these strategies are effective at reducing tumor burden. However, one main limitation of standard chemotherapy includes its ineffectiveness at treating patient relapse, as the majority of patients face cancer recurrence
As a result, these pitfalls have sparked interest in the investigation of the role of the cancer microenvironment in OvCa pathogenesis, which may offer insights into the adaptive nature of these cells, as well as provide optional areas to target.

Traditionally, the microenvironment consists of any biological component that interacts with tumor cells, which ranges from stromal cells, to extracellular matrix molecules, to cytokines. One of the major difficulties within cancer research is dissecting the molecular mechanisms underlying heterogeneous tumor growth, as well as understanding the interplay between stromal components and tumor cells, and whether stromal cells participate cooperatively with other cell types to influence cancer behaviour. Most studies typically delineate cancer-host interactions with the perturbation of a single molecule or cell type, however, communication of stromal cells with other stromal components should also be considered. It is clear that at least two hallmarks of cancer, angiogenesis and the ability of cancer cells to invade and metastasize, are dependent on the tumor microenvironment, which highlights the potential for disrupting the tumor-host interface for therapeutic intervention (35). It is predicted that these interventions will contribute to a new era of personalized medicine, as targeted therapies will be combined with standard clinical treatments, which are anticipated to improve patient survival.

In the following sections, an overview of the different roles of various microenvironment components and how they influence tumor progression will be discussed, in addition to the emerging therapies that target these interactions. Moreover, current conventional treatments will be re-examined, particularly the effect that microenvironment-targeting agents have on existing therapies, as well as their ability to improve patient outcome when used in combination.
Figure 1.1 – Pivotal events during ovarian cancer progression. In the early stages of EOC pathogenesis, malignant cells undergo neoplasia, and establish a primary tumor (1). Cancer cells may become migratory and invade the stroma (2), or shed into the peritoneal cavity and form multicellular aggregates (3). These aggregates may then generate cell adhesions to the peritoneal wall (4), breach this layer, and further metastasize. [Musrap, N. and Diamandis, E.P. Mol Cancer Res, 2012;10:1254-1264]

1.2.1 Inflammatory cytokine and chemokine networks in ovarian cancer

Numerous studies have highlighted the association between inflammation and cancer, particularly in the context of tumor progression (36). Consequently, this phenomenon posed by inflammatory cells and cytokine networks presents challenges for cancer treatment, rendering alternative strategies to complement existing therapies or to treat ovarian cancer recurrence with single administration. Before considering the impact of inflammatory cells to cancer development, it is important to identify the cytokine population within the ovarian cancer...
microenvironment, which forms an intricate network of soluble mediators that have a profound impact on tumor growth, angiogenesis, and more importantly, infiltration of leukocytes.

One of the most prominent cytokine members implicated in inflammation is tumor necrosis factor-α (TNF-α), which is constitutively expressed in ovarian tumors and stromal cells, such as macrophages (37). Interestingly, only tumors of the serous subtype displayed a positive association between TNF gene expression and tumor grade (37). TNF-α has a multifaceted role in cancer spread through its autocrine and paracrine actions (38), which are exerted through the stimulation of other cytokines and angiogenic factors (39). For instance, stable knockdowns of TNF-α in ovarian cancer cell lines resulted in reduced production of these factors, including chemokine (C-C motif) ligand 2 (CCL2), interleukin-6 (IL-6), chemokine (C-X-C motif) ligand 12 (CXCL12), vascular endothelial growth factor (VEGF), and migration inhibitory factor (MIF) (39). More importantly, reduced tumor growth and vascularization was observed in mice injected with TNF-α-deficient cancer cells, when compared to mock-transfected cells (39). In a similar study, Charles et al. utilized an ovarian cancer mouse model to study the regulatory properties of TNF-α on various cytokines in the tumor microenvironment (40). The authors demonstrated that TNF-α/TNFR1 signaling in CD4+ cells is fundamental for tumor growth, which was also associated with increased IL-17 levels in malignant ascites (40). Treatment with a TNF-α neutralizing antibody resulted in lower ascitic levels of IL-17 and plasma levels of IL-6, in addition to decreased tumor burden and leukocyte infiltrate after eight weeks, compared to control mice (40). To explore the applicability of these findings in humans, the authors examined IL-17 ascitic levels and TNF-α serum levels in ovarian cancer patients, and found a decrease in patients treated with the TNF-α antibody, Infliximab (40). A phase I clinical trial in patients displaying advanced cancer also resulted in
lower IL-17 serum levels after Infliximab treatment (Table 1.1) (40-41). Another TNF-α antagonist, Etanercept, which is a soluble p75 TNF receptor that inactivates TNF-α by competitive binding, was also assessed in phase I trials to evaluate its efficacy in treating recurrent ovarian cancer. In this study, two cohorts of 17 and 13 patients, respectively, were treated with dosages of 25 mg, two (cohort 1) and three (cohort 2) times a week (Table 1.1) (42). Six out of 18 patients who received a minimum of twelve weeks of therapy reached disease stabilization; 11 and 13 patients (cohort 1) displayed a drop in IL-6 and CCL2, respectively (42). Taken together, these findings reveal TNF-α as a viable target for EOC treatment.

In addition to TNF-α, high levels of several other pro-inflammatory cytokines have been identified in ascites fluid from ovarian cancer patients, including IL-6, IL-8, CCL2, and macrophage inflammatory protein-1β (MIP-1β) (43-44). To evaluate the prognostic significance of IL-6 and IL-8 levels in ascites, Lane et al. correlated levels to a number of clinical measures, including progression-free survival (44). Using multivariate analyses, the authors concluded that IL-6 could serve as a predictor of shorter progression-free survival. Similarly, in an alternate study, high serum IL-6 levels were also correlated with poor prognosis (45). A monoclonal IL-6 antibody, Siltuximab, was shown to effectively abrogate IL-6 signalling pathways by suppressing Stat3 phosphorylation, leading to a decrease of downstream antiapoptotic factors (46). Interestingly, Siltuximab combined with paclitaxel enhanced sensitivity and cytotoxicity in a paclitaxel-resistant cell line, SKOV-3_TR; however, these observations could not be recapitulated \textit{in vivo} (46). Therapeutic efficacy of this agent was assessed in a phase II clinical trial with 20 patients displaying advanced platinum-resistant ovarian cancer (47). Of these, one patient had a partial response, while seven patients reached
disease stabilization, in addition to exhibiting decreased plasma levels of several cytokines including CCL2, CXCL12, and VEGF, suggesting that they are regulated by IL-6 (47).

Emerging evidence has demonstrated that pro-inflammatory cytokines and chemokines form complex networks with each other, which collectively influence events that drive metastasis. Recently, Kulbe et al. delineated a link between three mediators of inflammation, TNF, CXCL12, and IL-6, and their paracrine actions on tumor angiogenesis and leukocyte infiltration (48). As expected, treatment with Infliximab led to decreased levels of CXCL12 and IL-6, thus, illustrating their interdependency, and also resulted in reduced tumor growth, vascularization, and infiltration of myeloid cells (48). These seminal studies illustrate the ability of cytokines to form complexes that promote cancer pathogenesis, which may be disrupted by direct targeting of one molecule.

1.2.2 The Role of Tumor-Associated Stromal Cells

The OvCa microenvironment encompasses a diverse subset of host cells that acquire an altered behaviour when recruited to the tumor site. In this section, we will discuss the roles of these various tumor-associated cells including macrophages, fibroblasts, adipocytes, and mesenchymal stem cells in OvCa progression, along with agents that have been developed to disrupt the tumor-host interface. Figure 1.2 depicts examples of interactions that occur between malignant tumor cells and various microenvironment components.
Figure 1.2 – Interactions between tumor cells and various microenvironment components. Several of these interactions are mediated through the exchange of soluble factors, or via cell adhesion mechanisms. [Musrap, N. and Diamandis, E.P. Mol Cancer Res, 2012;10:1254-1264]

1.2.2.1 Tumor-Associated Macrophages

The recruitment of tumor-infiltrating leukocytes (TILs) during cancer was naturally perceived to be the body’s immune response to a solid tumor; however, numerous studies have revealed that distinct leukocyte populations, with the exception of lymphocytes, are in fact, tumor promoting rather than tumor inhibiting. On the other hand, lymphocytic infiltrates are associated with favourable prognosis and have been correlated with improved rates of progression-free and overall survival of OvCa patients (49-50). For example, a series of immunohistochemical studies revealed that the presence of lymphocyte markers, T cell
intracellular antigen-1 (TIA-1), FoxP3, and CD20, could be indicators of positive prognosis for patients displaying high grade serous OvCa (51). This suggests that distinct populations of T cells are recruited to the tumor site and impose cytotoxic effects; however, many cancer cells escape detection by the immune system. Although it is not entirely known how tumor cells evade immune surveillance, it has been previously shown that this escape may be mediated through the immunosuppression of T cells by stromal cells in ascites, referred to as Hospicells (52). Hospicells produce an abundant supply of nitrous oxide, which suppresses CD4+ T cell proliferation and cytokine production, while conferring chemoresistance in cancer cells (52).

Immune infiltrates also include a rich supply of macrophages, which are recruited by tumor cells through their secretion of chemokines, particularly MCP-1 (also referred to as CCL2) (53). It is well-established that tumor cells and macrophages engage in a bidirectional interaction through the exchange of soluble mediators, which influence cell behaviour and phenotype. For instance, after being recruited to the tumor site, tumor cells induce changes in macrophage secretion of cytokines, chemokines, as well as matrix metalloproteinases (MMPs), such as MMP9, that enhance tumor growth in mice (54). Alterations in cytokine production activate a tumor-associated macrophage (TAM) M2 immunosuppressive phenotype, which is representative of those found in ovarian tumors (55). Polarization of monocytes and macrophages towards an M2 phenotype, which is marked by an increased expression of CD163, IL-10, CCL18, IL-8, chemokine (C-C motif) receptor 2 (CCR2), and chemokine (C-X-C motif) receptor 2 (CXCR2), can be stimulated by coagulation factor XII (FXII) or thrombin (56-57). Interestingly, when treated with either FXII or thrombin, conditioned medium (CM) from TAMs increased ovarian cancer cell invasiveness, with IL-8 being identified as the major chemoattractant mediating this invasion (56-57). Several lines of evidence indicate that
activation of the M2 phenotype can also be induced by CM from OvCa cells (58), or more specifically, leukemia inhibitory factor (LIF), IL-6, and colony stimulating factor-1 (CSF-1) (59). Consequently, CSF-1 is elevated in tumor cells, displaying higher expression in malignant tumors compared to benign neoplasms, and has also been associated with poor prognosis (28-30). Another study revealed that immunosuppressive TAMs could be converted back to immunostimulatory macrophages upon treatment with interferon-γ (IFNγ) (60). IFNγ-stimulated TAMs secreted less tumor-promoting mediators, inhibited the production of TAMs from monocyte precursors, and, more importantly, enhanced the proliferation of CD4+ T cells (60). As such, local administration of IFNγ at the tumor site may synergize with other antitumor immunotherapies, and enhance T cell-mediated destruction of tumor cells (60).

One attractive method for direct targeting of both tumor cells and TAMs with chemotherapeutic agents involves the folate receptor, which is expressed on both cell populations and has been used for the uptake of folate-linked drugs via receptor mediated endocytosis (61). Turk et al. utilized this strategy to construct folate-conjugated liposomes and measured its uptake ability by cancer cells and TAMs using an in vivo mouse model that recapitulates advanced staged OvCa (61). Overall, liposomes linked to folate showed a greater targeting capacity towards TAMs than tumor cells, which highlights the utility of liposome-linked to folate for the delivery of drugs to TAMs (61).

In another study, Geller et al. (2010) assessed the implications of paclitaxel and carboplatin-based chemotherapy on MCP-1 expression in an ovarian cancer cell line, MA-148 (62). Following administration of either drug, mRNA expression of MCP-1 increased in MA-148 cells, which were further confirmed in vivo through mRNA validation of mouse tumors following mouse exposure to the same chemotherapeutic regimen (62). Further studies are
needed to address the impact of MCP-1 on ovarian tumorigenesis, as it may be a marker of poor prognosis since it is an indicator of TAM recruitment and could possibly facilitate tumor recurrence (62).

Currently, few treatments developed against tumor-associated macrophages have shown promising potential, the best known being Trabectedin (34). Trabectedin binds minor grooves in DNA and prevents cell cycle progression, and has been shown to inhibit the differentiation of monocytes into macrophages (Table 1.1) (63). In vitro production of pro-tumoral mediators such as CCL2 and IL-6, potent stimulators of cancer progression, was decreased in TAMs and ovarian tumor cells when treated with Trabectedin (63). Previously, a randomized phase III clinical trial, OVA-301, assessed the efficacy of Trabectedin in combination with pegylated lysosomal doxorubicin (PLD) compared to PLD alone, in patients with recurrent OvCa following platinum-based chemotherapy failure (64). The authors concluded that Trabectedin/PLD combination improved progression-free survival and overall response rate in patients displaying a platinum-free interval of more than six months, compared to patients receiving PLD alone (64, 65). In a more recent preclinical study, it was shown that Trabectedin inhibited tumor growth in xenograft models of clear cell carcinoma of the ovary, which further demonstrates its potential as either a first-line or second-line therapy for certain subgroups of ovarian neoplasms (66). As such, patients exhibiting high amounts of immune infiltration in tumors may benefit from agents targeting activated TAMs.

1.2.2.2 Cancer-Associated Fibroblasts

Fibroblasts are essential components of connective tissue that are normally recruited and ‘activated’ during wound repair. In addition to their role in wound healing, fibroblasts have also been extensively linked to tumor progression, and are well-recognized as one of the major
constituents of tumor stroma (67). Contrary to their behaviour in wound repair, rather than regressing to their ‘inactive’ form, fibroblasts associated with cancers remain activated, similar to wounds that never heal (67). These fibroblasts undergo a desmoplastic reaction by forming reactive stroma, thus deemed cancer-associated fibroblasts (CAFs) or myofibroblasts (67). During carcinogenesis, CAFs are characterized by their increased deposition of extracellular matrix components such as collagens and are often distinguished from normal fibroblasts (NFs) by their altered phenotype and expression of two myofibroblastic markers, α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) (67-68).

Several studies have revealed the ability of CAFs to control the differentiation of epithelial cells through the secretion of cytokines and soluble factors (67). The resulting paracrine signalling between cancer cells and CAFs results in the release of growth and migratory signals that enhance the invasion of malignant cells and promote tumor progression (67). As such, further investigation of the paracrine signaling axis between cancer cells and fibroblasts may yield novel candidates for therapeutic targeting. Although the contribution of fibroblasts to the oncogenic microenvironment was previously elucidated, few studies have examined the connection and impact of CAFs to OvCa progression.

Evidence of fibroblast-to-myofibroblast transdifferentiation via cancer and fibroblast mediated crosstalk has been illustrated in a number of in vitro studies using well-established ovarian cancer cell lines. For instance, activation of normal ovarian primary fibroblasts and their conversion to a myofibroblast phenotype was observed upon their stimulation with transforming growth factor-β1 (TGF-β1) or conditioned medium from SKOV3 cells, a human ovarian carcinoma cell line (69). This stimulation also resulted in increased cellular reactive oxygen species (ROS) levels, causing an up-regulation of chloride intracellular channel 4
(CLIC4). Higher CLIC4 levels correlated with increased expression of α-SMA, which provided a solid indication of a myofibroblast phenotype (69). In a similar study, CM from a highly metastatic ovarian cancer cell line, HO-8910PM, induced FAP-1α expression in vitro, and identified TGF-β1 and IL-1β as putative paracrine signals that mediated this fibroblast activation (70). Interestingly, elevation of cell surface FAP-1α was found to promote proliferation, adhesion, and migration of HO-8910PM cells, and hence, may serve as a promising molecule for targeting CAFs (70). Thus far, few efforts have been made to target FAP, including a phase I study with the monoclonal antibody, Sibrotuzumab, which was used in 20 and 6 patients with metastatic colorectal carcinoma or non-small cell lung cancer, respectively (Table 1.1) (71). In this study, one colorectal cancer patient displayed stable disease for two years, however, no other tumor responses were observed in the remaining patients (71). More recently, a DNA vaccine generated against FAP was shown to suppress tumor growth and increase lifespan in a mouse model exhibiting colon cancer (72). Vaccinated mice demonstrated a 1.5-fold increase in lifespan, which reveals FAP as a candidate target for immunotherapy-based treatment (72).

In addition to myofibroblast differentiation from normal fibroblasts, human adipose tissue-derived mesenchymal stem cells (hADSCs) induced by lysophosphatidic acid (LPA) have also been shown to achieve CAF phenotypic status (73). Congruent with previous studies, treating hADSCs with conditioned medium from cancer cells or ovarian cancer patient ascites fluid resulted in the up-regulation of α-SMA expression in these cells (73). Moreover, stimulation by LPA also resulted in increased production of CXCL12 via TGF-β1 autocrine signaling in hADSCs, which was abrogated when pre-treated with an LPA receptor antagonist (73).
As alluded to earlier, in ovarian cancer, tumor cells and CAFs also participate in a reciprocal exchange of soluble components, which leads to the activation of particular signaling networks (67). For example, it was demonstrated that cytokines present within medium conditioned by an ovarian clear cell carcinoma cell line, ES-2, induced urokinase-type plasminogen activator (uPA) mRNA transcription in fibroblast cells, which is a protease implicated in cancer invasion and migration (74). Moreover, a recent study suggested that a pre-metastatic niche is created in the omentum through the activation and proliferation of normal fibroblasts (NFs) that are stimulated by the release of TGF-β1 from cancer cells (75). The establishment of the pre-metastatic niche would provide an altered microenvironment that enhances tumor invasion and implantation on peritoneal surfaces, through the secretion of hepatocyte growth factor (HGF) and matrix metalloproteinase-2 (MMP2) (75). An inhibitor of the TGF-β type I receptor, A83-01, abrogated TGF-β1 signaling and reduced the proliferation and activation of normal fibroblasts, as well as reduced α-SMA and MMP-2 expression in SKOV3/NF tumors (75). Such interventions should be considered for further development, as targeting elements of signal transduction pathways between cancer and stromal cells will lead to reduced levels of OvCa metastasis. In a parallel study assessing the contribution of CAFs to metastasis, using myofibroblast-specific markers, Zhang et al. concluded that CAFs were more abundant in disease during advanced stages and were associated with increased lymphatic vessel and microvessel density in addition to lymph node and omentum metastases (76). Interestingly, cancer-associated fibroblasts isolated from ovarian cancer patients induced more cancer cell migration than fibroblasts extracted from normal ovarian tissues (76).

Targeting CAFs or their associated autocrine-paracrine signaling loops appears promising for the development of future ovarian cancer therapies. More recently, an approach
using magnetic resonance imaging (MRI) and optical imaging tracked the recruitment of pre-labeled fibroblasts to the ovarian cancer stroma, which lined the outer rim of the tumor and co-localized with angiogenic vessels (77). Consequently, this imaging technique provides a non-invasive approach to target stromal cells for cellular therapy in the future (77).

1.2.2.3 Omentum-Derived Adipocytes

Adipocytes have often been classified as energy storing residents of adipose tissue; however, recent studies suggest that these fat-storing cells may serve other functions as well. Their heterotypic interactions with malignant tumor cells have been documented in breast, ovarian, colon, and gastric cancers (78). Tumor-promoting effects of adipocytes have been linked to their secretion of adipokines, hormones, and growth factors into the cancer microenvironment, which enhance cancer cell migration and invasion (79). A study conducted by Walter et al. revealed that normal adipose cells stimulated the migration and invasion of estrogen receptor (ER)-negative breast cancer cells (80). This effect was mediated via a cytoskeletal element, cofilin-1, and its regulation of IL-6 secretion in adipocytes (80). Another study revealed that during co-culture with breast cancer cell lines, adipocytes acquired an activated phenotype, characterized by increased production of proteases and cytokines, IL-6 and IL-1β, as well as delipidation and a loss of adipocyte-associated markers (79).

In a comprehensive study, Nieman et al. used fluorescent tracking of cancer cells in murine models to demonstrate the preferential migration of ovarian cancer cells to the omentum, an organ enriched in adipose cells (78). Similar to previous studies, this migratory behaviour was mediated by adipokines (IL-6, IL-8, MCP-1, tissue inhibitor of metalloproteinase 1 (TIMP-1), and adiponectin) secreted by adipocytes derived from the omentum (78). Interestingly, co-culture of adipocytes and ovarian cancer cells induced lipolysis in adipocytes,
resulting in the transfer of free fatty acids to cancer cells, which in turn, stimulated tumor cell proliferation through the generation of energy via β-oxidation. Fatty acid binding protein 4 (FABP4) was identified as a putative mediator in the transfer of lipids to cancer cells (78). As such, emerging therapies for personalized medicine will include those targeting mechanisms that enhance tumor metabolism, as in this case, lipid metabolism (78). In addition, hormones derived from adipose cells such as leptin, have been associated with an increase in ER-positive ovarian cancer cell growth, as ERα can be transcriptionally activated through the signal transducer and activator of transcription-3 (STAT-3) signaling pathway (81). These findings suggest that consideration of ER status, as well as the growth-promoting effects of adipocytes on cancer cells should be taken into account in ovarian cancer patients who are also obese (81).

1.2.2.4 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) have recently been recognized as active cellular components that are recruited to the tumor microenvironment, and their multipotency permits their differentiation into a variety of different cell types. Interestingly, Coffelt et al. have demonstrated that this recruitment may be partly stimulated by LL-37 (leucine, leucine-37), which is a pro-inflammatory peptide of human cationic antimicrobial protein 18, in addition to other migratory signals (82). In an OvCa xenograft model, human bone marrow-derived MSCs differentiated into CAFs, which was confirmed by the elevation of markers specific to fibroblast activation (54). In addition, mesenchymal stem cell-derived CAFs produced soluble protumorigenic factors, including IL-6, which enhanced tumor growth and proliferation (83). Combining cancer-associated MSCs with tumor cells in vivo and in vitro has been shown to result in increased expression of the bone morphogenetic protein (BMP) signaling network, which has several pathological roles in cancer progression (84). To determine the phenotypic
changes that are orchestrated during MSC-to-myofibroblast differentiation, Cho et al. treated adipose tissue-derived MSCs with exosomes isolated from two ovarian cancer cell lines, OVCAR-3 and SKOV-3 (85). Exosome-treated MSCs displayed an elevation of α-SMA expression, which, again, is indicative of an activated fibroblast phenotype, and also resulted in increased production of protumoral cytokines, CXCL12 and TGF-β (85).

Chemoresistance and unresponsiveness following application of standard therapies is common in most cancer patients, and usually results in cancer cells acquiring a “cancer stem cell (CSC)-like” phenotype. This phenotypic change is often associated with epithelial-mesenchymal transition (EMT), a key biological event implicated in cancer metastasis. As such, a metastatic cell line, OVA433 treated with cisplatin, expressed higher levels of EMT and stem cell markers, and enhanced activation of ERK2 signaling (86). Blockage of ERK2 signaling using a MEK inhibitor repressed EMT and CSC markers, suggesting that targeting this pathway may minimize tumor recurrence, by reducing mesenchymal characteristics that enhance tumor migration (86).

Human MSCs have been recently evaluated for their potential use as vehicles in cancer therapy, by exploiting their ability to preferentially migrate to and proliferate at tumor sites (87). For example, such efforts have been undertaken by using MSCs transduced with recombinant adenoviruses encoding endostatin, an inhibitor of angiogenesis (88). As a result, SKOV3 cells were able to induce migration of transduced MSCs, which, in turn, conferred anti-proliferative effects on cancer through secretion of endostatin (88). Similarly, Hu et al. used human umbilical blood mononuclear cell-derived MSCs as delivery vehicles for administration of interleukin-21 to ovarian tumors in mice, which has been shown to boost antitumor immunity in murine models (89). As such, this treatment hindered tumor growth in addition to prolonging
These data, taken together, provide supporting evidence for the application of MSCs as gene delivery vehicles as a feasible therapeutic strategy.

1.2.3 Targeting Extracellular Matrix Components

Alterations in extracellular matrix (ECM) components have been well described in the context of tumor adhesion and invasion. Malignant cells are constantly changing cell adhesion surface molecules in response to signals in their surroundings, and this will enhance their ability to disseminate locally. For these reasons, targeting the interactions between certain cell adhesion apparatuses and the ECM has been proposed, including cell membrane integrins (90-91). Integrin-related mechanisms have been shown to be essential at different stages of OvCa progression. For example, multicellular aggregate formation is mediated via β1-integrins and their subsequent attachment to the mesothelium is partially dependent on α5β1-integrins (92, 93). Efforts to antagonize α5β1-integrin interactions were implemented in phase II clinical trials, using a chimeric antibody, Volociximab (Table 1.1), in patients with platinum-resistant advanced OvCa, although clinical efficacy was not accomplished (94). Moreover, targeting of αvβ3–integrins with antibodies and radiolabelled nucleotides in xenografts has opened up new avenues and opportunities for therapeutic intervention (95-97).

Another efficient approach for targeting integrin-related attachment to ECM components involves the perturbation of a protein cross-linker, tissue transglutaminase (TG2) (98). TG2 facilitates the construction of integrin and fibronectin networks; therefore, inhibition would result in decreased cellular adhesion (98). In a recent study, Khanna et al. sought to block its transpeptidase activity by using a high-throughput screen of small molecule inhibitors and overall, seven compounds were able to inhibit cancer cell adhesion by at least 50% (98).
Further development and initiation of clinical trials to test these small molecule inhibitors may render alternative strategies that will minimize cancer spread.

Apart from integrins, other ECM adhesion molecules that contribute to cancer migration and invasion involve interactions between hyaluronan (HA) and versican, which can be blocked by small hyaluronan oligosaccharides (99). More importantly, there have been several documented studies demonstrating the pivotal role of HA in the adhesion of cancer cells to the peritoneum, which is one of the early events of OvCa progression (100). As a result, inhibition of this early step in metastasis may lead to improved patient outcomes.

### Table 1.1 – Microenvironment-targeting agents used in various cancer clinical trials.

<table>
<thead>
<tr>
<th>Drug/Agent</th>
<th>Type</th>
<th>Target/Mechanism of Action</th>
<th>Impact on Microenvironment Regulation</th>
<th>Stage in Clinical Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infliximab</td>
<td>Monoclonal antibody</td>
<td>Binds to TNF-α with high affinity; TNF-α blocker</td>
<td>Decreases levels of pro-inflammatory cytokines; Inhibits actions of TNF-α</td>
<td>Phase I</td>
</tr>
<tr>
<td>Etanercept</td>
<td>p75 TNF receptor fusion protein</td>
<td></td>
<td></td>
<td>Phase I</td>
</tr>
<tr>
<td>Siltuximab</td>
<td>Monoclonal antibody</td>
<td>Neutralizes IL-6</td>
<td>Inhibits functional activity of IL-6</td>
<td>Phase II</td>
</tr>
<tr>
<td>Trabectedin</td>
<td>Tetrahydro-isouquinoline alkaloid</td>
<td>Binds minor groove of DNA, preventing cell cycle completion; causes apoptosis</td>
<td>Inhibits monocyte-to-macrophage differentiation; decreases production of pro-tumoral cytokines</td>
<td>Phase III</td>
</tr>
<tr>
<td>Sibrotuzumab</td>
<td>Monoclonal antibody</td>
<td>Binds to FAP</td>
<td>Targets major constituents of tumor stroma</td>
<td>Phase I</td>
</tr>
<tr>
<td>Volociximab</td>
<td>Monoclonal antibody</td>
<td>Binds α5β1 integrins</td>
<td>Blocks attachment of cancer cells to the mesothelium</td>
<td>Phase II</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Monoclonal antibody</td>
<td>Binds all isoforms of VEGF-A</td>
<td>Angiogenesis</td>
<td>Phase III</td>
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</tbody>
</table>
1.3 Ovarian Cancer Metastasis

Unlike most solid tumors that typically metastasize via the intravasation and extravasation of blood vessels, ovarian carcinomas often disseminate locally throughout the abdominal cavity. There are two key events that are integral to the development of distant metastases, which include the invasion of the peritoneum and the formation of cancer spheroids. As mentioned above, various cell adhesion and extracellular matrix components play a fundamental role in these two events. In this section, a summary of main findings describing the early events of ovarian cancer metastasis will be discussed.

1.3.1 Ovarian Cancer Progression to the Peritoneum

In the previous section, we highlighted the influence that various stromal cells have on cancer cell behavior through various tumor-host interactions. One of the key components of the ovarian cancer microenvironment includes the peritoneum, which is a large membranous layer that surrounds abdominal organs, and is composed of a layer of mesothelial cells. The ability of cancer cells to breach this layer often leads to intraperitoneal dissemination, and therefore, cancer involvement with the peritoneum is one of the most important prognostic factors for OvCa. It is believed that cancer interaction with the mesothelium regulates changes in the expression of lipids and proteins that influence cancer cell motility, invasiveness and adhesion (101-103). In particular, it has been demonstrated that factors secreted by mesothelial cells, such as LPA, fibronectin, and TGFβ1p, enhance the motility and adhesion of various ovarian cancer cell lines (101, 103-106). More recently, it has been demonstrated that mesothelial cells isolated from the omentum can stimulate the adhesion and growth of various ovarian cancer cell lines through the release of paracrine signals, including IL-6 and IL-8 (107). During peritoneal
metastasis, several cancer cell adhesion mechanisms are used, including hyaluronan and CD44 interactions to the extracellular matrix (100, 108-110), as well as β1-integrins to mesothelial monolayers (92-93, 111). In a previous study, blocking CD44 resulted in a reduction in abdominal spread in a xenograft mouse model of ovarian cancer (93). A few studies have also identified other molecules that are implicated in ovarian cancer dissemination, and have noted their role in assisting cancer cells to interact with the peritoneum, such as versican, VCAM-1, P-cadherin, vitronectin, plasminogen activator inhibitor type-I, MMP2, and several integrins (93, 102, 112-118).

1.3.2 Ovarian Cancer Spheroid Formation

In recent years, the ability of cancer cells to form multicellular aggregates (also referred to as spheroids) has gained much attention, as they display an increased resistance to current chemotherapy agents (119), and are often present in ascites fluid of OvCa patients. Cancerous cells that shed from the primary tumor acquire anchorage-independent growth by forming aggregates in an effort to avoid anoikis (120-121). This resistance to apoptosis has been demonstrated to occur via cell cycle arrest, or by activation of ERK1/2 and PKC (121). These aggregates later implant onto the mesothelial layer of the peritoneum and then invade the basement membrane. It is evident that during the formation of spheroids, cancer cells require cell adhesion molecules to form attachments with one another, such as integrins and cadherins, along with various extracellular matrix components. In an early study examining spheroid behavior, it was shown that interactions between α5β1 integrins and fibronectin contribute to spheroid formation (92). The authors also further demonstrated that β1 integrin was essential for spheroid adhesion to laminin, fibronectin, and type IV collagen, as blocking it with a monoclonal antibody resulted in no attachment (92). Moreover, α2β1 integrins were later shown
to promote the disaggregation of spheroids on ECM, and were essential for the activation of matrix metalloproteinases 2 and 9, which help degrade the sub-mesothelial matrix during cancer cell invasion of local tissues (122). Other proteinases that have been documented to regulate spheroid formation and subsequent dissemination include membrane type I matrix metalloproteinase and kallikrein-related peptidase 7 (123-124). More recently, it was discovered that P-cadherin not only promotes the formation of multicellular aggregates, but also plays a role in spheroid attachment to the peritoneum (114). Interestingly, inhibition of P-cadherin resulted in a reduced number of tumor implants on the peritoneal lining of intraperitoneal xenograft mouse models (114). Aside from cadherins, tight junctions have also been implicated in spheroid structures, as claudin 4-deleted cells required more time to form compact spheroids (125). Other studies examining spheroid biology and characteristics have revealed that aggregates tend to be associated with an invasive or stem-like phenotype, often displaying an increased expression of mesenchymal genes (126-131). This finding coincides with the invasive behavior exhibited by MCAs. More recently, apart from roles in cell adhesion, integrins and talins were found responsible for the activation of a myosin and contractile force used to displace underlying mesothelial cells (132). Again, this highlights the ability of spheroids to invade the peritoneal lining and underlying matrices. Lastly, large glycoproteins, such as vitronectin, versican, and mucin 16, have been shown to be important in the early stages of spheroid formation (133-135).
1.4 Proteomic Approaches to Study Ovarian Cancer Biology

Proteins are the main structural and functional components of a cell, and therefore, make suitable targets for anticancer strategies (136). Efforts to dissect the ovarian cancer proteome have been supported by a vast array of proteomics technologies, including mass spectrometry. Not only has mass spectrometry aided in protein discovery and identification, but has allowed for the delineation of differential protein expression via various labeling and label-free approaches (137). In this section, an overview of high throughput mass spectrometry-based approaches and how it has been implemented in ovarian cancer research will be discussed.

1.4.1 Proteomics Initiatives in OvCa

The past decade has witnessed an increased interest in exploring the ovarian cancer proteome, with the majority of initiatives using MS-based technologies to identify disease biomarkers and to better understand mechanisms of drug resistance (138-139). Moreover, our lab has spent the last ten years developing protocols that can be used for mass spectrometry analysis of biological samples and proximal fluids. In a conventional MS-based experiment, proteins extracted from biological samples representing various disease conditions are denatured, reduced, and alkylated. These resulting proteins are then digested into peptides using specific enzymes, such as trypsin. To reduce sample complexity, the resulting peptides can be fractionated prior to injection into the mass spectrometer, which is done by chromatography or electrophoresis (140). Several different fractionation techniques are available including capillary electrophoresis, size exclusion chromatography, isoelectric focusing, reverse-phase chromatography, affinity chromatography, and ion-exchange chromatography (140). Within the mass spectrometer, peptides are ionized and fragmented by collision-induced dissociation. The mass-to-charge ratios of product ions can be used to determine the amino acid sequences of
representative peptides, which can be used to identify proteins through various bioinformatics software. A typical protocol often used for shotgun proteomics experiments is shown in Figure 1.3.

Overall, our group’s discovery efforts have led to the comprehensive proteomic profiling of various ovarian cancer cell lines, tissues, and ovarian cancer ascites fluid, resulting in a collection of potential OvCa biomarkers using shotgun and glycoproteomic techniques (141-143). Other efforts have examined tissues to capture subtype-specific markers capable of differentiating different classes of ovarian tumors (144-145). Moreover, proteomic profiling of serum has also led to the identification of three novel serum biomarkers that have been incorporated into the FDA-approved multivariate index assay, OVA1, which is used to discriminate suspicious pelvic masses from benign conditions (146-148).

Apart from an interest in biomarker discovery, there have been several comparative proteomic studies that have focused on understanding drug resistance (138). Given that the majority of patients develop platinum resistance, treating recurrent disease poses several challenges. As such, identifying markers of treatment response and mechanisms of resistance may bring about new markers for patient monitoring, as well as novel therapeutic targets. Several studies have generated and profiled drug resistant cell lines that were established from chemosensitive parental cell lines and have discovered protein expression changes in pathways involved in stress response, apoptosis, and cell cycle (149-154). Moreover, other groups have investigated differential protein expression changes in the intrinsic mitochondrial apoptotic pathway by using an organellar proteomic approach (155-156). Overall, these studies have shed light on changes that occur in molecular pathways involved in acquired drug resistance. However, further validation of these findings in patient samples and studies that address the
biological outcome of these protein expression changes are needed to determine their potential as clinical markers or as therapeutic targets.

Figure 1.3 – Typical protocol for mass spectrometry-based shotgun proteomics experiments. Proteins are extracted from samples and digested into peptides, which are then resolved and ionized in the mass spectrometer following an optional step of fractionation. Mass spectra are obtained and searched in databases to identify proteins.

1.4.2 Quantitative Proteomics

Most comparative proteomics experiments now incorporate some type of quantitative method of assessment to facilitate data interpretation and to extract meaningful information from such experiments. More importantly, quantifying differential protein expression between different sample conditions is necessary for making biological conclusions and shedding new light on disease pathology. Various strategies that are capable of accurately quantitating proteins have been introduced, each with its own advantages and disadvantages. The field of
quantitative shot-gun proteomics is generally defined by two different approaches, which are based on labeling and label-free techniques (157).

1.4.2.1 Labeling Approaches

Quantitation of proteins is often achieved through metabolic or chemical labeling of biological samples from different conditions, in which stable isotopes are used to differentiate proteins from each biological sample (137). Since isotopes have different masses, labeled ions will display a shift in mass-to-charge ratio. Therefore, peak ion intensities between labeled and non-labeled spectra can be compared and used to calculate relative abundances. The main labeling techniques used in clinical proteomics research include isotope-coded affinity tags (ICAT) (158) and isobaric tags for relative and absolute quantification (iTRAQ) (159), both of which have the advantage of being applied directly to patient samples. On the other hand, experiments involving the manipulation of cell culture models are often carried out using a metabolic labeling approach, referred to as stable isotope labeling by amino acids in cell culture (SILAC). One of the main advantages of labeling proteins is that it allows for multiplexing, and therefore, many samples can be simultaneously analyzed on the mass spectrometer, which results in better accuracy and reproducibility (137). On the downside, labeling approaches are more expensive, require additional optimization, and result in less proteome coverage in comparison to label-free methods (137).

1.4.2.2 Label-Free Quantitation

Label-free methods have proven to be a reasonable, cost-effective alternative to the above-mentioned strategies. There are two main label-free approaches for the quantitation of proteins, which include spectral counting and chromatographic peak areas (137). The method of spectral counting is based on the total number of spectra identified for a particular protein,
under the assumption that the number of mass spectra correlates with protein abundance (160). However, this method only becomes reliable when a higher number of spectra is identified. On the other hand, using areas under the curve of extracted ion chromatograms is based on the assumption that the areas of peak ion intensities are correlated with protein abundance (137). This method often requires a high-resolution mass spectrometer (137).
1.5 Rationale and Goals

1.5.1 Rationale

Ovarian cancer is a highly metastatic disease that is marked by low rates of patient survival. It is widely accepted that the implantation of cancer cells on the mesothelium and the formation of spheroids are critical to the progression of the disease, as they facilitate cancer spread to local abdominal organs, such as the liver and bowel. Due to the aggressiveness of the disease, current chemotherapeutic strategies often show only short-term success. More recently, several initiatives surrounding the development of therapeutic agents that target overexpressed molecular pathways in ovarian cancer, as well as angiogenesis, have witnessed minimal improvements in patient survival. Such limited success in these newer treatment regimens can be attributed to the fact that the underlying biology of OvCa metastasis still remains poorly understood. Targeting key molecules integral to the occurrence of events leading to ovarian cancer progression may better complement existing approaches. Our lab has spent the past decade designing and executing protocols for the high-throughput proteomics analysis of ovarian cancer cells and proximal fluids, with the hopes of discovering novel soluble markers that could be found in serum. In this study, we opted to utilize similar platforms to identify proteins important for cancer progression by using metastatic models of ovarian cancer.

1.5.2 Hypothesis

We hypothesize that during the progression of the disease, unique interactions occur between cancer cells and between ovarian cancer and mesothelial cells that lead to the differential expression of proteins involved in the adhesion of cancer cells to each other (forming spheroids) and to the mesothelium. Using in vitro co-culture model systems and in vitro models of multicellular aggregate formation, we will be able to characterize these
proteomic alterations using a comparative MS/MS-based approach. Identifying the altered expression patterns of proteins at the cancer-peritoneal interface and during spheroid formation may offer new insight into the mechanisms of cancer progression. In turn, these molecules may represent viable therapeutic targets, whose mechanism of action can be blocked to inhibit peritoneal dissemination and resistance to chemotherapy, and thereby, improve patient survival.

1.5.3 Objectives

Over the past few years, advances in proteomic technologies have led to the quantitative identification of proteins in various biological samples using different labeling and non-labeling approaches. More specifically, mass spectrometry has proven to be an efficient discovery tool for mining biological fluids and tissues in the search for soluble biomarkers that could be used for the early detection of various pathologies. However, recent studies have adopted this technique to identify proteins and disease-associated pathways that become altered during various stages of cancer pathogenesis, particularly through the use of co-culture model systems that reflect specific biological states (68, 105, 161). In the present study, we aimed to capture the differential protein expression that occurs during the metastatic progression of ovarian cancer. Our overall goal was to establish robust in vitro model systems and conduct “discovery” high-throughput proteomics to identify novel targets that may be elevated during cancer and mesothelial interaction, as well as proteins that are overexpressed during ovarian cancer spheroid formation. A summary of the specific aims presented in the subsequent chapters is as follows:
1) Identify novel proteins that may be elevated during cancer-mesothelial interaction.
   a. Conduct a comparative mass spectrometry analysis on conditioned media derived from ovarian cancer (OVCAR-5) cells, mesothelial (LP-9) cells, and a co-culture of the two cell lines
   b. Validate differentially expressed proteins in *in vitro* cultures
   c. Examine expression of promising candidates in clinical samples

2) Characterize proteomic alterations during OvCa multicellular aggregate formation
   a. Perform a quantitative MS-analysis on a cell line (OV-90) grown in anchorage-dependent and anchorage-independent conditions
   b. Validate candidates in *in vitro* models of OvCa multicellular aggregation
   c. Determine the role of promising candidates in multicellular aggregation

3) Establish an association between candidates in OvCa and perform pathway analysis of metastasis-associated proteins
   a. Determine a biological link among candidate proteins
   b. Perform bioinformatics to identify common themes and pathways among candidate proteins
Chapter 2 | Proteomic analysis of cancer and mesothelial cells reveals an increase in Mucin 5AC during ovarian cancer and peritoneal interaction


A link to the published paper can be found at

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2.1 Introduction

Among gynaecological malignancies, epithelial ovarian cancer is the leading cause of death and accounts for 5% of all cancer-related deaths in North American women (1). Given that patients are usually asymptomatic during early stages of the disease, the majority are often diagnosed at a late stage, when the survival rate is low. For the past few decades, the first-line treatment for advanced stage patients has consisted of cytoreductive surgery in combination with platinum-based chemotherapy (22). Although these treatments elicit an initial tumor response, malignant cells eventually develop resistance, ultimately leading to cancer recurrence. Considering that overall survival rates of ovarian cancer patients have improved little since the introduction of platinum-based drugs, there is a renewed interest in the development of more effective therapeutic agents that could complement conventional approaches (10).

Ovarian cancer is often marked as a molecularly heterogeneous disease that encompasses a diverse group of tumors, which vary both histologically and genetically. As a result, treatment of the disease has proven difficult as standard chemotherapy often elicits different patient outcomes (10). While a subset of patients present with slow growing tumors, the majority acquire rapid proliferating high-grade serous tumors, which are often characterized by late-stage presentation and intraperitoneal spread to abdominal visceral organs, which is promoted by soluble factors present within ascites fluid (10, 162-163). During progression of the disease, cancerous cells disseminate to the peritoneal cavity and implant on the peritoneum, which contains a thin membranous lining composed of mesothelial cells. After colonizing and breaching this layer, malignant cells are able to invade and metastasize to local organs. Since the attachment and invasion of the peritoneum is essential to the outcome of the disease, further
insight into molecular processes by which this occurs will add to our current understanding about the early events of metastasis, before the cancer becomes too difficult to treat.

Numerous studies have suggested that cancer-host interactions within the tumor microenvironment are partly responsible in promoting cancer invasion and metastasis (75, 164). In particular, cancer cell interaction with the mesothelium results in the differential regulation of lipids and proteins that enhance OvCa cell motility, attachment, and invasiveness (101-103, 108, 110, 165). For example, various cell adhesion and extracellular matrix components, including β1-integrin, VCAM-1, hyaluronan, and CD44, have been shown to facilitate cancer attachment and invasion of the peritoneum (93, 102, 108, 112, 165-166). However, the underlying mechanisms of this biological interaction still remain largely unknown, as there may be many other molecular factors that play a role. Therefore, increased knowledge of this tumor-host interface may lead to the discovery of novel therapeutic targets. In turn, abrogation of these targets may inhibit peritoneal dissemination and enhance patient survival.

In the past decade, high-throughput proteomics has been an efficient discovery tool for mining biological fluids and tissues in the search for soluble biomarkers that could be used for the early detection of various pathologies. However, recent studies have adopted this technique to identify proteins and associated pathways that become altered during various stages of disease pathogenesis, particularly, through the use of co-culture model systems that reflect specific biological states (68, 105, 161, 167). In the present study, we aimed to delineate differentially secreted proteins during ovarian cancer and mesothelial cell interaction by conducting a global secretome analysis using a mass spectrometry (LC-MS/MS)-based approach. In our attempts, we utilized a direct in vitro co-culture model of an ovarian cancer cell line, OVCAR-5, and a mesothelial cell line, LP-9, and compared the secretome
composition of this model to that of OVCAR-5 and LP-9 secretomes. Our proteomic analysis resulted in the overall identification of 2554 non-redundant proteins, whereby a subset was found to be differentially expressed in our co-culture model, which may reflect biological interactions at the cancer-peritoneal interface. Specifically, from our proteomic analysis, mucin 5AC (MUC5AC) was identified as our top candidate, which was also elevated in two other co-culture models (BG-1/LP-9 Co and OV-90/LP-9 Co) and in patient ascites fluid. Taken together, our approach reveals several proteins that are elevated during the interaction between ovarian cancer cells and the peritoneum. Further investigation of their role in OvCa pathogenesis is warranted.
2.2 Materials and Methods

2.2.1 Cell lines

The human ovarian cancer cell line, OVCAR-5, was obtained from the Fox Chase Cancer Centre (Philadelphia, PA). BG-1 cells were provided by Dr. Henri Rochefort (Montpellier, France), while the OV-90 (ATCC CRL-11732) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). All ovarian cancer cell lines were of the serous subtype. Primary human peritoneal mesothelial cells, LP-9, were purchased from the Coriell Institute for Medical Research (Camden, NJ). All ovarian cancer cell lines were grown in RPMI 1640 medium (Wisent) supplemented with 10% characterized fetal bovine serum (FBS) (Thermo Scientific). LP-9 peritoneal cells were grown in a 1:1 mixture of Ham’s F-12 medium/Medium 199 (Invitrogen) containing 10% FBS, 10 ng/mL epidermal growth factor (Reprokine Ltd.), and 0.4 ug/mL hydrocortisone (Sigma Aldrich). All cell lines were cultured in a humidified incubator adjusted to 37°C with an atmosphere of 5% CO2.

2.2.2 Establishment of monocultures and co-cultures of cancer and peritoneal cells

Proteomic Analysis

OVCAR-5 and LP-9 cell monocultures were established by culturing each cell line in three T175 cm² flasks using their respective growth media containing 10% FBS, as described above. Upon reaching 70% confluency, cells were washed three times with PBS (Wisent) and grown in 30 mL of chemically defined Chinese hamster ovary serum-free medium (Invitrogen) supplemented with 8mM L-glutamine (Invitrogen) for 48 hours. Co-cultures were also constructed in triplicates using T175 cm² flasks, as OVCAR-5 cells were plated over a confluent layer of LP-9 cells. Briefly, OVCAR-5 cells were washed, trypsinized, centrifuged (5
min at 450 x g), washed with PBS, resuspended in LP-9 media containing 10% FBS, and subsequently added to the monolayer of LP-9 cells. Cells were allowed to attach overnight before the co-cultures were washed three times with PBS and changed to serum-free media. After 48 hours, conditioned media (CM) was collected from each flask and centrifuged at 450 x g for 5 min to remove cellular debris. Total protein was measured using a Coomassie Blue (Bradford) total protein assay. Approximately 1mg of total protein from each replicate was subjected to further LC-MS/MS sample processing as described below.

**mRNA Expression Analysis**

Indirect one way co-cultures were constructed by using conditioned media from OVCAR-5 cells to stimulate LP-9 cells, and vice versa. Stimulations were done for approximately 24 hours, and cell pellets were collected and used for mRNA expression analysis.

Indirect two way co-cultures were also developed using cell culture inserts with pore sizes of 0.4 µm (Becton Dickinson and Company, NJ, USA). LP-9 cells were plated in six-well plates, which were then overlaid with cell culture inserts containing OVCAR-5, OV-90, or BG-1 ovarian cancer cells. After co-culturing for 24 hrs in normal growth media with 10% FBS, cell pellets were collected and washed, before undergoing total RNA extraction.

### 2.2.3 Sample processing for LC-MS/MS-based protein identification of secretomes

Conditioned media were dialyzed using a 3.5 kDa molecular weight cut-off porous membrane (Spectrum Laboratories, Inc., Compton, CA) in 4 litres of 1mM ammonium bicarbonate buffer at 4°C overnight. A total of three buffer exchanges were completed before freezing samples at -80°C. Frozen samples were lyophilized to complete dryness using a ModulyoD Freeze Dryer (Thermo Electron Corporation). Samples were then denatured with 8
M urea, reduced with 200 mM dithiothreitol at 50°C for 30 min, and alkylated with 500 mM iodoacetamide with shaking in the dark for 1 hour. Using NAP5 sephadex columns (GE Healthcare), samples were then desalted, frozen at -80°C, and lyophilized to complete dryness. Following lyophilization, samples were resuspended in 50 mM ammonium bicarbonate, water, and methanol. The samples were digested with trypsin overnight at 37°C using a 1:50 trypsin/total protein concentration ratio. Mobile phase buffer A (0.26 M formic acid, 10% acetonitrile; pH 2-3) was added to each digested sample, which were then subjected to strong cation exchange (SCX).

2.2.4 Strong cation exchange (SCX)-high performance liquid chromatography

Tryptic peptides were fractionated with an Agilent 1100 system using a one hour gradient of mobile phase A buffer, and peptides were eluted with the same buffer as mobile phase A SCX buffer with the addition of 1 M ammonium formate. Samples were then injected into a 500 µL loop that was connected to a PolySULFOETHYL aspartamide column containing an anionic polymer with pore sizes of 200 Å and a diameter of 5 µm (The Nest Group Inc., Southborough, MA). The fractionation was monitored at a wavelength of 280 nm, and fractions that were collected every two minutes from 24 to 50 minutes with a flow rate of 260 µL/min were used for further analysis. Fractions with a low peak absorbance were pooled, which resulted in a total of 12 fractions per sample replicate. Each fraction was diluted in order to obtain a final concentration of approximately 5% acetonitrile.

2.2.5 Mass spectrometry (LC-MS/MS)

Peptides were purified and extracted from SCX fractions using OMIX C18 Pipette Tips, and were eluted in 70% MS Buffer B (90% acetonitrile, 0.1% formic acid, 10% water, and
0.02% trifluoroacetic acid) and 30% MS Buffer A (5% acetonitrile, 0.1% formic acid, 95% water, and 0.02% trifluoroacetic acid). A total of 40µL of each fraction was loaded onto an EASY-nLC system (Proxeon Biosystems, Odense, Denmark), which was directly transferred online to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). Using a 5cm C18 analytic column, peptides from each fraction were resolved using a 90 minute gradient of MS Buffers A and B, in data-dependent mode. Peptides were subjected to one full MS1 scan (450-1450 m/z) in the Orbitrap (resolution 60,000), and six MS2 data-dependent scans in the linear ion trap mass analyzer. Using charge state screening, only charge states of +2 and +3 were selected for MS2 fragmentation.

2.2.6 Database search and protein identification

RAW files containing mass spectra of identified peptides were searched and analyzed on Mascot (Matrix Science, London, UK, version 2.2.0) to create DAT and MGF files. The resulting MGF files were analyzed in X!Tandem (Global Proteome Machine Manager, version 2006.06.01) using the International Protein Index (IPI) human database (version 3.62), in which XML files were generated. The resulting XML files along with DAT files were merged using Scaffold software (Proteome Software Inc., v. 2.06), which produced a list of proteins identified in each sample. Using the X!Tandem Log E and Mascot ion-score filters within Scaffold, we adjusted for false discovery rates (FDRs) of approximately 1% at the protein level. The FDR was calculated using the following formula: \( \frac{2 \times \text{false positives}}{\text{false positive} + \text{true positive}} \times 100 \), where false positives were proteins that were identified by sequences in the reverse database, and true positives were proteins that were identified by sequences in the forward database. Finally, protXML files were exported from Scaffold and uploaded into ProteinCenter (Proxeon Biosystems).
2.2.7 Candidate filtering and pathway analysis

From the protein lists generated, proteins identified in OVCAR-5 and LP-9 monoculture datasets were excluded from those present in the co-culture secretome, using comparison tools provided in Protein Center. To establish more stringent criteria, proteins that were unique to OVCAR5/LP9 co-cultures were filtered for two peptide hits, in order to increase the confidence in our candidates. Since cytosolic-derived proteins are released into conditioned media as a result of cell death during cell culture, secreted/membrane proteins were enriched for, by selecting for proteins that contain a signal peptide and those that were deemed ‘extracellular’ or ‘membrane’ according to Gene Ontology (GO) cellular localization annotations using Protein Center. Moreover, spectral counts of each protein were considered when selecting candidates, as proteins that had high spectral counts in the co-cultures and low counts in monocultures were also considered as potential regulators of OvCa progression. Generally, the remaining candidates were chosen if their spectral count in co-culture condition was at least two fold greater than the average spectral count in both monocultures. Protein Center was also used to categorize proteins according to their molecular and biological functions using assigned GO annotations. Protein networks of putative candidates were generated using Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com), which provided the top network functions of secreted proteins that displayed differential secretion during cancer-peritoneal interaction.

2.2.8 Cell migration assay

To determine the effect of cancer cell migration in response to treatment with LP-9 conditioned media, cell scratch assays were constructed by seeding OVCAR-5 cells in 6-well
plates. Upon reaching confluency, a scratch across the middle of each well was made using a pipette tip. Cells were then washed three times with PBS to remove cellular debris, and treated with either serum-free media or LP-9-derived conditioned medium for a period of 48 hours. Changes in cell migration were assessed by examining the ability of cancer cells to elicit wound repair, which was measured by calculating the mean wound length over time.

2.2.9 RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction (qPCR)

Purification of total RNA was performed using the RNeasy kit (Qiagen). The samples used for RNA extraction were as follows: OVCAR-5 cells stimulated with conditioned media from LP-9 cells, OVCAR-5 cells in serum-free conditions, LP-9 cells in serum-free conditions, and LP-9 cells stimulated with conditioned media from OVCAR-5 cells. cDNA was generated using a SuperScript First-Strand cDNA synthesis kit (Invitrogen), and subsequently used for qPCR to evaluate relative gene expression. TATA-binding protein (TBP) was used as a housekeeping gene to measure relative expression, as its expression was not expected to vary across the different experimental conditions. Gene expression analysis was performed on the following genes: PSCA, MUC4, CD109, LOXL4, LTBP1, PPP3CA, ITGB4, INHBA, HYAL1, COL6A3, LRG1, CAP1, PTPRK, CST6, MUC5AC, TFPI2, CXCL5, PLEC1, and GDF15. Forward and reverse primer sequences are shown in the table below:
mRNA expression analysis was also performed on OVCAR-5, OV-90, BG-1, and LP-9 cells grown as indirect two-way co-cultures using transwell inserts with 0.4µm pore sizes (Becton Dickinson). Quantitative PCR was performed using 1X SYBR Green PCR Master Mix (Applied Biosystems) and levels of mRNA transcripts were measured on a 7500 ABI system. Fold changes of gene expression between stimulated and control conditions were displayed as a heat map using FiRe version 2.2 (168).

### 2.2.10 MUC5AC enzyme-linked immunosorbent assay (ELISA) analysis in OvCa ascites and benign cyst fluids

All biological fluids were obtained with informed consent and Institutional Review Board approval, which include ovarian cancer ascites fluid from advanced stage ovarian cancer patients (n=8) and serous ovarian cyst fluid (n=10) from benign neoplasms. Levels of

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<th>Gene Symbol</th>
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<td>GDF15</td>
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<td>GCACTTCTGGCGTACGATCT</td>
</tr>
</tbody>
</table>

48
MUC5AC were measured using an enzyme-linked immunosorbent assay kit according the manufacturer’s instructions (Uscn Life Science Inc.).

2.2.1 Immunocytochemistry

LP-9, OVCAR-5, OV-90, and BG-1 cells were plated in 12-well plates in regular growth medium. Co-cultures of LP-9/OVCAR-5, LP-9/OV-90, and LP-9/BG-1 cells were constructed as described above. After reaching 80% confluency, all cell cultures were washed three times with PBS and grown in serum-free media for 2 days. Cells were then washed twice with PBS, and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Following fixation, cells were washed three times with ice-cold PBS and permeabilized with 0.2% Tween-20 for 10 minutes. After three 5-minute washes with PBS, cells were then treated with 1% BSA in PBST for a period of 30 minutes. Afterwards, cells were incubated with MUC5AC primary antibody (1:500, Abcam) in 1% BSA in PBST at 4 degrees Celsius overnight. The following day, all cells were rinsed three times for five minutes with PBS, and endogenous peroxidase was blocked with 3% H2O2 for 20 minutes. After washing, cells were incubated with horseradish peroxidase-conjugated secondary antibody in 1% BSA (1:1000) for 1 hour. Finally, cells were then rinsed three times with PBS, and incubated with DAB chromogenic substrate for approximately 10 minutes or until color developed. Cells were then washed and stored in PBS. No antibody controls for all monocultures and co-cultures were also performed, as well as an IgG1 isotype control for OVCAR-5/LP-9 co-cultures. Staining was visualized using a light microscope, and images were captured using the OLYMPUS Q-Color3 imaging system.
2.2.12 Immunohistochemistry

Ovarian cancer tissue microarrays (OV481 and OV808) containing fixed paraffin-embedded metastatic and primary ovarian cancer tissues were purchased from the US Biomax, Inc (Maryland, United States). To deparaffinize tissues, the TMAs were immersed in xylene, and were then rehydrated with ethanol. Endogenous peroxidase activity was blocked using hydrogen peroxide. After washing with PBS, antigen retrieval was performed using a trypsin solution in a humidified chamber. Slides were blocked and were incubated overnight with MUC5AC primary antibody (1:100). Following washes with PBS, slides were placed in secondary antibody for 1 hour using a BGX kit. Slides were then developed with DAB and counterstained with hematoxylin.

2.2.13 Statistical Analysis

All statistical significance tests on scratch assay and gene expression data were analyzed using independent t-tests (Minitab, v. 14). MUC5AC levels measured in ovarian cancer ascites and benign cyst fluids were compared using the Mann-Whitney U test (GraphPad Prism, v.6.03). Results comparing different conditions were considered significant if the p-value was less than or equal to 0.05. The Fisher exact test was calculated using SPSS Statistical Software.
2.3 Results

2.3.1 LP-9 conditioned media promote in vitro cancer cell migration

The progression of ovarian cancer is marked by enhanced cancer motility, as malignant cells adopt a migratory behaviour and travel through the extracellular matrix to distant metastatic sites. However, in this study, we were only interested in performing a global characterization of protein alterations that occur during cancer and peritoneal cell interaction. Alternatively, cancer cells can remain in ascites fluid and form multicellular aggregates, which preferentially attach to the peritoneum. Previous studies have established that media conditioned by mesothelial cells can increase the migratory potential of ovarian cancer cells, partially through the secretion of fibronectin as well as other unknown soluble factors (103, 104). Therefore, to assess whether this observation could be recapitulated with our cell lines, we constructed preliminary in vitro scratch assays by using LP-9 medium conditioned by LP-9 cells for 48 hours to stimulate confluent monolayers of OVCAR-5 cells that had been scratched with a pipette tip, creating a wound between the cells (Fig 2.1A). After 24 and 48 hours post-stimulation, there was a significant increase in wound closure by treated cancer cells, compared to non-stimulated cells, which was evaluated by calculating the mean wound length (p<0.05) (Fig 2.1A). As such, these observations support previous findings that suggest that soluble factors from mesothelial cells can either induce cancer cell migration, or stimulate cancer cells to secrete factors that lead to enhanced cell motility.

2.3.2 Proteomic profiling of monoculture and co-culture conditioned media

We sought to identify proteins that displayed elevated secretion during cancer-mesothelial interaction, which could provide biological insight into the mechanisms that
modulate peritoneal metastasis. Therefore, we conducted a comparative proteomic analysis of
the secretome, in which we compared the secretion of proteins identified in conditioned media
of a mesothelial cell line (LP-9) and an ovarian cancer cell line (OVCAR-5), to those present in
CM of LP-9/OVCAR-5 direct co-cultures using an experimental outline as shown in Figure
2.1B. Overall, our analysis resulted in the identification of 1435 proteins secreted by LP-9
cells, 1646 by OVCAR-5 cells, and 1586 by LP-9/OVCAR-5 co-cultures with a minimum of
one peptide (Figure 2.1C). Integrating all three datasets revealed a total of 2554 non-redundant
proteins, of which 189 proteins were specific to the co-cultures, as they were not detected in the
CM of either monoculture (Figure 2.1D). Moreover, in addition to identifying candidate
proteins that were exclusively present in the co-culture dataset, a subset of proteins displayed
lower secretion in monoculture secretomes compared to co-cultures based on spectral counting.
Figure 2.1 – Cell scratch assay and identification of proteins present in LP-9, OVCAR-5, and LP-9/OVCAR-5 co-culture conditioned media (CM). A) Cell scratch assay of OVCAR-5 cells treated with conditioned media from LP-9 cells and relative mean wound length at 0 h, 24 h, and 48 h post-treatment with LP-9 CM (*, p < 0.05, independent t-test). B) Experimental workflow used for protein identification. C) Total number of proteins identified with ≥1 peptides in the three replicates of each condition. D) Combining all identified proteins revealed 189 proteins unique to cancer–mesothelial co-cultures (minimum 1 peptide).

To limit our dataset of proteins to a smaller subset of candidates involved in cancer-peritoneal interaction, we applied several filtering criteria that would eliminate proteins that were not secreted or were likely to be false hits. Firstly, using the 189 proteins that were unique to the co-culture secretome, we filtered for proteins that were identified with a minimum of two peptides using tools provided in Protein Center, which narrowed down our initial list to 50 proteins (Figure 2.2A). Moreover, as it is well recognized that uncontrolled cell death occurs during regular cell culture growth conditions, the secretome contains several proteins that are typically deemed cytosolic. Thus, to remove these intracellular contaminant proteins, the
remaining proteins were categorized based on their cellular localization using Gene Ontology annotations available through Protein Center software. Specifically, proteins that were annotated as “extracellular” or “membrane” and/or contained a signal peptide were enriched for, which generated a total list of 36 proteins (Table 2.1). As mentioned earlier, differential secretion of proteins was also assessed by using normalized spectral counts. Secreted or membrane proteins that had lower spectral counts in monocultures compared to co-cultures were also considered as possible candidates, which resulted in 13 additional proteins that are summarized in Table 2.2. Overall, a total of 49 candidate proteins showed elevated secretion during cancer-peritoneal interaction. Further Gene Ontology classification revealed that the top biological processes included response to stimuli and metabolic processes, whereas top molecular functions of candidate proteins included catalytic activity and protein binding (Figure 2.3).

After delineating our list of candidate proteins, Ingenuity Pathway Analysis was used to uncover biological networks related to our candidates, as well as pinpoint potential protein-protein interactions. In summary, the top-associated network functions included molecular transport, protein trafficking, lipid metabolism, cancer, cell-to-cell signaling and interactions, and cell death and survival. Interestingly, candidates were linked to common pathways including TP53, TNF, and ESR1 (Figure 2.2B), which have all been implicated in ovarian cancer pathogenesis (39, 169-170).

2.3.3 Gene expression analysis of top candidates in OVCAR-5/LP-9 indirect one-way co-cultures

To determine whether the gene expression of our candidates correlates with our proteomic results, we performed real-time PCR on a subset of candidate proteins (PSCA,
MUC4, CD109, LOXL4, LTBP1, PPP3CA, ITGB4, INHBA, HYAL1, COL6A3, LRG1, CAP1, PTPRK, CST6, MUC5AC, TFPI2, CXCL5, PLEC1, and GDF15) on indirect co-cultures of LP-9 and OVCAR-5 cells. Briefly, conditioned media was collected from OVCAR-5 cells and was used to stimulate LP-9 cells, and vice versa. The expression of the above genes in each cell type was compared to cells grown in serum-free media, which is displayed as a heat map in Figure 2.2C with fold-change ratios. As expected, mRNA fold changes of some genes did not parallel with our proteomic data, which could be a result of post-translational processes such as protein degradation, half life, and shedding mechanism, or because direct cell contact between the two cell types may be required for gene activation. Of the genes analyzed, there was a significant increase in mRNA expression levels for HYAL1, LRG1, MUC5AC, TFPI2, and CXCL5 (p<0.05, independent t-test).
Figure 2.2 – Filtering, IPA analysis, and mRNA expression of selected candidates. A) Filtering candidates using two approaches: exclusion and spectral counting. B) Ingenuity Pathway Analysis clustered candidate proteins in networks belonging to molecular transport, cancer, cell-to-cell signaling and interaction, and cell death and survival. Gene and proteins are depicted as nodes (shaded/gray nodes represent upregulated proteins in our co-culture model; white nodes depict genes/proteins that were incorporated by Ingenuity Knowledge Base to build genes/proteins into networks). Nodes connected by solid lines indicate a direct relationship, whereas dotted lines depict an indirect relationship. C) mRNA expression of selected genes displayed as a heatmap. Ratios represent fold changes in expression of stimulated cells over control cells. Red corresponds to increased gene expression, whereas blue illustrates reduced expression (*, P ≤ 0.05, Student's t-test).
Table 2.1 – Secreted and membrane proteins identified exclusively in LP9/OVCAR5 co-culture supernatants (≥2 peptides). Each protein was present in at least two of the three replicates.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Symbol</th>
<th>Protein Description</th>
<th>No. unique peptides</th>
<th>Mean Spectral Count ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00216393</td>
<td>CLTA</td>
<td>Isoform Non-brain of Clathrin light chain A</td>
<td>4</td>
<td>2.3 ± 1.5</td>
</tr>
<tr>
<td>IPI00411680</td>
<td>PCMT1</td>
<td>Isoform 1 of Protein-L-isoaaspartate(D-aspartate) O-methyltransferase</td>
<td>2</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>IPI00639931</td>
<td>CAP1</td>
<td>Isoform 2 of Adenylyl cyclase-associated protein 1</td>
<td>10</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>IPI00216550</td>
<td>CD55</td>
<td>Isoform 1 of Complement decay-accelerating factor</td>
<td>3</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>IPI00306402</td>
<td>LOXL4</td>
<td>Lysyl oxidase homolog 4</td>
<td>3</td>
<td>1.7 ± 1.2</td>
</tr>
<tr>
<td>IPI00550451</td>
<td>PPP1CA</td>
<td>Serine/threonine-protein phosphatase PP1-alpha catalytic subunit</td>
<td>7</td>
<td>10.7 ± 4.0</td>
</tr>
<tr>
<td>IPI00643525</td>
<td>C4A</td>
<td>Uncharacterized protein</td>
<td>23</td>
<td>29.3 ± 4.7</td>
</tr>
<tr>
<td>IPI00303207</td>
<td>ABCE1</td>
<td>ATP-binding cassette sub-family E member 1</td>
<td>2</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>IPI00154451</td>
<td>MMS19</td>
<td>cDNA FLJ55586, highly similar to MMS19-like protein</td>
<td>3</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td>IPI00168847</td>
<td>HYAL1</td>
<td>Isoform 2 of Hyaluronidase-1</td>
<td>4</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>IPI00027422</td>
<td>ITGB4</td>
<td>Isoform Beta-4C of Integrin beta-4</td>
<td>5</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td>IPI00157567</td>
<td>PTPRK</td>
<td>Isoform 1 of Receptor-type tyrosine-protein phosphatase kappa</td>
<td>5</td>
<td>6.3 ± 4.0</td>
</tr>
<tr>
<td>IPI00791006</td>
<td>MUC4</td>
<td>Mucin-4 isoform a</td>
<td>7</td>
<td>4.7 ± 3.1</td>
</tr>
<tr>
<td>IPI00028670</td>
<td>INHBA</td>
<td>Inhibin beta A chain</td>
<td>5</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td>IPI0045536</td>
<td>CHID1</td>
<td>Isoform 3 of Chitinase domain-containing protein 1</td>
<td>3</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>IPI00019038</td>
<td>LYZ</td>
<td>Lysozyme C</td>
<td>2</td>
<td>4.3 ± 0.6</td>
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<tr>
<td>IPI00218830</td>
<td>NMT1</td>
<td>Isoform Short of Glycylpeptide N-tetradecanoyltransferase 1</td>
<td>2</td>
<td>1.7 ± 1.2</td>
</tr>
<tr>
<td>IPI00883772</td>
<td>GAA</td>
<td>lysosomal alpha-glucosidase preproprotein</td>
<td>12</td>
<td>12.3 ± 2.5</td>
</tr>
<tr>
<td>IPI00514894</td>
<td>KPNA6</td>
<td>Karyopherin alpha 6</td>
<td>2</td>
<td>2.0 ± 0</td>
</tr>
<tr>
<td>IPI00010338</td>
<td>F3</td>
<td>Tissue factor</td>
<td>4</td>
<td>2.3 ± 1.5</td>
</tr>
<tr>
<td>IPI00011284</td>
<td>COMT</td>
<td>Isoform Membrane-bound of Catechol O-methyltransferase</td>
<td>3</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td>IPI00029629</td>
<td>TRIM25</td>
<td>E3 ubiquitin/ISG15 ligase TRIM25</td>
<td>2</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>IPI00217952</td>
<td>GFPT1</td>
<td>Isoform 1 of Glucosamine--fructose-6-phosphate aminotransferase isomerizing. 1</td>
<td>2</td>
<td>1.0 ± 2.0</td>
</tr>
<tr>
<td>IPI00000728</td>
<td>USP15</td>
<td>Isoform 1 of Ubiquitin carboxyl-terminal hydrolase 15</td>
<td>3</td>
<td>1.7 ± 1.2</td>
</tr>
<tr>
<td>IPI00007321</td>
<td>LYPLA1</td>
<td>cDNA FLJ60607, highly similar to Acyl-protein thioesterase 1</td>
<td>2</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>IPI00016613</td>
<td>CSNK2A1</td>
<td>CSNK2A1 protein</td>
<td>2</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>IPI00032406</td>
<td>DNAJA2</td>
<td>DnaJ homolog subfamily A member 2</td>
<td>2</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Accession</td>
<td>Gene Symbol</td>
<td>Description</td>
<td>Count</td>
<td>Expression</td>
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<tr>
<td>-------------</td>
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<td>-----------------------------------------------------------------------------</td>
<td>-------</td>
<td>------------------</td>
</tr>
<tr>
<td>IPI00179415</td>
<td>PPP3CA</td>
<td>Isoform 1 of Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform</td>
<td>3</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>IPI00013446</td>
<td>PSCA</td>
<td>Prostate stem cell antigen</td>
<td>3</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>IPI00215899</td>
<td>SRPX</td>
<td>Isoform 2 of Sushi repeat-containing protein SRPX</td>
<td>2</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>IPI00103397</td>
<td>MUC5AC</td>
<td>Mucin-5AC (Fragment)</td>
<td>20</td>
<td>29.0 ± 6.6</td>
</tr>
<tr>
<td>IPI00218676</td>
<td>IL1RL1</td>
<td>Isoform B of Interleukin-1 receptor-like 1</td>
<td>2</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>IPI00893273</td>
<td>LTBP1</td>
<td>Latent-transforming growth factor beta-binding protein 1 isoform 5 precursor</td>
<td>16</td>
<td>9.0 ± 4.4</td>
</tr>
<tr>
<td>IPI00103480</td>
<td>LIPH</td>
<td>Lipase member H</td>
<td>2</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>IPI00217778</td>
<td>PLTP</td>
<td>Isoform 2 of Phospholipid transfer protein</td>
<td>3</td>
<td>12.7 ± 1.5</td>
</tr>
<tr>
<td>IPI00024650</td>
<td>SLC16A1</td>
<td>Monocarboxylate transporter 1</td>
<td>2</td>
<td>0.7 ± 0.6</td>
</tr>
</tbody>
</table>
Table 2.2 – Secreted proteins elevated in LP9/OVCAR5 co-cultures in comparison to cancer and mesothelial secretomes (based on average normalized protein spectral counts). Each protein was identified in all three co-culture replicates.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Symbol</th>
<th>Protein Description</th>
<th>Mean Spectral Count ± SD (LP9)</th>
<th>Mean Spectral Count ± SD (OVCAR5)</th>
<th>Mean Spectral Count ± SD (LP9/OVCAR5 Co)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00152540</td>
<td>CD109</td>
<td>Isoform 1 of CD109 antigen</td>
<td>5.3 ± 1.5</td>
<td>6.7 ± 1.5</td>
<td>35.0 ± 1.7</td>
</tr>
<tr>
<td>IPI00022200</td>
<td>COL6A3</td>
<td>Isoform 1 of Collagen alpha-3(VI) chain</td>
<td>3.3 ± 1.2</td>
<td>N/A</td>
<td>10.0 ± 6.9</td>
</tr>
<tr>
<td>IPI00022417</td>
<td>LRG1</td>
<td>Leucine-rich alpha-2-glycoprotein</td>
<td>N/A</td>
<td>4.0 ± 5.2</td>
<td>30.3 ± 2.9</td>
</tr>
<tr>
<td>IPI00019954</td>
<td>CST6</td>
<td>Cystatin-M</td>
<td>N/A</td>
<td>1.7 ± 2.1</td>
<td>17.3 ± 3.2</td>
</tr>
<tr>
<td>IPI00009198</td>
<td>TFPI2</td>
<td>Tissue factor pathway inhibitor 2</td>
<td>6.3 ± 2.5</td>
<td>N/A</td>
<td>10.7 ± 2.1</td>
</tr>
<tr>
<td>IPI00292936</td>
<td>CXCL5</td>
<td>C-X-C motif chemokine 5</td>
<td>6.0 ± 0</td>
<td>N/A</td>
<td>8.0 ± 1.7</td>
</tr>
<tr>
<td>IPI00014898</td>
<td>PLEC1</td>
<td>Isoform 1 of Plectin-1</td>
<td>57.7 ± 11.9</td>
<td>9.0 ± 6.1</td>
<td>102.3 ± 13.3</td>
</tr>
<tr>
<td>IPI00306543</td>
<td>GDF15</td>
<td>Growth/differentiation factor 15</td>
<td>4.7 ± 2.3</td>
<td>0.7 ± 1.2</td>
<td>17.3 ± 4.0</td>
</tr>
<tr>
<td>IPI00019590</td>
<td>PLAT</td>
<td>Isoform 1 of Tissue-type plasminogen activator variant 1</td>
<td>N/A</td>
<td>22.3 ± 18.0</td>
<td>115.3 ± 18.7</td>
</tr>
<tr>
<td>IPI00377045</td>
<td>LAMA3</td>
<td>Laminin alpha-3 chain variant 1</td>
<td>N/A</td>
<td>6.0 ± 10.4</td>
<td>27.7 ± 2.1</td>
</tr>
<tr>
<td>IPI00013890</td>
<td>SFN</td>
<td>SFN Isoform 1 of 14-3-3 protein sigma</td>
<td>0.3 ± 0.6</td>
<td>0.7 ± 0.6</td>
<td>10.3 ± 1.2</td>
</tr>
<tr>
<td>IPI00297487</td>
<td>CTSH</td>
<td>Cathepsin H</td>
<td>N/A</td>
<td>0.7 ± 0.6</td>
<td>8.3 ± 2.5</td>
</tr>
<tr>
<td>IPI00029273</td>
<td>MET</td>
<td>Isoform 1 of Hepatocyte growth factor receptor</td>
<td>N/A</td>
<td>13.3 ± 13.8</td>
<td>27.7 ± 1.5</td>
</tr>
</tbody>
</table>

*Note: N/A (not applicable): no proteins were detected in the sample
A Biological Process

B Molecular Function

Figure 2.3 – Gene Ontology classifications for secreted proteins in terms of A) biological process and B) molecular function. Each bar represents the percentage of proteins from the pool of candidates that belong to each GO category.

2.3.4 MUC5AC gene expression is increased in OVCAR-5/LP-9, OV-90/LP-9, and BG-1/LP-9 co-cultures (QPCR)

Based on our proteomic discovery data, mucin 5AC was our most attractive candidate as it displayed the highest spectral counts in cancer-mesothelial co-culture secretomes in comparison to all other candidates that were not identified in either cancer or mesothelial cell line conditioned media. Moreover, its gene expression was significantly elevated in indirect one-way co-cultures. Therefore, to evaluate its expression in other cell line models, gene expression analysis was performed in cells engaged in a two-way indirect co-culture, which is a more favorable system as both cell types share the same media and are able to exchange soluble factors. Specifically, OVCAR-5, OV-90, BG-1 and LP-9 cells were co-cultured together using transwell inserts of 0.4 µm pore sizes. Significant increases in MUC5AC expression were
observed in OVCAR-5, OV-90, and BG-1 cells that were co-cultured with mesothelial cells, as shown in Figure 2.4 (p<0.05, independent t-test). Similarly, MUC5AC also displayed increased expression in LP-9 cells that were co-cultured with either OVCAR-5 or OV-90 cells (p<0.05, independent t-test).

Figure 2.4 – Gene expression analysis of MUC5AC in cancer and peritoneal cells grown in co-culture. Transcript levels were measured in cells grown in monocultures and co-cultures (three biological replicates) in A) LP-9/OVCAR-5 B) LP-9/OV-90, and C) LP-9/BG-1 co-cultures (*, P ≤ 0.05, Student's t-test). All qPCR experiments were performed in technical triplicates.
2.3.5 MUC5AC is elevated in OvCa ascites fluid (ELISA)

Ascites fluid contains a rich milieu of secreted proteins and soluble factors that are shed by malignant cells and surrounding cells of the microenvironment. As such, proteins present in ascites may serve as potential mediators of the disease as well as provide insight into cancer progression. Levels of MUC5AC were assessed in patient ovarian cancer ascites fluid, as well as fluids from benign cyst neoplasms (Figure 2.5). Overall, the protein was found to be elevated in patient ascites fluid, compared to serous cyst fluid (p=0.021), which suggests that it becomes elevated during cancer. Classification of ascites and cyst fluid into low and high levels of MUC5AC also revealed a significant difference between the two groups (p = 0.001, Fisher’s exact test; sensitivity= 100%; specificity= 80%; predictive accuracy=88.9%).

![Figure 2.5](image)

**Figure 2.5** – Levels of MUC5AC were measured in ovarian cancer patient ascites (n = 8) and benign ovarian serous cyst fluid (n = 10) A) Significant differences in MUC5AC levels (ng/mL) were observed between the two conditions (P ≤ 0.05, Mann–Whitney test). B) 2 x 2 contingency table representing the distribution of ascites and serous cyst fluid cases with low and high levels of MUC5AC (P = 0.001, Fisher’s exact test; sensitivity = 100%; specificity = 80%; predictive accuracy = 88.9%). EOC, epithelial ovarian carcinoma.
2.3.6 Immunocytochemistry reveals elevated MUC5AC in cancer-peritoneal cell co-cultures

To confirm the upregulation of MUC5AC during cancer-peritoneal interaction, immunocytochemistry was performed, in which fixed cells were stained for the protein of interest (Figure 2.6). No antibody controls were also performed to ensure that there was no background staining present (data not shown). Staining of monocultures revealed low MUC5AC expression in the LP-9 cell line and an absence of staining in the OV-90, BG-1, and OVCAR-5 monocultures. In contrast, increased intensity of staining was observed in all three co-cultures, particularly in OV-90 and OVCAR-5 co-cultures, which suggests that MUC5AC becomes elevated during ovarian cancer attachment and growth on the mesothelium.

**Figure 2.6** – Immunocytochemistry analysis assessing MUC5AC expression in OVCAR-5 and LP-9; BG-1 and LP-9; OV-90 and LP-9 co-cultures and monocultures. MUC5AC expression was elevated in all co-cultures, while it displayed low expression in peritoneal cells, and absent in cancer monocultures. All images are displayed at x40 magnification.
2.3.7 Immunohistochemistry staining in primary OvCa and omental metastasis

Given that we identified MUC5AC to be specifically expressed during cancer and peritoneal interaction, we examined MUC5AC expression in primary ovarian cancer tissue and peritoneal metastasis using immunohistochemistry (Figure 2.7). MUC5AC was not observed in primary ovarian cancer; however, there was positive staining at the cancer-peritoneal interface in metastatic ovarian cancer that has invaded the omentum, which further suggests that MUC5AC is elevated during peritoneal metastasis.

![Image of immunohistochemistry staining](image1)

**Figure 2.7** – Immunohistochemistry staining of MUC5AC in A) primary ovarian cancer and B) omental metastasis. MUC5AC expression was not observed in primary ovarian cancer, but showed positive expression at the cancer-peritoneal interface.
2.4 Discussion

Along with other mechanisms of metastasis, such as the intravasation of cancer cells into blood and lymphatic cells and subsequent extravasation at distant sites, the formation of peritoneal implants from the adhesion of tumor cells to the mesothelium is also important for the establishment of distant metastases. Given that patients diagnosed at an advanced stage have a poor clinical outcome, an increased understanding of how tumor cells interact with peritoneum is essential for the development of therapies that prevent or target peritoneal attachment and invasion.

In this study, we sought to characterize proteomic changes that occur as a result of the interaction between cancer and mesothelial cells. Although previous studies have also incorporated the use of mass spectrometry to identify one or two mediators of peritoneal metastasis (105), our approach is the first to provide a global snapshot of all dysregulated proteins during the crosstalk of these two cell types. Specifically, LC-MS/MS was used for the proteomic profiling of conditioned media from an in vitro co-culture model between ovarian cancer (OVCAR-5) and peritoneal (LP-9) cells, and their respective monoculture secretomes. Overall, our analysis resulted in the identification of 2554 non-redundant proteins in all three experimental conditions, whereby a subset of proteins were identified solely in the co-culture secretomes. After applying a set of stringent filtering criteria, we were able to narrow down our candidates using two different approaches, which were based on spectral counting and monoculture exclusion. In total, 49 proteins displayed low secretion or absence in peritoneal or cancer cells alone in comparison to the co-culturing of the two cell populations. In order to confirm the feasibility of our approach, we examined whether proteins that have been previously linked to the peritoneal dissemination of ovarian tumors were identified through our
MS-based analysis. For example, to date, several molecules have been associated with EOC metastasis, including TGFβ1p, ITGβ1, VCAM1, MET, CD44H, ICAM-1, FN, CX(3)CL1, and mesothelin, which assist in the attachment of malignant cells to extracellular matrix (ECM) components or to mesothelial cells (93, 102, 105, 108, 111-112, 165, 171-172). As such, all of these proteins were observed in the conditioned media of our co-cultures, with some displaying increased secretion. Although the identification of these molecules strengthens the validity of our approach, we should also acknowledge the limitations of our experimental system. For instance, our co-culture model represents a two-dimensional system, and therefore, does not entirely recapitulate OvCa progression as it occurs in vivo, as it lacks the contribution of biological and cellular components belonging to the tumor microenvironment, including the underlying ECM. Moreover, given that OvCa is a heterogeneous disease, the use of one cell line is not sufficient to capture the entire proteome that is representative of all ovarian cancers, and therefore, proteomic secretions may be subjected to cell line biases. However, we postulated that because cancer-peritoneal attachment is a very specific interaction, similar proteomic alterations and molecules will be recruited, regardless of the cell line used, but how abundantly expressed they are will vary across different cell lines. Thus, we further evaluated our top candidate, MUC5AC, in two other comparable model systems using the ovarian cancer cell lines, OV-90 and BG-1. Interestingly, differences in expression were observed in mesothelial cells when they were co-cultured with BG-1 cancer cells, compared to those cultured with OVCAR-5 and OV-90 cells, which suggests that different cell lines have specific effects on mesothelial cells.

After analyzing the gene expression levels of our top candidates, we observed a significant elevation of mucin 5AC in both the ovarian cancer (OVCAR-5) and mesothelial
(LP-9) cell lines when they were stimulated with each other’s conditioned media (indirect one-way co-cultures). Other promising candidates that were elevated including HYAL1, LRG1, and TFPI2, have all been previously linked to ovarian cancer (173-175). In addition, increased expression of MUC5AC was also observed in two-way indirect co-culture models using OVCAR-5, OV-90, and BG-1 cell lines, as well as in patient ascites fluid and tissues representing omental metastasis, which indicates that it may have a putative role in the pathophysiology of the disease. However, further studies are required to evaluate whether MUC5AC has a direct role in promoting ovarian cancer progression, and whether it is a driver or passenger during metastasis. Furthermore, our immunocytochemistry analysis revealed increased MUC5AC expression in the co-cultures of all cell lines; however, the bulk of its secretion appears to be derived from peritoneal cells.

Interestingly, previous immunohistochemistry and biochemical studies have revealed an elevated expression of MUC5AC in ovarian tumor samples compared to normal tissue, primarily those of the mucinous subtype (176-177). However, little or no expression is often observed in ovarian serous carcinomas (178-179), suggesting that its induction may be mediated by the communication between cancerous cells and the mesothelium through the exchange of soluble factors during peritoneal metastasis (179). In our study, this theory is exemplified by the lack of MUC5AC expression in cancer cells cultured alone. However, in a recent study, mucin 5AC was elevated in the interstitial fluid and tumor lysates of endometrioid, mucinous, and serous ovarian carcinomas compared to control healthy ovarian tissue, but displayed marked heterogeneity among the subgroups of patients (177).

MUC5AC belongs to the mucin family of secreted and transmembrane glycoproteins, which have multifaceted roles in various diseases and are commonly dysregulated during
inflammation and cancer (180). Thus far, few transmembrane mucins have been associated with aberrant expression in ovarian cancer, particularly, mucin 16 (or CA125), which is elevated in ovarian cancer patient sera and is clinically used as a monitoring biomarker (181). Apart from its role in the clinic, one group has identified a binding domain for CA125 on mesothelin, a cell-surface protein expressed by both cancer and peritoneal cells (182). This binding domain may facilitate the adhesion of both cell types, and thus, has major implications for OvCa tumorigenesis (182). Interestingly, MUC4, a transmembrane mucin that is overexpressed in ovarian tumors, which has been shown to increase the motility and invasiveness of ovarian cancer cells through the induction of epithelial-to-mesenchymal transition, was also elevated in our co-cultures (183-185). To date, few studies have evaluated the pathophysiological association of MUC5AC with respect to ovarian cancer, as aberrant expression has mainly been linked to colorectal, pancreatic, and gastric carcinomas, in addition to the regulation of airway epithelial cells (178-179). Moreover, in these studies, its expression was often correlated with more aggressive and advanced stage cancers (178-179). As a result, mucin 5AC has been shown to enhance the invasive properties of cancer cells undergoing metastasis (180). In a recent study, the knockdown of MUC5AC in pancreatic cancer cells resulted in reduced adhesion, invasion, and metastasis, through the down-regulation of integrins, MMP-3 and VEGF (186). Moreover, in \textit{in vivo} xenograft studies, it was shown that the knockdown of MUC5AC suppressed tumor growth and tumorigenesis of pancreatic cancer, while when using \textit{in vitro} cell lines, MUC5AC was shown to inhibit TRAIL-induced apoptosis (187-188). From the above observations, it is evident that MUC5AC plays a major role in cancer progression, and the mechanisms by which it supports ovarian cancer metastasis should be further explored. Given that the adhesion and invasion of ovarian cancer to the peritoneum relies heavily on several
integrin molecules, it would be interesting to determine whether MUC5AC also regulates their expression, similar to what occurs in pancreatic cancer.

In addition to cancer, MUC5AC is also induced in human bronchial epithelial cells by the proinflammatory cytokines, TNF-α, IL-1β and IL-17A, through the activation of the NF-κB pathway, which are all known pathways/mediators that contribute to metastatic OvCa (189-192). Previous groups have demonstrated that pre-incubation of mesothelial monolayers with inflammatory cytokines including TNF-α, IL-1β, and IL-6 enhances their adhesion to colorectal cancer cells and alters their own morphology (171, 193-194). Given that TNF-α is actively produced by ovarian cancer cells, and is a potent stimulator of MUC5AC, we postulate that one mechanism by which TNF-α increases the adhesion between cancer and peritoneal cells may be mediated through the release of MUC5AC; however, whether its induction acts in a similar way in ovarian cancer has yet to be elucidated with additional experiments.

Overall, in the present study, we performed a comprehensive proteomic analysis to characterize alterations in protein secretion that occur during ovarian cancer-peritoneal interaction. Our findings provide evidence that MUC5AC becomes elevated during the direct co-culturing of cancer and mesothelial cells. As such, future efforts should aim to delineate its functional relevance in terms of cancer cell migration and invasion of the mesothelium, using appropriate in vitro and in vivo model systems, in addition to determining the underlying mechanisms that cause its induction.
Chapter 3 | Comparative proteomics of ovarian cancer aggregate formation reveals an increased expression of calcium-activated chloride channel regulator 1

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3.1 Introduction

For several years, ovarian cancer (OvCa) has been the most lethal gynecological malignancy among women in North America, where it accounts for 5% of all cancer-related deaths (1). Unfortunately, in most cases, the disease is not detected until advanced stages, at which point the cancer has progressed considerably. Despite recent efforts made towards the development of personalized treatment strategies for ovarian cancer, patient survival rates have barely improved since the arrival of platinum-based chemotherapy, with an overall average 5-year survival rate of 44% (1, 10). Meanwhile, the standard treatment for late stage patients continues to be cytoreductive surgery followed by chemotherapy (22). Although this approach may lead to a partial or complete remission, the probability that residual cancer cells not removed during surgery will develop resistance is high, and will eventually lead to recurrent disease (22). Beyond this point, the cancer is rendered incurable. Given that the majority of patients develop high-grade tumors, which are characterized by late stage presentation and intraperitoneal spread, there is an urgent need to understand OvCa metastasis and identify key molecules that drive tumor progression.

The formation of metastatic implants on the peritoneum relies on the detachment and spread of cancerous cells from the primary tumor (195). During this process, detached cells remain suspended in ascites fluid within the peritoneal cavity and form multicellular aggregates (MCAs), also known as spheroids, which obtain an anchorage-independent behavior that is resistant to apoptosis (92, 195). As such, recent emphasis has been placed on delineating the underlying biology of spheroid growth and formation given that these 3D models are more representative of in vivo conditions, and share similar biological features to solid tumors (196-197). More importantly, it has been shown that MCAs facilitate ovarian cancer metastasis, by
breaching the mesothelium and causing widespread peritoneal dissemination (111, 122, 132, 198). Numerous studies have highlighted the ability of spheroids to acquire chemoresistant, and stem-like properties, both of which have major implications for disease outcome (126, 129) (127-128, 196). Thus far, it is believed that interactions between various cell adhesion molecules and extracellular matrix components contribute to the formation of MCAs, including integrins, fibronectin, and cadherins (92, 114, 122, 132, 199). Interestingly, studies have also shown that gene and protein expression can differ between cancer cells grown in monolayers versus those within multicellular aggregates (128, 200). Thus, a global comprehensive proteomics analysis that compares the proteome of the two cell populations may add to our current understanding about OvCa progression, as well as aid in the identification of novel therapeutic targets.

Over the past decade, advances in proteomic technologies have led to the quantitative identification of proteins in various biological samples using different labeling and non-labeling approaches (138). Such strategies offer several advantages, as they allow for a direct quantifiable comparison of proteins between samples to be performed, rather than being restricted to a qualitative analysis. In this study, a quantitative proteomics-based approach using stable isotope labeling of amino acids in cell culture (SILAC) (201) coupled to mass spectrometry (LC-MS/MS) was used to identify differentially expressed proteins in ovarian cancer cells (OV-90) cultured as aggregates (anchorage-independent, OV-90AI) compared to those cultured as monolayers (anchorage-dependent, OV90-AD). In total, 1897 proteins were quantified, as 37 and 25 proteins were overexpressed and underexpressed, respectively, in aggregate-forming cells compared to cells grown as monolayers. From our analysis, calcium-activated chloride channel regulator 1 (CLCA1) was significantly elevated during MCA
formation, which was confirmed using other cell line models. By using chloride channel blockers, in addition to siRNA knockdown of CLCA1, we further demonstrated that CLCA1 has an effect on cell aggregation. Taken together, our findings reveal novel proteins that facilitate spheroid formation, which may serve as potential therapeutic targets for the treatment of OvCa.
3.2 Materials and Methods

3.2.1 Cell Lines

The human ovarian cancer cell lines, OV-90 (serous) (CRL-11732), TOV-112D (endometrioid) (CRL-11731), and ES-2 (clear cell) (CRL-1978) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown and maintained in RPMI 1640 medium (Gibco) supplemented with 10% characterized fetal bovine serum (FBS) (Thermo Scientific). All OvCa cells were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO₂.

3.2.2 Cell Culture/SILAC-Labeling

OV-90 cells were seeded into T25 flasks, and were cultured using a modified version of RPMI 1640, which initially had lacked arginine and lysine amino acids (Athena ES, Baltimore MD), but had been spiked with either ‘heavy’ amino acids (L-Arg6 [¹³C] and L-Lys8 [¹³C and ¹⁵N]) (Cambridge Isotope Laboratories), or ‘light’ amino acids (arginine [¹²C] and lysine [¹²C and ¹⁴N]) (Sigma). The resulting SILAC media was then supplemented with 10% dialyzed FBS (Life Technologies) and additional L-proline amino acids were also added to reduce arginine isotope- conversion to proline in the cells (202). Cells were then grown in ‘heavy’ and ‘light’ conditions for a period of five doubling times to ensure adequate labeling efficiency. During the last passage, ‘light’ and ‘heavy’ cells were each transferred to three T25 flasks and grown to confluence. ‘Heavy’ labeled cells were used to generate cell line aggregates, using the hanging drop method (203, 204), whereas ‘light’ labeled cells were maintained in adherent conditions. Briefly, heavy-labeled cells were suspended from the top of a petri dish plate that contained 10-15 mL of PBS. Each condition was conducted in three experimental replicates. After a period of
two days, cell pellets from each replicate was collected, washed twice with PBS, and stored at -80°C.

Monolayers and multicellular aggregates of OV-90, TOV-112, and ES-2 cells used in mRNA and western blot analyses were generated in the same method as described above, with the exception that regular RPMI supplemented with 10% FBS was used.

3.2.3 Sample Preparation and Strong Cation Exchange of SILAC-labeled cells for Mass Spectrometry

Cell pellets were resuspended and lysed in 250 μL of 0.1% RapiGest (Waters Inc, Milford, MA) in 25 mM ammonium bicarbonate, vortexed, and were then sonicated for 30 seconds. The resulting lysates were then centrifuged at 4°C for 15 minutes at 10,000 x g to remove cellular debris. The total protein concentration of each sample was measured using a Coomassie (Bradford) protein assay and cell lysates from ‘heavy’ and ‘light’ labeled cells were combined in a 1:1 total protein ratio. The samples were then denatured at 80°C for 15 min, reduced with 10 mM dithiothreitol, and alkylated with 20 mM iodoacetamide for 1 hr. Proteins were digested overnight at 37°C with trypsin using a trypsin: total protein ratio of 1:50 (Sigma). Samples were acidified with 1% trifluoroacetic acid to cleave RapiGest, which was then removed by centrifugation.

Samples containing tryptic peptides were diluted with mobile phase buffer A (0.26M formic acid, 10% acetonitrile) to a final volume of 500μL. The entire sample was then loaded onto a PolySULFOETHYL A column containing an anionic polymer with pore sizes of 0.02μm and a diameter of 5μm (The Nest Group Inc., MA). The column was connected to an Agilent 1100 High Performance Liquid Chromatography system. Peptides were fractionated and eluted using a one-hour gradient of a buffer that consisted of mobile phase A with the addition of 1M
ammonium formate. The separation was monitored at a wavelength of 280 nm and fractions were collected every two minutes from 24 to 50 minutes with a flow rate of 200 µL/min. The last two fractions, which displayed the lowest peak absorbance, were pooled, resulting in a total of 12 fractions per replicate. Fractions were then diluted to achieve a final acetonitrile concentration of 5%.

3.2.4 Mass Spectrometry

Peptides from each fraction were purified and extracted using OMIX C18 Pipette Tips, and were eluted in 70% MS Buffer B (90% acetonitrile, 0.1% formic acid, 10% water, and 0.02% trifluoroacetic acid) and 30% MS Buffer A (5% acetonitrile, 0.1% formic acid, 95% water, and 0.02% trifluoroacetic acid). Approximately 40 µL of each fraction was loaded onto an EASY-nLC system (Proxeon Biosystems, Denmark), which was coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, CA). Peptides were first loaded onto a 3cm C18 trap column, and eluted peptides were then resolved using a 5cm analytical C18 column with a 90 minute gradient of MS Buffers A and B, using data-dependent mode. Peptides were subjected to one full MS1 scan (450-1450 m/z) in the Orbitrap (60,000 resolution), and six MS2 scans in the linear ion trap mass analyzer. Using charge state screening, only ions with charge states of +2 and +3 were selected to undergo MS2 fragmentation.

3.2.5 Data Analysis and Protein Quantitation

RAW files generated with XCalibur software (v. 2.05, Thermo Fisher Scientific) that contained mass spectra of all identified peptides were analyzed using MaxQuant software (v 1.2.2.5) and searched against the International Protein Index (IPI) human database (v 3.71). The
maximum number of modifications per peptide was set to 5, along with a maximum of two missed cleavages. Both a peptide and protein false discovery rate of 1% was selected for, and a minimum of 1 unique peptide was chosen for protein identification. Re-quantification and match between runs were specified. The resulting heavy/light ratios were used for further study and selection of candidates.

Using the data output produced by MaxQuant software, the standard deviation (95% confidence interval) of Log 2 transformed ratios was computed. The z-score and p-values were calculated based on the assumption that the log ratios were zero. The resulting p-values were then corrected for multiple testing using the Benjamini-Hochberg method (205), which was used to control the false discovery rate (FDR). Proteins with adjusted p-values (q-values) less than 0.05 were chosen as possible candidates.

International Protein Index (IPI) numbers of overexpressed and underexpressed candidates were loaded into ProteinCenter, and categorized according to their molecular and biological functions using predicted GO annotations. Top cellular and molecular functions of candidate proteins were determined using Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com).

3.2.6 mRNA Expression Analysis

Total RNA was extracted from cell pellets using an RNeasy Kit (Qiagen). A SuperScript First-strand cDNA synthesis kit (Invitrogen) was used to synthesize complementary DNA for subsequent qPCR reactions. GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was used as an endogenous control to normalize the relative expression of the top six SILAC candidate genes: SLC1A5, SERPIND1, MAOB, CLCA1, FN1, and CES1. Forward and reverse primer
sequences: GAPDH, TCTCCTCTGACTTCAACAGCG (forward) and ACCACCTGTGTGCTTAGCCAA (reverse); SLC1A5, TCCTCCTCAACCAGCAAAAAACCC (forward) and CCACGCCATTATTCTCCTCCAC (reverse); SERPIND1, GTGGAGTCCCTGAAGTTGATGG (forward) and CCTTCCTCGTTCACTGTGATCG (reverse); MAOB, GTGAAGCAGTGTGGAGGCACAA (forward) and TTCACCTCGGTCTCCAAGGAGGT (reverse); CLCA1, CCACTCCTATGACAACAGCC (forward) and GTGATTGAGGCGGGTACCAGT (reverse); FN1, ACAACACGGGTGACTGAGAC (forward) and GGACACAACGATGCTTCCTGAG (reverse); CES1, AATCCACTCTCCGAAAGGCAACT (forward) and GACAGTGTGGTCTGGTCTCTCCT (reverse). QPCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems), and were carried out on a 7500 ABI system. Relative gene expression was calculated using the comparative Ct method (206).

3.2.7 Western Blot

Cell pellets (OV-90 and OV-90S; TOV-112D and TOV-112DS; ES-2 and ES-2S) were lysed with 1x Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM of PMSF (phenylmethanesulfonyl fluoride) and were sonicated for 30 seconds. The samples were then centrifuged at 15,000 x g for 10 minutes to remove any cellular debris. The total protein concentration was determined using the Bradford protein assay and equal amounts of total protein from each sample were loaded onto an SDS-PAGE gel (BioRad). After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (BioRad) and were blocked overnight at 4°C. A primary rabbit polyclonal antibody against CLCA1 (Santa Cruz) was used to examine protein expression in cell lysates.
3.2.8 Treatment with Chloride Channel Blocker, Niflumic Acid (NFA)

Niflumic acid (Sigma) was prepared in DMSO as a 1000x stock solution, which allowed for the final concentration of DMSO used in experimental stimulations to be low. Cells were grown in six well plates and were pre-treated with 100µM of NFA for a period of 5 hours before generating MCAs, while control cells were maintained in regular growth media. After 5 hours, treated and control cells were grown in anchorage-independent conditions for a period of 48 hours in either regular media or media containing 100µM NFA before the aggregates were collected and imaged.

3.2.9 Cell Viability Assay

To determine whether niflumic acid had an effect on cell viability, an alamarBlue assay (Invitrogen) was performed. Briefly, cells (OV-90, TOV-112D, and ES-2) were seeded into 96-well plates for 24hrs, and different concentrations of NFA were used to treat the cells, which was performed in triplicates. After 24 hours, 1/10th volume of alamarBlue reagent was added to the cell culture medium, and the cells were incubated at 37°C for 2 hrs. The reagent was detected by measuring absorbance at 570 nm using a plate reader, and differences in cell viability were assessed.

3.2.10 siRNA Transfections for CLCA1

siGENOME SMARTpool siRNAs for CLCA1 (Dharmacon) and non-targeting siRNA pools were purchased from GE Healthcare and transfections were performed according to the recommended protocol. Briefly, cells (OV-90, TOV-112D, and ES-2) were seeded in six well plates, and were transfected with target and non-target oligonucleotides using DharmaFECT1
Transfection Reagent with a final siRNA concentration of 50nM. After 3 days, the cells were collected and the ability to form aggregates was examined, by culturing cells in MCAs as described above. Transfected cells were also collected to assess the protein expression of CLCA1 using western blot analysis.

3.2.11 Statistical Analysis

All statistical significance tests on gene expression, western blot, and cell viability data were analyzed using independent t-tests (Minitab, v. 14). Results were considered significant if the p-value was less than or equal to 0.05.
3.3 Results

3.3.1 MS-identification of proteins differentially expressed during MCA formation

To identify proteins that displayed differential expression during ovarian cancer cell line aggregate formation, we conducted a comparative proteomics analysis, in which we quantitatively compared the expression of proteins identified in lysates of cells grown as monolayers to those grown as aggregates. As such, SILAC was used to label proteins in each condition by growing cells (OV-90) in media containing ‘heavy’ and ‘light’ arginine and lysine isotopes (Figure 3.1A & B). For each protein identified, a heavy/light (H/L) ratio was generated using MaxQuant software, which represents the abundance of a particular protein in each condition. Overall, our analysis resulted in the quantification of 1897 proteins with a minimum of one peptide hit. To identify candidates that were differentially regulated between the two conditions, we chose proteins whose q-values were less than 0.05, which represent p-values corrected for multiple testing (Benjamini-Hochberg, FDR of 5%). As a result, with this definition, 37 and 25 proteins were overexpressed and underexpressed, respectively, in aggregate-forming cells compared to cells grown as monolayers, using cut-off ratios of 1.8 and 0.58 (Figure 3.1C and Tables 3.1 and 3.2). Proteins that displayed ratios between 0.58 - 1.8 were not considered to show a significant change in protein expression. To confirm the accuracy of our ratios, we examined H/L ratios of housekeeping proteins, which are expected to display the same level of protein expression in both conditions. Overall, various control proteins displayed H/L ratios close to 1, including GAPDH (0.98), RPL27A (0.98), and RPS20 (0.91). Mass spectra of peptides for promising candidates were further examined and six proteins
(SLC1A5, SERPIND1, MAOB, CLCA1, FN1, and CES1) were chosen for further study (Figure 3.2).

Gene Ontology annotations available through Protein Center software were used to classify candidate proteins based on their biological processes and molecular functions, which included metabolic processes and nucleotide and protein binding, respectively. Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) was also used to reveal top molecular and cellular functions related to our candidates, which included small molecule biochemistry, lipid metabolism, molecular transport, and cell death and survival (Table 3.3).
Figure 3.1 – A) OV-90 cells were grown in anchorage-dependent and –independent conditions using the hanging drop method. Images are displayed at x40 magnification. B) Schematic representation of the SILAC-based proteomic workflow used to identify differentially expressed proteins. C) Graphical plot illustrating the number of quantified proteins with various heavy/light ratios. Proteins that displayed ratios above 1.8 and below 0.58 were considered statistically significant.
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<sup>1</sup>q-value: adjusted p-value corrected for multiple testing (Benjamini-Hochberg method)
Figure 3.2 – Heavy and light spectra of representative peptides from housekeeping proteins (GAPDH and RPS20) and candidate proteins (CLCA1, CES1, FN1, SLC1A5, SERPIND1, and MAOB) that were further validated in other cell line models.
Table 3.3 – Molecular and cellular functions of proteins that are a) up-regulated and b) down-regulated during MCA formation.

a) Molecular & Cellular Functions of Over-Expressed Proteins

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b) Molecular & Cellular Functions of Under-Expressed Proteins

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3.3.2 Gene expression validation of top candidates using in vitro models of aggregate formation

Quantitative PCR was used to examine the mRNA expression of top SILAC candidates in three ovarian cancer cell line models of MCA formation, including OV-90, TOV-112D, and ES-2. Each cell line was grown in anchorage-dependent and –independent conditions as described above, and relative expression of the above genes was compared in cells grown as MCAs and monolayers (data not shown). Of the genes examined, both CLCA1 (Figure 3.3A) and CES1 (data not shown) showed significant increases in mRNA expression when cells were grown as MCAs (p<0.05, independent t-test), which indicates that they may play an important role in OvCa progression.

3.3.3 Validation of CLCA1 protein expression in cell line models of aggregate formation

The top candidate identified in our proteomic discovery, CLCA1, displayed a 8-fold increase in OV-90 cells that were grown in anchorage independent conditions. To confirm the increase in protein expression of CLCA1 during the formation of multicellular aggregates, western blotting was performed on three cell lines that were grown as monolayers and as aggregates (OV-90/OV-90S; TOV-112D/TOV-112DS; ES-2/ES-2S). Overall, there was a significant increase in expression of CLCA1 during aggregate formation in two cell lines, OV-90 and ES-2 (p<0.05, independent t-test), with no difference observed in TOV-112D cancer cells (Figure 3.3B).
Figure 3.3 – A) Relative CLCA1 mRNA expression in cell lines cultured as monolayers and spheroids. B) Western blot validation of CLCA1 in cells grown in anchorage-dependent and – independent conditions (*, $p \leq 0.05$, independent t-test).
3.3.4 CLCA1-blocker prevents multicellular aggregation

To assess the role of CLCA1 in aggregate formation, cancer cells were treated with niflumic acid (NFA), which is a blocker of calcium-activated chloride channels and inhibits CLCA1 function (207, 208). Briefly, OV-90, TOV-112D, and ES-2 cells were pre-treated with 100µM of NFA for a period of 5 hours along with untreated cells, before creating OvCa cell line aggregates. After a period of 48 hours, it was shown that blocking chloride channels resulted in a reduced ability for cancer cells to form aggregates (Figure 3.4B). To confirm that the observed effects on cell aggregation were due to NFA’s blocking ability and not due to decreased cell viability, an alamar blue assay was performed. Cells in monolayer were treated with the same concentration of NFA used in the above experiment for a period of 24 hours, which resulted in no significant change in cell viability (Figure 3.4A).

![Graph showing cell viability](image)

**Figure 3.4** – Treatment of cancer cell lines with niflumic acid (NFA). A) Cells did not display a significant change in cell viability when treated with 100µM NFA for 24 hours. B) Cells displayed decreased cell aggregation when treated with NFA (48 hours). All images are displayed at x40 magnification.
3.3.5 siRNA knockdown and MCA formation with CLCA1 siRNA-transfected cells

The protein expression of CLCA1 was assessed in siRNA-transfected OV-90, TOV-112D, and ES-2 cells using western blot analysis. Seventy-two hours after CLCA1 siRNA transfection, the protein expression of CLCA1 had decreased, compared to cells transfected with non-targeting siRNAs (Figure 3.5A). To examine the effect of CLCA1 on cell aggregation, CLCA1 siRNA-transfected and control cells were used to form aggregates as described above. After a period of 24 hours, the ability to form compact aggregates was reduced (Figure 3.5B).

A)

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**Figure 3.5** – A) Western blot of control and CLCA1 siRNA-transfected OV-90, TOV-112D, and ES-2 cells. B) Multicellular aggregates generated with transfected cells. All images are displayed at x40 magnification.
3.4 Discussion

The formation of multicellular aggregates in ascites fluid of ovarian cancer patients plays a major role in ovarian cancer metastasis and has important implications for cancer therapy. Recent studies have highlighted that cell behavior and gene expression profiles vary between cells grown in monolayers versus those that are grown as MCAs (128, 209). Given that these differences result in an increased invasiveness and chemoresistance, a better understanding of how these spheroids form could lead to more effective approaches to treat the disease. As such, unraveling proteomic alterations during the transformation of cancer cells into a more dominant and malignant phenotype may result in the identification of novel therapeutic targets that may improve patient survival.

In this study, we undertook a comparative mass spectrometry-based approach to identify changes in proteomic expression during anchorage-independent and -dependent growth of the ovarian cancer cell line, OV-90. This cell line was chosen as our model system since its ability to form MCAs has been well established (162). Overall, our analysis resulted in the identification of 1897 proteins, of which 37 were overexpressed and 25 were under-expressed in MCA forming cells. The majority of proteins identified did not display differential expression as they fell in between our chosen cut-off ratios. Interestingly, one of the top SILAC candidates, fibronectin 1 (FN1), has been previously described as promoter of spheroid formation through its interaction with β1-integrin, which further strengthens our findings (129).

We do acknowledge some limitations of our study. For instance, we included one cell line for our proteomic discovery, which may result in cell line biases. Given the complexity of OvCa, it would be expected that proteomes would differ among various cell lines. To account for this drawback, we evaluated our top candidates in other cell line models. Furthermore,
although MCAs are believed to reflect solid tumors, our *in vitro* model lacks the contribution of the surrounding tumor microenvironment, including external signals that would be present in ascites fluid.

To confirm our preliminary data, we analyzed the mRNA expression of our top candidate proteins in three cell line models (OV-90, TOV-112D, and ES-2), which revealed a significant elevation of calcium-activated chloride channel regulator 1 (CLCA1) in two of the three cell lines (OV-90 and ES-2) when cultured as multicellular aggregates. Further, protein expression validation using western blot analysis corresponded with our gene expression data, as CLCA1 also displayed increased protein expression in OV-90 and ES-2 MCAs, with little or no difference observed in TOV-112D cell line aggregates. These differences observed between cell lines may be due to disease heterogeneity, or could be attributed to the fact that the cell lines are representative of different ovarian cancer subtypes.

Given that multicellular aggregates are formed within ovarian cancer ascites fluid, it is reasonable to expect that proteins identified in our analysis, particularly those that are extracellular or membrane bound, would be detected in these proximal fluids as well. A few of our top candidates were also identified in previous proteomic analyses of ovarian cancer ascites (210-213), including FN1 and SERPIND1. Although, CLCA1 was not found in these fluids, several chloride channel-related proteins, such as chloride intracellular channel protein (CLIC) 1, CLIC4, and CLIC5, were detected (211-213). These observations suggest that these proteins may have important roles in cancer progression.

To determine whether CLCA1 has a role in cell aggregation, experiments that blocked its activity or reduced its expression were performed. In both cases, treating cells with a chloride channel blocker, NFA, or using siRNAs to knockdown CLCA1 resulted in a reduced
ability of cells to form aggregates. Interestingly, decreased aggregation for siRNA experiments was observed after a period of only 24 hours, as reduced cell aggregation was not observed after 48 hours. This finding indicates that other proteins are also involved in OvCa cell line aggregate formation.

CLCA1 is a transmembrane protein that belongs to a family of ion channels that regulate calcium-dependent chloride conductance, and has been implicated in numerous biological processes (214-215). These include epithelial secretion, cell-cell adhesion, mucus production, and apoptosis (215). Various chloride channels have been implicated in playing a role in ovarian cancer tumorigenesis, by promoting tumor-stroma interactions, and facilitating cancer metastasis (216). For instance, in a recent study, it was shown that chloride channel blockers, including NFA, were able to decrease proliferation, adhesion, and invasion of an ovarian cancer cell line, A2780 (217). Such findings support our results, and indicate that CLCA1 plays a major role in cancer metastasis. In addition to OvCa, aberrant expression has also been reported in colorectal cancer, as CLCA1 was shown to regulate the proliferation and differentiation of a colon cancer cell line (218).

CLCA1 has also been implicated in other diseases, as studies have documented an increased level in inflammatory airway conditions, such as asthma, where it plays a role in mucus production (219). In particular, CLCA1 has been associated with the hypersecretion of mucin 5AC (MUC5AC), as both genes are induced in upper airway mucosal explant tissue upon stimulation with TNF- α (207-208, 220). In these studies, treatment of bronchial epithelial cells with the chloride channel blocker, NFA, resulted in decreased MUC5AC mRNA expression; however, the mechanism by which CLCA1 regulates MUC5AC has yet to be elucidated (207, 220). We have previously reported an increased expression of MUC5AC
during ovarian cancer-peritoneal interaction (221). Whether CLCA1 controls the expression of MUC5AC in ovarian cancer requires further investigation.

In summary, we have conducted a comparative proteomics analysis, which revealed several proteins that display differential expression during cancer metastasis. Our findings provide new insight into the mechanisms of MCA formation, as we have identified proteins that may contribute to ovarian cancer pathogenesis. Further investigation into the role of CLCA1 in ovarian cancer biology is needed, as future efforts should gear towards understanding the effects of CLCA1 on cell survival and cancer resistance to chemotherapeutic drugs, in addition to assessing its role in cell aggregation *in vivo*. 
Chapter 4 | Investigating the association between CLCA1 and MUC5AC and further delineation of the role of CLCA1 in ovarian cancer progression
4.1 Introduction

Ovarian cancer is an aggressive disease that is most often diagnosed during advanced stages. Given the drawbacks of current chemotherapy, which include drug resistance and poor response in the less common OvCa subtypes, the need for more effective therapies has been brought to surface. In recent years, this need has lead to the design and application of a wide variety of agents that range from targeting angiogenic mechanisms of the tumor microenvironment to interfering with homologous recombination repair machinery (26, 30). As each of these new therapies are intended for specific subclasses of patients, other treatments that could complement existing approaches are still essential. A better understanding of the underlying biology surrounding OvCa metastasis may lead to the identification of key molecules that are integral to the progression of the disease, which therefore, may serve as practicable therapeutic targets.

As highlighted in earlier sections, spheroid growth and formation, as well as cancer interaction with the peritoneum, have become attractive areas to design targets for novel therapeutic interventions. Interestingly, similar cell adhesion molecules and proteins have been implicated in both of these processes. For instance, cell adhesion components, including β1-integrin, VCAM-1, hyaluronan, and CD44, have been shown to promote cancer attachment and invasion of the peritoneum (93, 102, 108, 112, 165), while interactions between various cell adhesion molecules and extracellular matrix components contribute to the formation of MCAs, including integrins, fibronectin, and cadherins (92, 114, 122). More recent findings have revealed that cancerous cells and spheroids may breach the peritoneum by using cytoskeletal elements to exert force on mesothelial cells, resulting in their displacement (132). Despite the
considerable efforts that have been made towards understanding ovarian cancer biology, there is a long way before these findings could be translated into effective therapeutic approaches.

In the previous chapters, a detailed description of two comparative proteomic approaches used to discover differentially expressed proteins during ovarian cancer metastasis were described. Through these exercises, two proteins, mucin 5AC (MUC5AC) and calcium-activated chloride channel regulator 1 (CLCA1), were identified as being important for ovarian cancer progression, and therefore, were further studied. More specifically, MUC5AC was found to be elevated during cancer and peritoneal cell interaction, whereas, CLCA1 was identified being important for ovarian cancer cell aggregation.

MUC5AC is part of the mucin family of transmembrane and secreted glycoproteins, which have been implicated in various diseases and display aberrant expression during inflammatory-related diseases and cancer (180). For instance, mucin 16 (or CA125) is highly elevated in the serum of advanced staged ovarian cancer patients and is used as a monitoring biomarker (181). Few studies have evaluated the involvement of MUC5AC with respect to ovarian cancer, as its dysregulation has mainly been linked to colorectal, pancreatic, and gastric carcinomas, and the regulation of airway epithelial cells (178-179). In a recent study, the knockdown of MUC5AC in pancreatic cancer cells resulted in reduced adhesion, invasion, and metastasis, through the down-regulation of integrins, MMP-3 and VEGF (186). From these observations, it is evident that MUC5AC plays a major role in cancer progression.

Meanwhile, CLCA1 is a transmembrane protein that belongs to a family of ion channels that regulate calcium-dependent chloride conductance, and has been implicated in numerous biological processes, such as epithelial secretion, cell-cell adhesion, mucus production, and apoptosis (215). Currently, there have only been a few studies that have examined CLCA1
protein function. It has been shown that CLCA1 undergoes auto-cleavage that results in two cell surface-associated subunits, and that this cleavage is necessary for the activity of calcium-activated chloride channels (214, 222). However, mechanistic details surrounding human CLCA1 function and its associated pathways remain poorly understood. Interestingly, it was previously shown that CLCA2 contains interacting binding domains for beta 4 integrin and adheres to it during lung colonization of breast cancer cells; however, CLCA1 failed to interact (233). In a recent study, it was demonstrated that chloride channel blockers, including niflumic acid (NFA), were able to decrease proliferation, adhesion, and invasion of an ovarian cancer cell line, A2780 (217). Such findings support our result and indicate that CLCA1 plays a role in cancer metastasis.

As reviewed earlier, there have been many reports that have linked CLCA1 with mucin production in various inflammatory airway conditions, including asthma, cystic fibrosis, and chronic obstructive pulmonary disease (219, 222). In particular, CLCA1 has been associated with the hypersecretion of mucin 5AC (MUC5AC), as both genes were induced in upper airway mucosal explant tissue upon stimulation with tumor necrosis factor (TNF)-α (207-208) (220). In these studies, treatment of bronchial epithelial cells with the CLCA1 blocker, NFA, resulted in decreased MUC5AC mRNA expression; however, the mechanism by which CLCA1 regulates MUC5AC has yet to be elucidated (207, 220). Given the strong association between CLCA1 and MUC5AC documented in the literature, we therefore wanted to explore this potential relationship in ovarian cancer. Below we propose a general model to illustrate how CLCA1 may regulate MUC5AC expression in ovarian cancer (Figure 4.1). In this study, we examined the expression of MUC5AC in cells grown in monolayers or as multicellular aggregates. We also examined MUC5AC expression in spheroids that were generated with cells transfected
with CLCA1 siRNA. In addition, we further explored the role that CLCA1 may play in the adhesion ability and cell viability of various ovarian cancer cell lines.

**Figure 4.1** – Proposed model of how CLCA1 may regulate MUC5AC expression. Cell-cell interaction via multicellular aggregate formation may lead to the increased expression of CLCA1, which is processed into two cell surface-associated subunits of 90 kDa and a group of 37-41 kDa proteins. These cleavage products might activate receptors belonging to the MUC5AC pathway, such as the epidermal growth factor receptor (EGFR). [CaCC, calcium-activated chloride channel]
4.2 Materials and Methods

4.2.1 Cell lines

OV-90, TOV-112D, and ES-2 ovarian cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cells were grown and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, and maintained in a humidified incubator at 37°C with an atmosphere of 5% CO₂. To produce multicellular aggregates, cells were grown in suspension using the previously described hanging drop method (203, 204). Briefly, cells were suspended from the cover a petri dish for a period of 48 hours to allow for spheroid formation. Both monolayers and spheroids were collected, washed twice with PBS, and underwent RNA or protein extraction for gene or protein expression analysis.

4.2.2 siRNA Knockdown of CLCA1.

siGENOME SMARTpool siRNAs for CLCA1 (Dharmacon) containing a pool of four siRNAs (GCACGAGUAUCUUUGUUUA, GUACAUACCUGGCUGGAUU, GAACAAAGCUCACAAGUAUAU, and AAAGUUAGCUGAAUAUGGA) and non-targeting siRNAs pool #1 (Dhamarcon) were purchased from GE Healthcare and transfections were performed as per manufacturer’s instructions. OV-90, ES-2, and TOV-112D cells were seeded in 24-well plates, and transfected with siRNA oligonucleotides using DharmaFECT1 Transfection Reagent. After 3 days, the control and CLCA1-knockdown cells were grown in anchorage-independent conditions using the hanging drop method as described above. Multicellular aggregates were collected and washed twice with PBS, which were then used for mRNA expression analysis.
4.2.3 RNA extraction and gene expression analysis

Total RNA was collected from control and CLCA1-knockdown spheroids using an RNeasy Extraction kit (Qiagen), as well as cDNA was synthesized using a SuperScript First-strand cDNA synthesis kit (Invitrogen). Quantitative PCR was performed to measure CLCA1 and MUC5AC gene expression. mRNA from monolayers and spheroids of OV-90, TOV-112D, and ES-2 cells from our previous study were used to measure MUC5AC expression. QPCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) and ran on a 7500 ABI system. The relative gene expression was assessed using the comparative Ct method (206), and GAPDH was used an endogenous control to normalize gene expression.

4.2.4 Western Blot

Equal amounts of total protein lysates from monolayers and multicellular aggregates of OV-90, ES-2, TOV-112D cells from the above study were loaded onto 4–15% precast polyacrylamide gels (BioRad), electrophoresed for 45 minutes at 200 volts, and proteins were transferred onto PVDF membranes. Membranes were either blocked for 1.5 hours at room temperature or overnight at 4°C with 5% blocking solution. Membranes that were blocked for 1.5 hours were incubated with a primary mouse anti-MUC5AC antibody in 1% skim milk, whereas, the membrane blocked overnight was incubated with a primary β-actin antibody in 1% skim milk for 1 hour at room temperature. The membranes were washed for a total of six times with 1X TBS-T. Membranes were then incubated with corresponding horseradish peroxidase conjugated-secondary antibodies in 1% milk for 1 hour at room temperature. Membranes were washed for a total of six times with 1X TBS-T, before detection with ECL western blotting detection reagent.
4.2.5 Cell adhesion assay

96-well plates were coated with 10 µg/mL of fibronectin overnight at 4°C. Afterwards, the plates were incubated with 0.2% BSA for one hour at 37°C to block non-specific binding sites. Approximately 4 x 10^5 cells were plated in each well with RPMI medium that either contained or did not contain 100 µmol/L NFA, and were allowed to attach for 1.5 hours. Unattached cells were removed from the wells by aspiration, and the relative abundance of the adhered cells was assessed using alamarBlue. Changes in optical density were analyzed by measuring absorbance at 540 nm.

4.2.6 Cell toxicity assay

OV-90, ES-2, and TOV-112D cells were seeded in 96-well plates and grown overnight. Cells were adapted to serum-free media before treatment with 0.1µM paclitaxel, with or without the 100µM CLCA1 inhibitor, NFA. After incubation for 72 hours, cell viability was assessed using alamarBlue reagent.

4.2.7 Statistical analysis

All independent t-tests performed to assess statistical significance were analyzed with GraphPad Prism 6.0. Findings were considered significant if the p-value was less than or equal to 0.05.
4.3 Results

4.3.1 Expression of MUC5AC is elevated in ovarian cancer multicellular aggregates

Given the association of CLCA1 with MUC5AC expression, we performed quantitative PCR to assess whether there is an increase in MUC5AC when three ovarian cancer cell lines (OV-90, TOV-112D, and ES-2) were grown as aggregates. Interestingly, MUC5AC displayed significant increases in mRNA expression upon anchorage-independent growth (p<0.05, independent t-test) in comparison to cells that were grown as monolayers (Figure 4.2). Protein expression of MUC5AC was also examined in the three cell line models, whereby increased protein expression was observed in one cell line (TOV112-D) (Figure 4.3).

**Figure 4.2** – Gene expression analysis of MUC5AC in ovarian cancer spheroid formation. Transcript levels were measured in cells grown as monolayers and multicellular aggregates in A) OV-90, B) TOV-112D, and C) ES-2 cell lines. (*, p ≤ 0.05, student's t-test; Error bars represent SD). All quantitative PCR experiments were performed in technical triplicates.

**Figure 4.3** – Western blot analysis of MUC5AC in OV-90, TOV-112D, and ES-2 cell monolayers and OV-90S, TOV-112S, and ES-2S line ovarian cancer spheroids. β-actin was used a housekeeping control.
4.3.2 Expression of MUC5AC is decreased in CLCA1-knockdown ovarian cancer spheroids

To assess whether CLCA1 can regulate MUC5AC expression, we used CLCA1 siRNA to knock it down in OV-90, ES-2, and TOV-112D cancer cell lines (Figure 4.4A). CLCA1 was efficiently knocked down in OV-90 and TOV-112D cells (Figure 4.4). Using the control and CLCA1 siRNA-transfected cells, multicellular aggregates were generated and MUC5AC mRNA expression was measured. Decreases in MUC5AC expression was observed in OV-90 and TOV-112D aggregates containing CLCA1 siRNA-transfected cells compared to controls (Figure 4.4B).

![Gene expression of A) CLCA1 and B) MUC5AC in OV-90, TOV-112D, and ES-2 multicellular aggregates that were generated with cells transfected with control and CLCA1 siRNA.](image)

**Figure 4.4** – Gene expression of A) CLCA1 and B) MUC5AC in OV-90, TOV-112D, and ES-2 multicellular aggregates that were generated with cells transfected with control and CLCA1 siRNA.
4.3.3 Effect of CLCA1 on cancer cell adhesion to fibronectin

The effect that CLCA1 has on cancer cell adhesion to components of the extracellular matrix was examined using the CLCA1 inhibitor, NFA. Briefly, cancer cells were added to fibronectin-coated plates in the absence or presence of NFA. Overall, in 2 of 3 cell lines, cell adhesion was reduced after 1.5 hours when cells were treated with NFA compared to the control condition as illustrated in Figure 4.5 (p<0.05).

![Graph showing cell adhesion to fibronectin](image)

**Figure 4.5** – The effect of NFA on the adhesion of cancer cells to fibronectin. Cells were either cultured in RPMI medium alone, or with 100 µM NFA and were allowed to attach to fibronectin-coated plates for 1.5 hours. (*p ≤ 0.05, student's t-test; Error bars represent SD). All experiments were performed in technical triplicates.
4.3.4 Effect of CLCA1 on paclitaxel chemoresistance

The ability of CLCA1 to promote chemoresistance was examined by determining cell viability of paclitaxel-treated cells in the presence or absence of a CLCA1 inhibitor. After a period of 72 hours, cells treated with NFA in addition to 0.1µM paclitaxel exhibited reduced cell viability as shown in Figure 4.6 (p<0.05). All cell lines displayed a 30-40% reduction in viability when treated with NFA in combination with paclitaxel. This data suggests that CLCA1 may promote chemoresistance in ovarian cancer multicellular aggregates.

Figure 4.6 – Effect of NFA on cell viability of paclitaxel-treated cells. OV-90, TOV-112D, and ES-2 cells were treated with 0.1µM paclitaxel for 72 hours in the absence (control) or presence of NFA. (*p ≤ 0.05, student's t-test; Error bars represent SD). All experiments were performed in technical triplicates.
4.4 Discussion

Ovarian cancer is a heterogeneous disease that is marked by late stage diagnosis and poor overall survival rates. Unfortunately, details surrounding the biology related to ovarian cancer metastasis are not well known. In an effort to better understand the disease, we previously used high throughput proteomics to identify metastasis-specific proteins in in vitro models that represent disease progression. In our attempts to characterize metastatic OvCa, two proteins, MUC5AC and CLCA1, were uncovered as being potential mediators of cancer progression. Interestingly, various studies in chronic airway diseases have revealed an association between the two proteins, and have suggested that CLCA1 may induce mucin 5AC expression, leading to increases in its secretion. As a result, this observation prompted us to further investigate whether there is a potential link between MUC5AC and CLCA1 expression in ovarian cancer.

Given that CLCA1 is overexpressed in ovarian cancer multicellular aggregates, we speculated that if there was a link between CLCA1 and MUC5AC, we should observe a similar increase in expression of MUC5AC. Upon formation of spheroids, all three lines exhibited an increase in MUC5AC mRNA expression; however, these results were not consistent at the protein level. This may be due to the fact that MUC5AC is a secreted protein, and therefore, assessing its expression in cell lysates is not practicable, as it does not reflect the total amount of protein that was produced as most of it may have been secreted. Moreover, aggregates that were generated with CLCA1 siRNA-transfected cells displayed a decreased expression of mucin 5AC compared to aggregates transfected with control siRNA in OV-90 and TOV-112D cell lines. If CLCA1 controls MUC5AC expression, it would be expected that changes in MUC5AC expression would reflect CLCA1 knockdown efficiency. Interestingly, this was observed among
ES-2 CLCA1 siRNA-transfected cells, in which the expression of CLCA1 and MUC5AC was only slightly reduced.

Previous studies have demonstrated that chloride channels have an effect on the attachment of cancer cells to the extracellular matrix, as chloride channel inhibitors have been able to reduce the adhesion rate of ovarian cancer cells by at least 20% (217). In the previous chapter, we also observed an overexpression of fibronectin during multicellular aggregate formation. As such, the effect of CLCA1 on the adhesion ability of cancer cells to fibronectin was examined, in which the adhesion rate was only reduced by 5% after 1.5 hours. Lastly, we discovered that cells treated with NFA exhibited decreased cell viability upon treatment with chemotherapeutic drugs. As a result, CLCA1 may contribute to the chemoresistant properties of spheroids.

In summary, the purpose of this study was to establish whether CLCA1 controls the expression of MUC5AC as demonstrated in other diseases. Our findings suggest that CLCA1 can regulate MUC5AC expression, and has an effect on the binding of cancer cells to fibronectin, as well as cell viability. Further efforts are needed to address the mechanisms by which CLCA1 induces MUC5AC, and to what extent this plays a role in spheroid growth and formation in vivo.
Chapter 5 | Systems biology approach for investigating the molecular relationships between cancer metastasis-specific candidate proteins
5.1 Introduction

Ovarian cancer is a heterogeneous disease, resulting from dynamic changes in gene and protein function and regulation. As cancer metastasis is governed by changes in cell behavior and phenotype, a global snapshot of dysregulated pathways related to proteins implicated in the disease is needed in order to fully understand cancer progression. Systems biology approaches and the development of bioinformatics tools have become popular since the advent of high-throughput technologies. This is mostly because both genomic and proteomic experiments often generate large amounts of data, which can be difficult to interpret. Since proteins interact with one another, the assembly of proteins into networks based on molecular relationships is one way to simplify and extract meaningful information from such studies (223). The construction of visual networks obtained through data integration and predicted protein-protein interactions can provide more insight into the biology of the disease under investigation, as well as uncover biomarkers or therapeutic targets (224). Moreover, they can aid researchers in formulating new hypotheses (225). These networks often entail nodes that are connected by lines (referred to as edges), in which individual proteins or genes represent nodes, and edges represent relationships between the two connected entities (225).

In addition to protein-protein interactions, networks can be created based on biological processes and molecular functions pertaining to the dataset of interest, which use gene ontology (GO) terms to represent the data. Such analysis often results in the construction of enrichment maps, and allows proteins to be grouped together based on their functional characteristics and overrepresentation of GO terms.

In the previous chapters, we have described two high-throughput proteomics studies in which we generated three lists of proteins that are differentially expressed during ovarian cancer
metastasis. In particular, we identified 49 proteins that were elevated during cancer-peritoneal interaction, and 37 and 25 proteins that displayed increased and decreased expression, respectively, upon OvCa MCA formation. In this study, we took a bioinformatics approach to dissect pathways and networks related to these metastasis-specific proteins in an effort to understand ovarian cancer metastasis as a whole, using publicly available tools. More specifically, we utilized two well-referenced tools, Cytoscape (227-228) and Ingenuity Pathway Analysis (Ingenuity® Systems; IPA, http://www.ingenuity.com), to conduct our analysis. Overall, this study enables us to identify common themes and pathways among the candidates we identified in our two previous studies.
5.2 Methods

5.2.1 Dataset selection

In our efforts to better understand perturbed networks in metastatic ovarian cancer, we used our proteomic-derived datasets from our prior mass spectrometry experiments. This included 49 proteins that were differentially secreted during co-culturing of ovarian cancer (OVCAR-5) and mesothelial cells, as well as 37 and 25 proteins that were overexpressed and under-expressed, respectively (via SILAC), during multicellular aggregate formation in OV-90 cancer cells.

5.2.2 Enrichment map profiling

Enrichment analysis of overrepresented GO terms was performed using the BiNGO (v2.44) plugin (226) in Cytoscape (v3.2.0) (227-228). A separate analysis was performed for GO ‘biological process’, ‘molecular function’, and ‘cellular component’ terms for all three datasets. Statistical analysis of overrepresented GO terms was performed using the hypergeometric test, and corrected for multiple hypothesis testing using the Benjamini and Hochberg False Discovery Rate (FDR) correction at a significance level of 0.05. Data containing the enriched GO annotations were exported from BiNGO and were visualized using the Enrichment Map plugin v2.0.1 (229) in Cytoscape. The following parameters were used to construct the networks: Jaccard coefficient of 0.25, FDR of 10%, and a q-value (adjusted p-value) of 0.05.
5.2.3 Bioinformatics prediction of protein-protein interactions between cancer metastasis-specific proteins

To identify protein-protein interaction networks among candidate proteins, Ingenuity Pathway Analysis was used (Ingenuity® Systems; IPA, http://www.ingenuity.com). The gene symbols of candidate proteins were uploaded into the software and a core analysis was performed. Proteins were searched against IPA’s curated Ingenuity Knowledge Base to identify networks and pathways that are relevant to the uploaded genes. Interactive networks were constructed based on IPA’s algorithms. A separate analysis was carried out for each dataset.
5.3 Results

5.3.1 Enrichment analysis of differentially expressed proteins during ovarian cancer metastasis

Using high throughput proteomics, we were able to identify 49 differentially expressed proteins during cancer-peritoneal interaction and 62 during multicellular aggregate formation. To get a visualization of overrepresented GO pathways among our datasets, these proteins were uploaded and analyzed using the BiNGO plugin within Cytoscape. Accordingly, we were able to determine enriched GO terms (which are represented by red circles) for proteins involved in ovarian cancer metastasis, which were displayed as an enrichment map (Figures 5.1-5.3). In the co-culture dataset, enriched GO terms included those that were related to cell junction assembly, response to stress, and enzyme inhibitor activity (Figure 5.1). Meanwhile, steroid dehydrogenase activity and asparagine biosynthesis were enriched molecular functions among overexpressed and under-expressed SILAC candidates, respectively (Figures 5.2 and 5.3).

5.3.2 Protein-protein interaction network analysis

IPA was used to identify pathways that were related to candidate proteins. The top molecular and cellular functions of co-culture candidates include molecular transport, protein trafficking, and cell-to-cell signaling and interactions. Candidates were linked to common pathways including MAPK, ERK1/2, PDGF, and VEGF (Figure 5.4). Meanwhile, the associated molecular functions of proteins that were elevated during cancer spheroid formation include lipid metabolism, molecular transport, and small molecule biochemistry, and were linked to pathways involving ubiquitin C, CFTR, and interleukin 13 and 6 (Figure 5.5). Finally, under-expressed proteins during MCA formation were associated with lipid metabolism, small
molecule biochemistry, and vitamin and mineral metabolism molecular functions, and were intertwined in networks related to Akt, VEGF, Erk1/2, NFkB, and MAPK (Figure 5.6).

**Figure 5.1** – Enrichment map of gene ontology A) biological process B) molecular function and c) cellular component terms among the 49 proteins that were differentially expressed during ovarian cancer-peritoneal interaction. Red circles indicate overrepresented GO terms that were determined to be statistically significant (p<0.05).
Figure 5.2 – Enrichment map of gene ontology A) biological process B) molecular function and c) cellular component terms among the 37 SILAC proteins that were overexpressed during multicellular aggregation. Red circles indicate overrepresented GO terms that were determined to be significant (p<0.05).
Figure 5.3 – Enrichment map of GO A) biological process B) molecular function and c) cellular component terms among the 25 SILAC proteins that were under-expressed during multicellular aggregation. Red circles indicate overrepresented GO terms that were determined to be significant (p<0.05).
Figure 5.4 – Protein-protein interaction network of co-culture candidates that were overexpressed during cancer-peritoneal interaction. Proteins are represented as nodes (shaded nodes represent upregulated proteins; white nodes depict genes/proteins that were added by Ingenuity Knowledge Base to build genes/proteins into networks). Nodes connected by solid lines indicate a direct relationship, whereas dotted lines depict an indirect relationship.
Figure 5.5 – Protein-protein interaction network of SILAC candidates that were overexpressed during multicellular aggregate formation. Proteins are represented as nodes (shaded nodes represent upregulated proteins; white nodes depict genes/proteins that were added by Ingenuity Knowledge Base to build genes/proteins into networks). Nodes connected by solid lines indicate a direct relationship, whereas dotted lines depict an indirect relationship.
Figure 5.6 – Protein-protein interaction network of SILAC candidates that were under-expressed during multicellular aggregate formation. Proteins are represented as nodes (shaded nodes represent downregulated proteins; white nodes depict genes/proteins that were added by Ingenuity Knowledge Base to build genes/proteins into networks). Nodes connected by solid lines indicate a direct relationship, whereas dotted lines depict an indirect relationship.
5.4 Discussion

The purpose of this chapter was to use systems biology approaches to better understand proteins that were differentially expressed during our models of ovarian cancer metastasis. Specifically, we performed an enrichment analysis of overrepresented gene ontology terms related to the biological process, molecular function, and cellular component associated with candidate proteins. Among the co-culture dataset, cell junction assembly, response to stress, and enzyme inhibitor activity were enriched molecular processes; whereas steroid dehydrogenase activity and asparagine biosynthesis were enriched among SILAC candidates. As a proof of concept, enriching for gene ontology cellular component terms validated the accuracy of our enrichment analysis. Since co-culture candidates were previously selected for by filtering for secreted, extracellular, and membrane proteins, we would expect that these GO terms would be enriched in our analysis, which is what we observed. Likewise, since our SILAC proteomic experiments were performed on cell lysates, we did not observe any enrichment of ‘extracellular’ GO terms, which again, is what would be expected.

Like any other experimental method, it is important to note that systems biology approaches are still subject to limitations and caution should be taken when using this type of analysis. For instance, there are the chances that false positive interactions could be included in the pathway, or that these interactions are not pertinent to the tissue or disease under question (223). Therefore, in silico- based studies always require experimental validation. Another drawback of systems biology approaches is that they are meant to represent pathways that are occurring within a cell at a specific time point. However, proteins are not static entities, as they undergo spatial and temporal changes and are influenced by the surrounding tumor
microenvironment. This is especially the case in ovarian cancer, as cell surface molecules and cell phenotypes are constantly changing during metastasis.

Overall, we observed both common (Akt, PDGF, ERK1/2, VEGF, p38 MAPK) and different pathways among proteins that were identified in the two experimental models. The difference in molecular pathways could suggest that perhaps different cell mechanisms are used during cancer-peritoneal adhesion and MCA formation. On the other hand, given that ovarian cancer is a molecular heterogeneous disease, a difference in biological mechanisms used between cell lines (OVCAR-5 and OV-90) would be expected. A better analysis would have been to conduct our discovery initiatives using the same cell line or multiple cell lines in both models. Only then, would we be able to determine whether similar pathways are recruited throughout these particular steps of cancer metastasis.
Chapter 6 | Summary and Future Directions
6.1 Overview

Widespread peritoneal dissemination is a defining feature of late stage ovarian cancer, which is facilitated by cancer attachment to the mesothelium and the formation of spheroids in ascites fluid. Current chemotherapy approaches are effective for localized disease of specific subtypes, but offer little therapeutic advantage for disseminated cancer. As of now, it is well known that cancer adhesion to the peritoneal lining and the formation of spheroids are led by a subset of cell adhesion molecules; however, blockade of these components does not completely inhibit these metastatic events, suggesting that other factors are involved. The rationale of this study was to better understand ovarian cancer metastasis, with the hopes of identifying potential therapeutic targets. Using in vitro model systems of ovarian cancer pathogenesis, we were able to identify numerous proteins that may play a role in ovarian cancer progression using high-throughput mass spectrometry. First, we performed a comprehensive proteomics analysis of a co-culture model of ovarian cancer and mesothelial cells, in which we identified various proteins that were differentially regulated during cancer-peritoneal interaction. We further validated one protein, MUC5AC, and confirmed its differential expression at the cancer-peritoneal interface. As similar cell adhesion proteins have been implicated in both processes, we next performed a comparative quantitative proteomics analysis of a cell line grown as monolayers and as spheroids. After identifying a subset of overexpressed proteins, we determined that CLCA1 is increased during multicellular aggregation, and plays a role in spheroid formation. CLCA1 was also shown to promote cell adhesion to fibronectin, and played a role in promoting cell viability in paclitaxel-treated cells. In addition, we demonstrated that CLCA1 was able to regulate MUC5AC expression, as CLCA1-knockdown cells exhibited reduced expression of MUC5AC. This body of work was the first study to recognize MUC5AC
and CLCA1 as potential mediators of ovarian cancer metastasis, and was also the first to globally characterize proteomic alterations during two important stages of OvCa progression. Lastly, we identified a putative link between MUC5AC and CLCA1 in ovarian cancer.
6.2 Summary of Key Findings

The main goals of this study were to 1) identify novel proteins that are elevated during cancer-mesothelial interaction 2) delineate proteomic alterations during ovarian cancer multicellular aggregate formation and 3) determine whether CLCA1 induces MUC5AC in ovarian cancer, in addition to identifying pathways among candidate proteins.

An overview of methodology and the major findings of each objective are summarized below:

1) Identify novel proteins that may be elevated during cancer-mesothelial interaction.

- We performed a comprehensive proteomics analysis of an *in vitro* model system that represents ovarian cancer-peritoneal interaction. The secreted proteome of a co-culture model containing ovarian cancer (OVCAR-5) and mesothelial (LP-9) cells was compared to their respective monoculture secretomes, in which 49 proteins of 2554 identified were differentially expressed.
- The relative mRNA expression of the top candidates was assessed using cells that were grown in indirect co-cultures.
- A significant increase in MUC5AC expression was displayed in three different co-culture models including OVCAR-5 and LP-9, BG-1 and LP-9, and OV-90 and LP-9.
- Elevated levels of MUC5AC were detected in ovarian cancer ascites fluid in comparison to benign cyst fluids.
- Immunocytochemistry analysis confirmed increased expression of MUC5AC in cancer-peritoneal co-cultures.
- Immunohistochemistry analysis of ovarian cancer tissues derived from omental metastases showed positive staining of MUC5AC at the cancer-peritoneal interface.
2) Characterization of proteomic alterations that occur during ovarian cancer spheroid formation.

- We conducted a quantitative proteomic analysis of a cell line model (OV-90) of ovarian cancer multicellular aggregate formation. Using a label-based approach, we were able to quantify differential protein expression between cells grown as monolayers (anchorage-dependent growth) and as multicellular aggregates (anchorage-independent growth). In this study, 37 and 25 proteins were over-expressed and under-expressed, respectively, in spheroid-forming cells.

- Gene and protein expression of CLCA1 was increased in multicellular aggregate formation of three ovarian cancer cell lines (OV-90, TOV-112D, and ES-2).

- Treatment of ovarian cancer spheroids with a CLCA1 inhibitor or knock down of CLCA1 resulted in a reduced ability of cancer cells to form aggregates.

3) Establishing an association between MUC5AC and CLCA1 in ovarian cancer

- *MUC5AC* mRNA expression was elevated in ovarian cancer MCAs compared to monolayers

- siRNA knockdown of CLCA1 in ovarian cancer cell line aggregates resulted in decreased expression of MUC5AC

- Upon treatment with a CLCA1 inhibitor, cancer cells displayed a slight decrease in the ability to adhere to fibronectin

- Cancer cells showed a reduction in cell viability during chemotherapy treatment with paclitaxel in the presence of a CLCA1 inhibitor
4) Systems biology approach for establishing relationships between cancer metastasis-associated proteins.

- Among the co-culture dataset, cell junction assembly, response to stress, and enzyme inhibitor activity were enriched gene ontology terms for molecular processes
- Steroid dehydrogenase activity and asparagine biosynthesis were enriched molecular processes during multicellular aggregate formation
- Cellular pathways, including Akt, PDGF, ERK1/2, VEGF, and MAPK, were common between both experimental model systems

Overall, the goal of this study was to use high-throughput proteomics technology to better characterize and understand metastatic ovarian cancer. In our attempts, we obtained several novel findings, as we identified proteins that may be implicated in ovarian cancer progression. Our findings suggest that MUC5AC and CLCA1 are differentially expressed during the disease, and actively participate in cancer adhesion to the peritoneum and spheroid formation. It is evident that cancer is not a single gene disease, as several pathways become disrupted and dysregulated during its course. As such, one of the main strengths of our approach is that by using high-throughput methods, we can examine global protein expression changes, which gives us a complete picture of biological transformations occurring within the cell.

As with most studies, we do recognize that there are significant limitations and drawbacks of our research approach. The main limitation is that our study focuses on *in vitro* models, and therefore may lack clinical applicability. To account for this, we incorporated three-dimensional culture models in our methods, which are now recognized as biologically
relevant systems that are highly representative of \textit{in vivo} tumor growth conditions (197, 230-231). Therefore, we have reason to believe that our findings will be translatable into the clinical setting. As such, the findings presented in this thesis lay the groundwork for future studies, and should be taken into consideration in further investigations.
6.3 Future Considerations

The knowledge obtained from the work presented in this dissertation has shed new insight into the pathobiology of ovarian cancer, specifically, features associated with peritoneal metastasis. However, the information that has accumulated from these studies is far from complete, as our findings allow for further exploration. For instance, through both of our discovery approaches, we identified many candidate proteins that displayed altered expression, which may have important roles at either the cancer-peritoneal interface, or during multicellular aggregate formation. Further characterization of these proteins warrants future investigation to determine whether they are important in the metastatic spread of ovarian cancer.

The bulk of experiments were conducted using *in vitro* model systems, with minimal clinical validation or translation. As such, more clinical validation would be desirable to assess the therapeutic value of our potential therapeutic targets. For instance, instead of using established cell lines, primary cancer cells could be isolated from ovarian cancer patient ascites fluid, and grown in anchorage-independent growth conditions. This would provide a clinical component that may complement our previous observations. Moreover, it would also be fundamental to recapitulate these findings using *in vivo* mouse models of ovarian cancer peritoneal metastasis.

The majority of studies examining CLCA1 structure and function have been conducted in inflammatory airway diseases. To date, it has been shown that CLCA1 undergoes autocleavage, resulting in two cell surface-associated subunits that are necessary for the activity of calcium-activated chloride channels (214, 222). Moreover, there are numerous reports that CLCA1 regulates mucin expression; however, the mechanism by which this occurs is not known. Further studies are needed to investigate the mechanistic role of CLCA1 in the
formation of multicellular aggregates. Given that CLCA1 is located at the cell surface, identification of unknown interacting partners would provide insight into the biological and physiological function of CLCA1, as well as its mechanistic role in spheroid formation.

Although the link between CLCA1 and MUC5AC has been previously proposed in other diseases, there have been no reports that this occurs in ovarian cancer. As such, this prompted us to investigate if CLCA1 regulates MUC5AC in our experimental model, in which we observed a decreased expression of MUC5AC in CLCA1-knockdown cells. It is essential to determine how this occurs by examining the effect of CLCA1 on MUC5AC-associated molecular pathways, such as Notch and EGFR (232).

Given that CLCA1-knockdown ovarian cancer cells displayed a reduced ability to form spheroids, we believe that this change in phenotype is related to changes in cell adhesion molecules at the cell surface. Therefore, the expression and reorganization of cell adhesion proteins implicated in ovarian cancer, such as integrins and cadherins, as well as the changes in MCA binding to the peritoneum should be investigated. Lastly, previous studies have shown that multicellular aggregates are associated with an invasive phenotype and express stem-like genes (126-127). Therefore, it would also be valuable to examine changes in the expression of these invasive genes.
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