AMPK Signalling As A Regulator Of Autophagy In A Model Of Ovarian Tumour Dormancy

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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Abstract
One of the hallmarks of epithelial ovarian cancer (EOC) metastasis lies in the process of spheroid formation, whereby tumour cells aggregate into 3D structures. Previous literature suggests that as EOC cells form spheroids they undergo bioenergetic stress, activate AMP-activated protein kinase (AMPK) signaling, and thereby force cells to enter a metabolically quiescent state. We have previously shown that EOC spheroids upregulate autophagy, a process that provides energy during starvation conditions. Herein, I examined the role of AMPK-mediated signaling regulation of autophagy in a model of ovarian tumour dormancy. Attenuation of AMPK signaling in EOC spheroids resulted in reduction of autophagic flux in all EOC lines although this reduction may occur in an LC3 and p62 independent manner. Interestingly, I identified that AMPK activation in spheroids is likely regulated by CAMKK-beta. The results of this study help further our understanding of the complex mechanisms driving autophagy in late-stage ovarian cancer.

Key Words: Epithelial Ovarian Cancer, Tumour Dormancy, Spheroid, AMPK, Autophagy
Co-Authorship Statement

All chapters were written by Jeremi Laski

The COV318 and FT190 mCherry-eGFP-LC3B lines were established by Dr. Bipradeb Singha

The iOvCa147-MA cell line was established by Dr. Gabriel E. DiMattia

The RNA isolation and purification for Clariom™ S analysis was performed by Dr. Adrian Buensuceso. Clariom™ S analysis was performed by the TCAG Microarray Facility, The Hospital for Sick Children, Toronto ON.
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Dedication

To Alex,
For your unwavering support, understanding and love.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA-Carboxylase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>ARID1A</td>
<td>Gene encoding AT-rich interactive domain-containing protein 1A</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy Related Protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BECN1</td>
<td>Gene encoding Beclin 1</td>
</tr>
<tr>
<td>BME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>BRAF</td>
<td>Gene encoding B-Raf protein</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Gene encoding Breast cancer type 1 susceptibility protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAMKKβ</td>
<td>Calcium/Calmodulin dependent protein kinase kinase</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GABARAP</td>
<td>Gamma-Aminobutyric Acid Receptor-Associated Protein</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>HGSOC</td>
<td>High Grade Serous Ovarian Cancer</td>
</tr>
<tr>
<td>KRAS</td>
<td>Gene encoding Kras</td>
</tr>
<tr>
<td>LAS</td>
<td>Leica Application Suite</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule Associated Protein1A/1B Light Chain</td>
</tr>
<tr>
<td>LGSOC</td>
<td>Low Grade Serous Ovarian Cancer</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver Kinase B1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mechanistic Target of Rapamycin Complex 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian Surface Epithelium</td>
</tr>
<tr>
<td>p62</td>
<td>Sequestosome 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-Derived Xenografts</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose Phosphate Pathway</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression Free Survival</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PIK3CA</td>
<td>Gene encoding phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
</tr>
<tr>
<td>PRKAA1/2</td>
<td>Gene encoding AMPK catalytic subunit α1/2</td>
</tr>
<tr>
<td>PTEN</td>
<td>Gene encoding Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene diFluoride</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Media</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Gene encoding p62/Sequestosome 1</td>
</tr>
<tr>
<td>STIC</td>
<td>Serous Tubal Intraepithelial Carcinoma</td>
</tr>
<tr>
<td>STK11</td>
<td>Gene encoding LKB1</td>
</tr>
<tr>
<td>TAK1</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase 7</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline-Tween 20</td>
</tr>
<tr>
<td>TGM2</td>
<td>Transglutimase 2</td>
</tr>
<tr>
<td>TP53</td>
<td>Gene encoding Tumour Protein 53</td>
</tr>
<tr>
<td>ULA</td>
<td>Ultra-Low Attachment</td>
</tr>
<tr>
<td>ULK1</td>
<td>Uncoordinated 51-like Kinase 1</td>
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<tr>
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<td>Wnt Signaling Pathway</td>
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Chapter 1

Introduction

1.1 Ovarian Cancer

1.1.1 Epidemiology and Clinical Treatment of Epithelial Ovarian Cancer (EOC)

Ovarian cancer is the most deadly gynecologic malignancy in women. In Canada, the death to incidence ratio is approximately 0.64, making it the fifth highest ratio of all cancers (Canadian Cancer Society, 2017). The most prevalent form of ovarian cancer is epithelial ovarian cancer (EOC), which accounts for more than 90% of all diagnosed ovarian cancer cases. Stromal and germ-cell tumour incidence is much less common, and are estimated to be approximately 3.5% each (Chen et al., 2003).

EOC’s high mortality rate can be attributed to a variety of factors, particularly the lack of definitive symptoms that prevent women from identifying a cause for concern, potentially mistaking abdominal discomfort and bloating for generalized post-menopausal symptoms instead. Current diagnostic tests are limited to physical pelvic exams, ultrasound and CA-125 serum tests. The CA-125 serum biomarker is known to have poor sensitivity in early stages of disease progression, making it difficult for physicians to establish an accurate diagnosis (Nossov et al., 2008). As a result, the majority of EOC cases usually present with late-stage disease (stage III-IV) with significant tumour burden. Extensive surgical debulking procedures must often be coupled with cytotoxic chemotherapy in hope of slowing disease progression. Nevertheless, rates of recurrence remain exceptionally high, with relapsed cancers exhibiting chemo-resistant phenotypes (Vaughan et al., 2011).

The current standard for treatment for EOC comprises of cytoreductive surgery followed by platinum and taxane-based chemotherapy. The two most commonly used drugs are carboplatin and paclitaxel. Carboplatin acts as an alkylating agent, where through the addition of an alkyl group to DNA bases, DNA strand fragmentation occurs. Carboplatin
additionally cross-links DNA strands, preventing DNA separation for transcription and translation (Shaloam and Tchounwou, 2014). Paclitaxel functions through hyper stabilization of the microtubule structure. By arresting microtubule disassembly, paclitaxel-treated cells are unable to remodel their cytoskeleton, or undergo mitosis (Schiff et al., 1979).

The major limitation of the abovementioned chemotherapeutics is their specificity towards highly mitotic cells. As such, less proliferative cells are capable of withstanding chemotherapy treatment and recurring as secondary forms of disease. In patients with initial high tumour burden, neoadjuvant therapy may be indicated, where chemotherapy is administered prior to surgery in hopes of shrinking overall tumour size before debulking begins (Mahner et al., 2016). Nevertheless, regardless of treatment plan, nearly all patients will present with chemo-resistant disease, highlighting the importance of developing strategies to improve overall clinical outcomes.

1.1.2 Origins and Subtypes of Epithelial Ovarian Cancer

Initially, the ovarian surface epithelium (OSE) was considered to be the cell type of origin for nearly all EOCs. The foundations of this theory lay in the mechanism of follicular rupture during menstruation described in the “incessant ovulation” hypothesis proposed by Fathalla in 1971. The incessant ovulation hypothesis postulated that with repetitive menstrual cycles and mechanical disruption of the OSE through follicular rupture, remnants of the OSE could become trapped as ovarian cysts. Due to their proximity to the stroma, the ovarian cysts would be exposed to estrogen rich extra-follicular fluid, which could promote tumorigenesis (Fathalla, 1971). This theory was widely accepted throughout the end of the 20th century and supported by epidemiological evidence indicating a negative correlation between oral contraceptive usage and ovarian cancer incidence (Purdie et al., 2003).

However a paradigm shift occurred in 2001, when a Dutch research group identified that fallopian tube intraepithelial carcinomas shared both molecular and histological features with high-grade serous ovarian cancers (HGSOCs). Although no neoplastic changes were
observed in the patients’ ovaries, the mutational status of BRCA1, a tumour suppressor gene, suggested that these women were already predisposed to developing ovarian cancer despite not showing any outwardly signs of the disease (Piek et al., 2001). This was the first study to suggest that the ovary may not be the site of origin for EOC, and propelled research into the field of serous tubal intraepithelial carcinoma (STIC) lesions. Subsequent research identified that these STIC lesions exist not only in women carrying the BRCA1 mutation, but can occur in women without any mutations at the BRCA1 locus (Aziz et al., 2001). By this time, pathologists already had reported large heterogeneity between patient EOC samples, with stark differences in histology between tumours. Strong supporting evidence for EOC heterogeneity arose from a gene profiling study of 50 primary ovarian cancer samples in 2005. Using a microarray approach, researchers were able to identify unique gene expression signals that outlined similarities between serous ovarian tumours with fallopian tube cells. Additionally, mucinous-like neoplasms were found to have strong genetic correlations with colonic mucosa, whereas endometrioid and clear cell-like neoplasms possessed genetic markers for the endometrium (Marquez et al., 2005). All this data combined pointed towards multiple sites of origin for EOC that are both genetically and histologically distinct from the ovaries themselves.

Under current guidelines, EOC is now classified into four main histologically distinct subtypes; serous, mucinous, endometrioid and clear cell. Serous carcinomas are now thought to be derived from the fallopian tube epithelium and can be further categorized as being either High-Grade Serous Ovarian Cancers (HGSOCs) or Low-Grade Serous Ovarian Cancers (LGSOCs). Although these tumours are classified as serous in origin, both mutational status and aggressiveness are different between the two subtypes, with high-grade neoplasms being substantially faster growing than their low-grade counterparts (Chen et al., 2013). Consequently, when studying the biology of EOCs, it is important to identify the specific subtype of EOC under examination.
1.1.3 Genomic Profile of HGSOC and other EOC subtypes

Epidemiologically, HGSOCs account for almost 70% of all EOCs, making them the most common form of ovarian cancer. One of the most striking characteristics of this EOC subtype is that nearly all HGSOCs (>95%) exhibit mutations in TP53, a well-characterized tumour suppressor gene. Moreover, these tumours exhibit high levels of chromosomal instability with vast amounts of copy number aberrations (CNAs) (Domcke et al., 2013). Interestingly, Domcke’s study identified that cell lines most frequently used and cited when studying HGSOC are most likely not representative of this histotype. This finding strongly reaffirms the importance of selecting biologically relevant cell lines when studying HGSOC.

The genomic profile of HGSOCs differs sharply to that of other EOC subtypes, which are wildtype for TP53 but exhibit mutations in several other genes involved in critical cellular signaling pathways. A large proportion (over 66%) of LGSOCs possess mutations within KRAS and BRAF. Although mutually exclusive, these mutations allow for the dysregulation of the mitogen-activated protein kinase (MAPK) pathway, ultimately promoting cellular invasiveness (Oikonomou et al., 2015). Clear cell tumours exhibit mutations within the ARID1A and PIK3CA genes, which are responsible for chromatin remodeling and cellular growth respectively. Mucinous tumours commonly present with KRAS mutations, whereas endometrioid tumours have high levels of mutations within ARID1A, CTNNB1, and PTEN genes (Kurman and Shih, 2011). Aberrations in these genes impact transcription pathways, either through chromatin remodeling or through the WNT/β-catenin pathway (Wu et al., 2007). These differences in mutational burden throughout EOC subtypes further exemplify the complexity of EOC and the multiple intersecting signaling pathways governing tumour progression.

Although several EOC subtypes have been described above, the scope of this thesis lays within the HGSOC field. As such, cell lines characterized with TP53 mutations and serous-like phenotype were used as the model of study.
1.1.4 Mechanism of Metastasis in EOC

Contrary to most other cancers, EOC’s mechanism of metastasis is unique, as it rarely involves the circulatory system. Instead, metastasis occurs through the dissemination of cancerous cells throughout the peritoneal cavity suspended in ascites fluid. Although a low level of peritoneal fluid is commonly found within all people, a pathological buildup of this fluid is referred to as ascites. A significant portion of ovarian cancer patients will present with this ascites buildup that is therapeutically managed either through paracentesis or surgery (Lengyel, 2010). The unpredictability and variability of ascites accumulation contributes to the difficulty in managing late stage ovarian cancer patients. Furthermore, the microenvironment of ascites fluid is highly variable between patients. Oxygen concentration, leukocyte infiltration and overall nutrient availability are some of the many factors that can differ between ascites samples, increasing the complexity of the metastatic process (Kipps et al., 2013).

To identify potential diagnostic markers within ascites samples, work has been conducted in characterizing both the proteome and metabolome within primary ascites samples. Shender and colleagues identified 424 proteins unique to malignant ovarian ascites samples relative to control cirrhotic (non malignant) ascites. Interestingly, significantly lower levels of glucose were observed in EOC derived ascites compared to non-malignant ascites, a phenomenon associated by the authors with the Warburg effect, the tendency of cancer cells to preferentially utilize glycolysis over oxidative phosphorylation even under high oxygen conditions. Supporting this claim is the finding of significantly higher levels of Transglutimase 2 (TGM2) in malignant EOC ascites (Shender et al., 2014). The role of TGM2 in modulating the Warburg effect has been shown by activating the glycolytic pathway through the NF-κB signaling axis in breast cancer cell lines (Kumar et al., 2014). The promising differences observed between malignant and nonmalignant ascites warrant further study. As high-throughput screening methodologies improve, further characterization of EOC ascites samples will be essential in uncovering potential therapeutic targets.
The mode of metastasis in EOC follows a particular dissemination pattern. Cells from primary tumour sites are capable of detaching into the peritoneal space in multicellular aggregates commonly referred to as spheroids (Figure 1.1). These spheroids can move to other areas within the abdominal cavity where they reattach, thus promoting metastasis.

![Ovarian Cancer Metastasis via Ascites and Peritoneal Carcinomatosis](image)

**Figure 1.1. Model of peritoneal carcinomatosis**

Mode of metastasis in ovarian cancers. Cells can detach from a primary tumour site and become suspended in ascites fluid throughout the peritoneal cavity. Aggregate clusters of cells are referred to as spheroids, which can subsequently reattach to secondary peritoneal surfaces, thus promoting metastasis. Filtered ascites-derived spheroids can be visualized immediately with phase contrast microscopy (top right image). Metastatic foci attached to peritoneal structures (bottom right image courtesy D. Lanvin) exemplify the vast number of disseminating ovarian cancer cells throughout the abdomen.

Both native single cells and spheroids have been repeatedly isolated from patient ascites and monitored in suspension cultures, supporting the intraperitoneal carcinomatosis model (Frankel et al., 1997; Burleson et al., 2006; Shepherd et al., 2007). Epithelial to mesenchymal transition (EMT) has been identified as one of the key initiation events for spheroid formation and proposed as a mechanism which confers viability to the
suspended cells (Mao et al., 2013; Jagsi et al., 2017). EOC spheroids are additionally capable of evading cell-detachment mediated apoptosis (anoikis) by becoming metabolically quiescent and decreasing proliferation (Peart et al., 2015). Increased levels of autophagy have been reported as well and shown to contribute to the viability of spheroids during periods of nutrient deprivation while suspended in ascites. (Correa et al., 2014;). These changes in cellular maintenance processes allow EOC spheroids to exhibit enhanced chemotherapy resistance relative to adherent cells (Espérance et al., 2008). The increased chemo resistant phenotype of EOC spheroids outlines the necessity for sensitizing these cells to chemotherapy as a potential therapeutic for reducing metastatic potential.

1.1.5 Modeling Metastasis *in vitro* and *in vivo*

To mimic the metastatic process *in vitro*, adherent EOC cells can be trypsinized and seeded into ultra-low attachment (ULA) dishes. The neutrally charged and hydrophilic coating of the plastic allows for the aggregation of cells in suspension without the need for agitation of the suspension culture. Various plate formats can be employed, with round-bottom ULA plates allowing for the aggregation of uniform spheroids that can be held consistent throughout experimental manipulation. Moreover, the use of round-bottom plates allows for the tracking of single spheroids over long periods of time (days to weeks), providing reproducible data in monitoring general spheroid formation. Suspension cultures not only work for established cell lines, but can be used for primary patient derived spheroids filtered from ascites samples (Shepherd et al., 2007).

To emulate dissemination of EOC cells throughout the peritoneal cavity, patient-derived xenografts (PDXs) can be used in murine models. Intraperitoneal injections of primary or immortalized EOC cells propagate cells throughout the abdominal cavity. Mice subsequently develop ascites and can be monitored for overall tumour burden (Sanja Sale, 2009; Liu et al., 2017). The most obvious deficit in the abovementioned methods is the lack of a functioning immune system in the mice, preventing researchers from
recapitulating the native EOC environment. Nevertheless, the outlined models provide necessary preclinical evidence for subsequent research during clinical trials.

1.2 AMP-activated Protein Kinase (AMPK)

1.2.1 Structure and Overall function of AMPK

The serine/threonine kinase, AMP-activated Protein Kinase (AMPK) serves as a bioenergetic stress sensor in nearly all mammalian systems. AMPK activation has been characterized as a major regulator of catabolic and anabolic processes although its functions are tissue specific (Hardie, 2014).

Following nutrient deprivation, increased cytosolic levels of adenosine monophosphate (AMP) and adenosine diphosphate (ADP) trigger AMPK phosphorylation at the threonine 172 residue (T172). Phosphorylation of AMPK can occur through one of the upstream kinases, Liver Kinase B1 (LKB1), calmodulin dependent protein kinase kinase beta (CAMKKβ) or mitogen-activated protein kinase kinase kinase 7 (TAK1) (Herrero-Martín et al., 2009). AMPK additionally possesses auto phosphorylation activity, helping amplify its signaling to downstream effectors.

AMPK activation can occur in three distinct (although not mutually exclusive) manners. AMP binding to the γ subunit promotes phosphorylation of the T172 residue by upstream kinases. Similarly, binding of AMP promotes allosteric activation. Finally, both AMP and ADP binding induce conformational change in protein structure ultimately preventing T172 dephosphorylation by protein phosphatases (Davies et al., 1995; Gowans et al., 2013).

The tissue specificity of AMPK lies in the multiple possible isoform combinations. AMPK exists as a heterotrimeric protein consisting of one catalytic (α) and two regulatory (β, γ) subunits. Up to 12 different isomeric configurations are possible making AMPK a variably expressed protein (Stapleton et al., 1996).
1.2.2 Pathways Under AMPK Control

AMPK is considered to be a master regulator of metabolism due to the numerous downstream effectors under its control (Figure 1.2). The two primary signaling branches can be categorized based on catabolic and anabolic pathway modulation. Following AMPK phosphorylation, catabolic pathways are activated to help increase overall ATP availability. Pathways responsible for fatty acid oxidation, autophagy as well as glycolysis are all increased, helping generate new sources of ATP. Similarly anabolic pathways are shut off, helping conserve what limited energy resources are left. Protein and lipid synthesis, gluconeogenesis as well as cellular proliferation pathways are all downregulated, placing the cell into a metabolically dormant state. Following increases of ATP levels, AMPK phosphorylation levels are reduced, allowing the cell to return to a more anabolic state.
Figure 1.2. AMPK is the cellular master energy sensor and regulates numerous anabolic and catabolic pathways

Following cellular stress, AMPK becomes phosphorylated at T172 (black dot), activating downstream catabolic processes (autophagy, lipid metabolism, glucose metabolism), while concurrently shutting off anabolic processes (protein synthesis, lipid synthesis, gluconeogenesis). Cellular proliferation is concurrently reduced when AMPK is activated.
1.2.3 Duality of AMPK Function in Cancer

AMPK activation has long been implicated in numerous cancers although its functions seem to be context dependent. A large body of literature indicates the function of AMPK as being tumour suppressive. Examinations of flash frozen tumour samples from numerous cancers correlated increased AMPK activation with improved progression-free survival (PFS) rates. The studies included head and neck, colorectal, gastric, lung and kidney cancers, highlighting the expansive tissue types exhibiting increased AMPK activation (Zadra et al., 2015). Supporting evidence for the tumour suppressive properties of AMPK came from Chou and colleagues who identified that AMPK knockdown promotes EMT. This finding was validated in both breast and prostate cancer cells, where EMT is considered a hallmark for metastasizing cells (Chou et al., 2014). AMPK has been additionally implicated in modulating tumorigenesis in an antagonistic manner with a known oncogene. Global knockout of the AMPK catalytic subunit (α1) promoted lymphoma development in transgenic mice overexpressing c-myc (Faubert et al., 2013). Faubert’s et al. work further identified that loss of AMPK signaling enhanced the Warburg effect in colorectal carcinoma cells, a common characteristic of highly glycolytic cancer cells. The authors found that the tumour suppressive properties of AMPK functioned through the downregulation of HIF-1α and related glycolytic genes, ultimately generating an “anti-Warburg” effect.

Although the use of AMPK activators has been cited as possessing anti-neoplastic effects, Vincent and colleagues found that the mechanism for cytotoxicity of these agonists often occur in an AMPK-independent manner (Vincent et al., 2015). As such, caution should be used when using agonists to elucidate AMPK-mediated anti-neoplastic effects.

The evidence outlining AMPK’s tumour suppressive role is substantial, however there have been a number of studies implicating a tumour-permissive role for AMPK as well. During metastasis, cells are subjected to high levels of hypoxia, reactive oxygen species (ROS), reduced nutrient availability and overall stress associated with substrate detachment. AMPK activation has been found to reduce cytotoxic ROS, thereby
permitting cancer cell survival through increasing NADPH levels generated by the pentose phosphate pathway (PPP). Lack of AMPK activation in LKB1-deficient A549 cells following glucose deprivation was found to increase cell death (Jeon et al., 2012) suggesting that cancer cells require some levels of AMPK to maintain energy homeostasis. More recently, high levels of AMPK activation were identified in native EOC spheroids, which are subject to decreased nutrient availability and hypoxia in ascites (Peart et al., 2015). Overall, these studies suggest that AMPK may actually contribute to preserving cancer cell viability during periods of heightened stress.

Ultimately, there appears to be a duality for the role of AMPK in cancer progression. During early stages of tumour development, AMPK seems to serve a tumour suppressive role by restricting overall cell growth and proliferation. In metabolically challenged cancer cells, activated AMPK may allow for cell survival until more favorable conditions are established, thus promoting metastasis.

1.3 Autophagy

1.3.1 Overview

Autophagy is a highly conserved, lysosomal degradative pathway. Activation of this complex signaling cascade allows for the turnover of long-lived proteins, and excess or aberrant organelles (He and Klionsky, 2010). A separate but equally important function of autophagy is to provide alternate sources of energy during periods of starvation. Activated during cellular stress (nutrient deprivation, infection, hypoxia), autophagy provides fresh sources of amino acids and ATP in metabolically challenged cells. Several sub-types of autophagy exist, including mitophagy, chaperone-mediated autophagy and xenophagy, that allow for the targeted degradation of specific organelles or pathogens (Dikic and Elazar, 2018). However, the most commonly studied (and the focus of this thesis) is macroautophagy. Macroautophagy (herein described as autophagy) is the process responsible for bulk degradation of cytoplasmic cargo. Dysregulation of autophagy has long been implicated in numerous pathologies, most notably in
neurodegenerative disease (Pickrell and Youle, 2015; Komatsu et al., 2006; Ichimura et al., 2008; Klionsky, 2006). More recently, autophagy has been found to mediate cancer progression with both tumour-suppressive and tumour-promoting functions (White, 2015).

1.3.2 History of Autophagy Research

The term “autophagy” was coined from the greek words “auto” meaning self and “phagy” meaning to eat. First described by Christian de Duve in 1963 following his discovery of lysosomes, the autophagy field was brought to the mainstream scientific stage by Yoshinori Ohsumi in the 1990s. Ohsumi’s characterization of 15 autophagy related proteins (ATGs) in yeast outlined essential proteins necessary for autophagy induction (Ohsumi, 2001). Subsequent work identified homologs in various model systems, although a large proportion of ATGs are highly conserved amongst eukaryotic cells. Interestingly, evidence from numerous sources indicates tissue specificity and differences in mediators of autophagy regulation in various pathologies, highlighting the complexity of this dynamic process (Herzig and Shaw, 2018; Hardie, 2014; Carling et al., 1996).

1.3.3 Degradation of Cytoplasmic Cargo

Autophagic degradation of cargo occurs through the induction of a double-membrane structure called a phagophore. Although the original source of phagophore is not fully understood, it is believed to originate from the endoplasmic reticulum or mitochondria. The phagophore can engulf cargo and subsequently fuse with the lysosome, forming the autolysosome. The acidity of the autolysosome (pH 4.5-6.5) (Maulucci et al., 2015) and lysosomal hydrolases contribute to the degradation of the cargo, which is broken down and ultimately released back into the cytoplasm through various permeases located within the double membrane (Lim and Zoncu, 2016).
1.3.4 Molecular Mechanisms of Autophagy Induction

Numerous signaling axes intersect to modulate autophagy regulation, with core machinery groups being sequentially recruited to complete autophagosome maturation (Figure 1.3).

Four main complexes are considered to be the main modulators of autophagy: (i) the Unc51 like autophagy activating kinase (ULK1), (ii) the Class III PI3K (PI3K C3) complex, (iii-iv) the ubiquitin-like ATG12-ATG5-ATG16 and Microtubule-Associated Protein 1 Light Chain 3 A/B/C (LC3) complexes.
Figure 1.3 Autophagy induction occurs in numerous sequential steps.

Following (A) cellular stress, AMPK activates the ULK1 complex and concurrently inhibits mTOR signaling to initiate autophagy. (B) Phagophore recruitment occurs with the recruitment of the Class 3 PI3K complex. (C) The ATG12/5/16 multimeric complex helps bring mature LC3-II to the phagophore membrane allowing for the engulfment of the cytoplasmic cargo. The subsequent (D) fusion of the lysosome generates the (E) autolysosome structure, where lysosomal hydrolases can degrade autophagic cargo and transport the degraded constituents back into the cytoplasm via permeases.
Various autophagy initiating pathways have been identified, the best-characterized being the mammalian target of rapamycin (mTOR) pathway. Originally identified in yeast (Noda et al., 1998), the mammalian homolog mTORC1, has been found to be a key repressor of autophagy (Hosokawa et al., 2009). With adequate nutrient and energy supply, mTORC1 repressively phosphorylates the ULK1 complex, preventing autophagy induction (Hosokawa et al., 2009). However, following nutrient starvation or rapamycin treatment, mTORC1 becomes inactive, thus allowing the ULK1 complex to activate the downstream PI3K complex. Activation of the PI3K complex promotes vesicle nucleation and expansion, thus starting the initial induction of the phagophore (Axe et al., 2008). The subsequent steps for vesicle expansion are dependent on a series of ubiquitin-like conjugation systems. PI3K activity promotes the assembly of the multimeric ATG12-ATG5-ATG16 complex through sequential activity of the E1 enzyme (ATG7), E2 enzymes (ATG10/3) and the E3 complex (ATG12-5) (Geng and Klionsky, 2008). Recruitment of the ATG12-ATG5-ATG16 complex to the phagophore membrane helps shape the autophagosome structure and directs the lipidation and binding of LC3 to the autophagosome (Fujita et al., 2008). LC3 has been identified as an essential protein for promoting autophagosome elongation and curvature whose loss disrupts autophagic flux (Lee and Lee, 2016). During autophagosome development, LC3 is cleaved by ATG4 to create LC3-I. The ATG7/3 complex subsequently lipidades LC3-I at the C-terminal glycine residue by adding a phosphatidylethanolamine group, generating LC3-II (Kabeya et al., 2004). Following LC3 recruitment to the phagophore, the ATG12-ATG5-ATG16 complex dissociates and the mature autophagosome fuses with the lysosome to degrade its cargo.

1.3.5 Experimentally Measuring Autophagy

Autophagy is a dynamic process, therefore interpreting autophagy markers can be challenging. Currently, assessment of autophagic flux is performed by immunoblot analysis of both LC3 and p62 levels. Being recruited to autophagosomal membranes, LC3 is proteolytically cleaved at its C-terminus followed by lipidation to generate LC3-II, making it an excellent marker for monitoring the progression of autophagosome development (Tanida, et al., 2008). Due to its ubiquitin-binding domain, p62 is known to
function as a mediator protein, targeting ubiquitinated proteins as well as LC3 to the autophagosomal membrane (Takamura et al., 2011). Accumulation of p62 protein levels is indicative of reduced autophagic flux due to inadequate lysosomal clearance (Klionsky et al., 2015). To accurately assess autophagy dynamics, LC3 and p62 levels should be interpreted in tandem. A high LC3-II: LC3-I ratio and a low level of p62 can be considered to be indicative of high autophagic flux, whereas an inhibition of this process would result in reduced conversion of LC3-I into LC3-II as well as an accumulation of p62.

Alternative methods for assessing autophagic flux include live-cell reporters such as the mCherry-eGFP-LC3B construct and transmission electron microscopy. During autophagosomal development and lysosomal fusion, the eGFP fluorescence becomes quenched due to the high acidity of the lysosome. Monitoring the change in overall fluorescence from green to red helps establish whether autophagy is running to completion. Although this system is predominantly used with confocal fluorescence microscopy to count autophagosomal punctae (Klionsky et al., 2015), we have adapted the reporter usage to fit our 3D model system of spheroid formation. In our model, we examine overall spheroid colour shift as a method of identifying autophagic flux disruptions. An alternative method to assessing autophagy is through transmission electron microscopy. By identifying and counting individual autophagosomes, researchers can infer if autophagy is running to completion. An accumulation of cytosolic autophagosomes suggests the cell’s inability to turnover these structures thus indicating a reduction in autophagic flux (Klionsky et al., 2015).

To monitor autophagy effectively, more than one assay should be performed to ensure accurate assessment of the autophagic state of the cell. Positive controls such as Chloroquine (CQ) a well-characterized lysomotropic agent (Maulucci et al., 2015; Morgan et al., 2014), can be used to ensure readouts for autophagic disruption are consistent.
1.3.6 Duality of autophagy induction in cancer

Early reports indicated a tumour suppressive role for autophagy in cancer. In the context of prostate, breast and ovarian cancer, BECN1, a gene encoding an important component of the PI3K complex, was reported to be lost monoallelically in up to 75% of tumour samples (White, 2015). However, the proximity of BECN1 to the BRCA1 locus, a known tumour suppressor, makes it difficult to elucidate whether loss of BECN1 truly contributes to cancer progression (Aziz et al., 2001; Piek et al., 2001). Interestingly, Correa and colleagues identified that BECN1 is actually dispensable for autophagy induction in ovarian cancer even though it is lost monoallelically in up to 70% of ovarian tumour samples (Correa et al., 2015).

The tumour suppressive role of autophagy emerged from studies employing ATG knockout models. In 2011, Takamura and colleagues identified that a liver-specific knockout of ATG7 in mice induces tumorigenesis in hepatocytes by promoting inflammation and accumulation of toxic ROS species (Takamura et al., 2011). Aberrant ROS levels had been previously characterized as playing a key role in mediating tumorigenesis with autophagy impairment potentiating the tumour phenotype (Karantza-Wadsworth et al., 2007).

Another important protein involved in tumour progression is p62 (otherwise known as sequestosome-1/ SQSTM1). High levels of p62 have been reported in various cancers and proposed as a contributing factor in tumorigenesis by increasing endoplasmic reticulum stress and DNA damage (Moscat and Diaz-Meco, 2009). Due to its ubiquitin-like binding domain, p62 acts as a scaffolding protein for LC3 during autophagosome formation. Autophagy deficiency has been shown to contribute to aberrant accumulation of p62 (Takamura et al., 2011), thus highlighting a tumour suppressive role for autophagy.

Although a large body of literature suggests a tumour suppressive role for autophagy, studies have identified that in certain contexts, autophagy is tumour promoting. During the process of metastasis, cells are often placed under large amounts of stress whether
that is through nutrient deprivation, hypoxia or substrate-detachment. A recycling process
such as autophagy may be beneficial in promoting cell survival during metabolic
depletion events. Indeed, autophagy has been shown to be upregulated in hypoxic
tumours (Spowart et al., 2012) and activated as a response to high levels of ROS
generated by cancer cells (Bellot et al., 2009). Hypoxic treatment of colorectal
adenocarcinoma cells induces autophagy as a cell survival mechanism (Bellot et al.,
2009), suggesting that avascular tumours can utilize autophagy as a strategy to confer
viability during hypoxic stress. From a more global perspective, punctate LC3B
expression in solid tumours has been correlated with poor patient outcomes in various
tissues including lung, prostate, ovaries, endometrium and pancreas (Klump et al., 2011).
The numerous types of cancers in which LC3B is highly expressed strengthen the notion
of autophagy being a tumour-promoting phenomenon.

Ovarian cancer spheroids are often suspended in nutrient deficient ascites fluid with
limited energy resources. Moreover, due to their compact morphology and lack of
vasculature, these spheroids are subjected to high levels of hypoxic stress (Aboagye et
al., 1995). Recently, Correa and colleagues identified that ovarian cancer spheroids are
highly autophagic relative to their adherent counterparts. Furthermore, Correa identified
that autophagy inhibition reduces spheroid viability, outlining a tumour-promoting role
for autophagy and suggesting a potential therapeutic target for ovarian cancer metastasis
(Correa et al., 2014).

It has also been shown that cancer therapy such as chemotherapy or radiation can induce
autophagy (Liu et al., 2010; Lomonaco et al., 2009). Due to high rates of chemotherapy
resistant-disease in ovarian cancer (Espérance et al., 2008), autophagy inhibition seems to
be a viable treatment modality to limit metastatic burden in ovarian cancer.
1.4 Rationale, Hypothesis and Objectives
We have previously shown that as EOC cells form spheroids they undergo bioenergetic stress, activate AMPK signaling, and force cells to enter a metabolically quiescent state (Peart et al., 2015). We have also demonstrated separately that EOC spheroids up-regulate autophagy as a cell survival mechanism (Correa et al., 2014). Although AMPK signaling has been listed as an activator of autophagy (Hardie, 2011), it has never been investigated in ovarian cancer spheroids. Herein, I sought to examine whether AMPK can modulate autophagy induction as a cell survival mechanism in EOC spheroids. I hypothesized that AMPK signaling was necessary and sufficient to induce autophagy as a cell survival mechanism in EOC spheroids. To investigate this hypothesis, AMPK activity was attenuated in spheroids using the AMPK inhibitor, Compound C, or with siRNA-mediated PRKAA1/2 silencing. Spheroids were also treated with STO-609, a selective inhibitor of CAMKKβ, to block downstream AMPK phosphorylation. Attenuating p-AMPK levels through either pharmacological inhibition or siRNA-mediated knockdown reduced autophagic flux in all EOC spheroids as visualized by fluorescence microscopy. However, I observed no change in spheroid viability following AMPK knockdown. It appears additionally that AMPK-mediated autophagy inhibition may occur in an LC3/p62 independent manner. Interestingly, I identified that CAMKKβ regulates AMPK activation and autophagic flux in spheroids. The results of this study will further our understanding of the mechanisms controlling autophagy in EOC, and may provide support for developing new treatment strategies for metastatic disease.
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2 Chapter 2
AMPK as a Modulator of Autophagy in Epithelial Ovarian Cancer Spheroids

2.1 Introduction
Ovarian cancer is the most deadly gynecologic malignancy in women. In Canada, the death to incidence ratio is approximately 0.64, making it the fifth highest ratio of all cancers (Canadian Cancer Society, 2017). A particular subset of this disease, epithelial ovarian cancer (EOC), is responsible for over 70% of all diagnosed ovarian cancer cases (Bowtell et al., 2015). The high mortality rates from EOC can be attributed to a variety of factors, particularly the lack of definitive symptoms that prevent women from identifying a cause for concern, potentially mistaking abdominal discomfort and bloating for generalized post-menopausal symptoms instead. Additionally, current diagnostic tests are limited to physical pelvic exams, ultrasound and CA-125 serum tests. The CA-125 serum biomarker is known to have poor sensitivity in early stages of disease progression, making it difficult for physicians to establish an accurate diagnosis (Nossov et al., 2008). As a result, the majority of EOC cases will present as late-stage disease with significant ascites buildup, where extensive surgical debulking procedures must often be coupled with cytotoxic chemotherapy in hope of slowing disease progression. Nevertheless, rates of recurrence remain exceptionally high, with the relapsed cancers exhibiting chemoresistant phenotypes (Vaughan et al., 2011). As such, gaining further understanding of the mechanisms that govern EOC disease progression is of utmost importance in developing new therapeutics against this type of malignancy.

A unique hallmark of EOC metastasis lies in the process of spheroid formation (Shield et al., 2009). EOC cells are capable of detaching from the primary tumour site and becoming suspended in peritoneal fluid located within the abdominal cavity. These cells can subsequently form aggregates known as spheroids, which spread throughout the abdominal cavity and ultimately reattach in new locations, giving rise to secondary tumours. Tumour spheroids are known to possess enhanced chemo-resistance (Espérance
et al., 2008), making them a promising model in which to study chemo-resistance mechanisms.

Previous literature suggests that as EOC cells form spheroids they undergo bioenergetic stress, forcing the cells to enter a metabolically quiescent state, facilitating survival by activating AMP-activated protein kinase (AMPK) signaling (Peart et al., 2015). A serine/threonine kinase, AMPK serves as a bio-energetic stress sensor in nearly all mammalian systems, and functions as a regulator of cell survival during starvation (Hardie, 2014). Following nutrient deprivation, increased levels of adenosine monophosphate (AMP) and adenosine di-phosphate (ADP) trigger AMPK phosphorylation at the threonine 172 residue (T172). Although AMPK phosphorylation has been characterized as a major regulator of inducing catabolic processes while concurrently down-regulating anabolic processes, its exact functions are often tissue specific (Hardie, 2014). Our previous reported increased levels of p-AMPK in EOC spheroids highlight the necessity of characterizing the function of this signaling axis in ovarian cancer.

It has also been separately shown that EOC spheroids upregulate macroautophagy (described here as autophagy), a lysosomal process allowing for the degradation and recycling of intracellular nutrients and damaged organelles (Correa et al., 2014). Dual roles have been suggested for autophagy in mediating cancer progression. Although in some cases autophagy can function as a tumour suppressive mechanism, a large proportion of studies have identified autophagy as a tumour promoting process, particularly in late stage disease (White, 2015). Various cancer cells undergoing cellular stress, particularly during metastasis and periods of hypoxia, are capable of upregulating autophagy and recycling their intracellular constituents ultimately providing alternative nutrient sources (Makeover, et al., 2008). This is thought to provide cancer cells with a mechanism to circumvent apoptosis and anoikis during metastasis.

One of the overarching signalling pathways linking metabolism homeostasis and autophagy regulation is AMPK and its associated proteins. Although listed as a canonical
activator of autophagy, AMPK-mediated signalling regulation of autophagy has never been characterized in EOC. Here we sought to examine the effect of AMPK signaling on autophagy induction in a spheroid model of ovarian tumour dormancy and metastasis. Herein, we demonstrate that loss of AMPK signalling reduces autophagic flux in EOC spheroids, although with no observed effect on spheroid cell viability.
2.2 Methods

2.2.1 Cell Culture

All work with patient materials has been approved by the Western University Research Ethics Board (Approval # 3818). Work was conducted with EOC cell lines; iOvCa147-MA, OVCAR8 and COV318, and are classified as high-grade serous EOC. The iOvCa147-MA line was generated by intrapertioneal injection of iOvCa147 cells into immunocompromised mice, which subsequently formed tumours and ascites fluid. This ascites fluid was collected and re-plated into tissue culture conditions, generating the iOvCa147-MA line. A non-malignant fallopian tube epithelial cell line FT190 (gift from R. Drapkin) was used as a control cell line (Karst and Drapkin, 2012). Cell lines were cultured in either DMEM (iOvCa147-MA, COV318, FT190) or RPMI (OVCAR8), supplemented with 10% fetal bovine serum (FBS) (Wisent) unless otherwise specified. Cells were either grown under adherent conditions on tissue culture plastic (Sarsted) or grown in suspension cultures using Ultra-Low Attachment (ULA) dishes (Corning) as performed previously (Correa et al., 2015).

2.2.2 siRNA knockdown

RNA interference-mediated knockdown was achieved using Dharmacon siGenome SMARTpool reagents: Non-targeting control pool #2 (D-001206-14-05), PRKAA1 (D-001206-14-05), PRKAA2 (M-005361-02-0005). Cells were seeded onto 6-well adherent plastic plates, 300,000 cells/well (iOvCa147-MA), 100,000 cells/well (OVCAR8) and allowed to attach overnight. The next day siRNA and DharmaFect transfection reagents (T-2001–02) were both diluted in 200 µL of serum free media. Following 5 min incubation, siRNA-containing medium was added to DharmaFect-containing medium and incubated for 20 minutes. Antibiotic free media was added to the media complex to a final volume of 2 ml/well and plated onto the cells. Cells were subsequently left in transfecting media for 3 days, after which the cells were trypsinized and seeded into non-adherent ULA dishes for 48 hours for protein isolation and fluorescence microscopy.
2.2.3 Generation of mCherry-eGFP-LC3B clones

OVCAR8 cells were plated into 6-well adherent plastic culture vessels at a density of 100,000 cells/well. Cells were transfected with the pBABE-puro-mCherry-eGFP-LC3B plasmid (gift from Jayanta Debnath, Addgene plasmid # 22418; RRID: Addgene_22418), using the Lipofectamine 2000 reagent according to the manufacturer’s protocol (MAN0009872, Invitrogen, Carlsbad Ca). Following 72 hours, cells were placed in puromycin-supplemented (1 µg/ml) media for 72 hours. Puromycin-resistant fluorescent clones were selected using cloning rings and expanded clonal populations expressing high levels of green fluorescence were chosen for subsequent experiments. The COV318 and FT190 cell lines were generated in an identical fashion as described above however, following puromycin selection, transfected cells were pooled in suspension conditions using ULA dishes and selected on the basis of high red fluorescence.

2.2.4 Live Fluorescence Microscopy

OVCAR8, COV318 and FT190 mCherry- eGFP-LC3B cells were imaged with a Leica (DMI 4000B) fluorescence microscope. All image processing (overlay) was performed on Leica Application Suite (LAS). Fluorescent images were imaged using the GFP and Y3 filter cubes. All images are presented in their original format with no adjustments in colour or exposure correction.

2.2.5 IncuCyte® ZOOM live cell imaging

Following siPRKAA1/2 transfection, OVCAR8 mCherry-eGFP-LC3B spheroids were seeded at 4000 cells/well in 96-well round-bottom ULA plates. Each individual well of cells was imaged using the IncuCyte® ZOOM live cell imaging system (Sartorius, phase, GFP, RFP) at 2-hour intervals for 72 hours. Images were subsequently stitched together to generate time-lapse videos. All images/videos are presented in their original format with no adjustments in colour or exposure correction.
2.2.6 Protein Isolation

*Adherent Culture Isolation:* Cells were washed with ice cold Phosphate Buffered Saline (PBS) and placed in modified radioimmunoprecipitation assay (RIPA) lysis buffer as described previously (Correa et al., 2014). Cells were subsequently scraped and let to lyse on ice for 30 minutes.

*Spheroid Culture Isolation:* Spheroid cell suspensions were collected and centrifuged at 2400 RPM for three minutes. The cell pellet was washed with PBS and placed in a modified RIPA lysis buffer for 30 minutes on ice.

2.2.7 Immunoblotting

Total protein concentration was quantified by Bradford assay with bovine serum albumin (BSA) used as standards. Protein samples were diluted to a working concentration of 30 µg protein per sample and were resolved by 8 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% w/v BSA in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). Membranes were subsequently probed with protein specific primary antibodies at a 1:1000 dilution and incubated overnight at 4°C. Following primary antibody incubation, membranes were incubated for 1 hour at room temperature with a peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (1:10000 in 5% BSA). Protein detection was achieved through enhanced chemiluminescence using Luminata Forte (Millipore Sigma) and imaging was performed using the Chemidoc™ MP 7 System (Bio Rad). Densitometric analyses were subsequently performed using ImageLab™ software with tubulin used as a loading control. Certain membranes requiring assessment of both phosphorylated and total levels of particular proteins were stripped with stripping buffer (19.8 ml H₂O, 5 ml 10% SDS, 1.5 ml Tris-HCl pH 6.6, 177 µL BME) and subsequently blocked and re-probed with the appropriate antibody.
2.2.8 Antibodies and Other Reagents

Primary antibodies were used to detect threonine-172 phosphorylated AMPKα (40H9), total AMPKα (D63G4), p62 (2775S), LC3B (5114S) (Cell Signaling Technology, Danvers, MA), tubulin (T9026) (Sigma, Mississauga, ON). Secondary antibody: anti-rabbit (NA934V GE Healthcare, Chicago IL). The following pharmacologic agents were used: Compound C (P5499), Chloroquine (6628), Metformin (D150959-5G) (Sigma, Mississauga, ON), AICAR (10010241), ST0-609 (15325), Oligomycin (11342) (Cayman Chemical, Ann Arbor, MI) at concentrations as indicated in the text.

2.2.9 Trypan Blue Viability Assay and Cell Counting

Cell viability counts were performed using the Trypan Blue Exclusion method. Cells were trypsinized and the resulting suspension was centrifuged at 2400 rpm for 3 min. Suspensions were washed with PBS and centrifuged at 1000 rpm for 3 min. To disaggregate clusters of cells, 50 µl of trypsin (0.25%) was added to each pellet and left to dissociate for 10 minutes. Following dissociation, 50 µl of FBS was added to inactivate the trypsin. Prior to loading the cell suspension into the BioRad cell counting slide, 100 µl of Trypan Blue Reagent (Gibco, #1520-061) was added to the solution. All cell counts were performed on the BioRad TC10™ automated cell counter.

2.2.10 Alamar Blue Viability Assay

Cells were plated at a density of 2000 cells/well into 96-well flat bottom ULA dishes and allowed to aggregate as spheroids for 2 days. Alamar Blue reagent (1:10 dilution in media) was added to wells containing media and cells. Fluorescence excitation and emission was measured at 560 and 590 nm after 6 hours using the Wallac 1420 Victor 2 spectrophotometer. Viability readings are presented as normalized fluorescence intensity (arbitrary units).

2.2.11 Clariom™ S Assay Transcriptome and Gene Ontology Analysis

OVCAR8 cells were grown in adherent conditions for 48h or under ULA conditions for 4h and 24h at which point RNA was isolated and collected using the RNeasy prep kit
Sample purity was assessed using the Nanodrop™ spectrophotometer (Thermo Fisher Scientific). RNA samples were diluted to a working concentration of 100 ng/µl in water and sent to the TCAG Microarray Facility, The Hospital for Sick Children (Toronto, ON) for Clariom™ S analysis. Raw data processing was conducted using the Transcriptome Analysis Console Software (Thermo Fisher Scientific). Gene ontology analysis was performed to assess changes in autophagy-related transcript abundance. For full autophagy gene ontology list see Appendix A.

2.2.12 Statistical Analysis

All graphs were generated using Graphpad Prism 8 (La Jolla, California). Data are expressed as mean ± SEM. All statistical analyses (Student’s t-test and ANOVA (analysis of variance) with Dunnett’s multiple comparison test) were performed using Graphpad Prism 8. Results were deemed significant at P<0.05.
2.3 Results

2.3.1 Cell detachment and spheroid formation does not alter key autophagy or AMPK gene expression

Currently, assessment of autophagic flux is performed by assessing protein levels of both microtubule-associated protein 1A/1B-light chain (LC3) as well as p62 (sequestosome1). Being recruited to autophagosomal membranes, LC3 is proteolytically cleaved at its C-terminus followed by lipidation to generate LC3-II, making it an excellent marker for monitoring the progression of autophagy (Tanida, et al., 2008). Due to its ubiquitin-binding domain, p62 is known to function as a mediator protein, targeting ubiquitinated proteins to the autophagosomal membrane. Accumulation of p62 protein levels is indicative of reduced autophagic flux (Klionsky et al., 2015). Prior to assessing protein status of these key protein markers, autophagy transcriptome analysis was performed to determine whether any changes in AMPK, LC3 or p62 transcript levels could be attributed to loss of cell attachment. To elucidate what autophagy related transcriptome changes occur during loss of cell attachment and spheroid formation, we performed Clariom™ S transcriptome profiling followed by Gene Ontology (GO) analysis of 421 known autophagy-related genes (GO:0006914, Qiagen Autophagy RT Profile Array Gene List) in the OVCAR8 cell line.

OVCAR8 cells suspended in ULA conditions for 4 hours as well as 24 hours were compared to a sub confluent 48h adherent cell control group. Neither of these data sets yielded significant changes in expression of AMPK catalytic subunits (PRKAA1 & PRKAA2), LC3B (MAP1LC3B) or p62 (SQSTM1) (Figure 2.1). This data implies that any changes we may observe in protein expression levels between adherent and spheroid conditions are due to post-translational processing rather than mRNA production or processing. Interestingly, very few autophagy related genes are differentially expressed between adherent and spheroid conditions. Within the 4-hour spheroid condition, only 11 genes were found to be differentially expressed (Supp. Table 2.1) whereas only 2 genes were differentially expressed in the 24-hour spheroid condition (Supp. Table 2.2). Furthermore, differentially expressed genes found in the earlier time point are not
maintained within the 24-hour group, with two new genes appearing during the later stage of spheroid formation. Overall, as expected, altered autophagy regulation in spheroids is unlikely to occur at the level of gene expression.
OVCAR8 cells were grown in adherent conditions for 48h or under ULA conditions for 4h and 24h at which point RNA was isolated and sent for Clariom™ S Assay transcriptome analysis. No significant difference (NS) was observed between adherent (48h) and spheroid (4h, 24h) conditions of two AMPK catalytic subunits (PRKAA1/2), LC3B (MAP1LC3B) and p62 (SQSTM1) (n=3).

Figure 2.1. AMPK, p62 and LC3B mRNA transcript profile does not change following spheroid formation in OVCAR8 cells.
2.3.2 EOC cells exhibit concurrent increase in phosphorylated AMPK and LC3 processing during spheroid formation

In order to identify AMPK phosphorylation kinetics as well as to characterize autophagy induction, iOvCa147-MA cells were seeded in ULA conditions and isolated at various time points. Following immunoblot analysis, we identified increased levels of LC3 processing and increased p-AMPK at T172 in later stage spheroids (24-72 hours) relative to adherent control cells (Figure 2.2). One of the most significant time points for both high levels of p-AMPK as well as LC3 processing was the 48 hour time point, this data led us to perform all subsequent spheroid experiments at the 48-hour time point. Additional time course experiments conducted in the OVCAR8 cell line further validated the 48-hour time point as optimal for evaluating spheroids (data not shown).
Figure 2.2. iOvCa147-MA spheroids display increased phospho-AMPK and LC3 processing when compared to adherent control cells.

Cells were trypsinized and seeded in ULA plates for up to 72 hours after which protein was isolated for (A) immunoblotting (B) phase contrast imaging of spheroid formation (scale bar=250 µm). Sub-confluent 72-hour adherent cells cultured in regular tissue plastic were used as a control. (C) Densitometric analysis for p-AMPK/AMPK, LC3-II/ I, LC3-II levels. Tubulin was used as loading control. Data presented are the mean (n=3) ± SEM. Significance tested by one-way ANOVA followed by Dunnett’s multiple comparison test. Asterisks indicate significant differences relative to control (*P<0.05).
2.3.3 AMPK knockdown potently inhibits autophagic flux in EOC spheroids but does not alter p62 or LC3 processing

To elucidate the role of AMPK signalling regulation of autophagy, we performed siRNA-mediated knockdown of two AMPK catalytic subunits (α1 and α2) in both iOvCa147-MA and OVCAR8 spheroids. AMPK exists as a heterotrimeric protein consisting of one catalytic (α) and two regulatory (β, γ) subunits. Although up to 12 different isomeric configurations are possible, there are only two known catalytic subunits that are encoded by the genes PRKAA1, PRKAA2 (Stapleton et al., 1996). Combined knockdown of both catalytic subunits allowed us to control for any potential variations in differential gene expression in our cell lines of study, control for compensation and maximize attenuation of AMPK activity. Following transfection in adherent conditions, cells were trypsinized and seeded into ULA conditions for 48 hours, at which point protein was collected for immunoblot analysis. To our surprise, AMPK knockdown in iOvCa147-MA or OVCAR8 48-hour spheroids did not significantly alter LC3 or p62 processing relative to siNT control spheroids. There was no significant difference in LC3 processing, LC3-II or p62 levels (Figure 2.3). This was quite intriguing, as AMPK has been implicated in various models of study as a canonical activator of autophagy, and its loss severely inhibits autophagic flux (Egan et al., 2010; Kim et al., 2011; Hardie, 2011). No significant difference in spheroid cell viability was observed between the AMPK knockdown and control as determined by Trypan blue exclusion (Figure 2.3). Our viability data further corroborate previously reported negligible effects of AMPK knockdown on overall spheroid viability (Peart et al., 2015).

To further investigate the effect of AMPK knockdown on autophagic flux in EOC spheroids, we used OVCAR8 cells transfected with an eGFP-LC3B fluorescent autophagy reporter construct. Following AMPK knockdown, OVCAR8 cells were seeded as spheroids and assessed using live-cell fluorescence microscopy. Interestingly, a buildup of green fluorescence was observed in spheroids following AMPK knockdown indicating a block in autophagic flux (Supp. Figure 2.3). However, as autophagy is a very dynamic process, the use of a single colour reporter poses certain limitations. It is difficult to truly monitor autophagic progression from early-to-late stage autolysosome
maturation with this single fluorescence reporter construct. To address this issue, we stably transfected the OVCAR8 cell line with the mCherry-eGFP-LC3B reporter. Following autophagosome fusion with the acidic lysosome, the pH-sensitive eGFP signal is quenched, whereas the mCherry signal remains unaffected (Figure 2.4A). Highly autophagic cells will exhibit predominantly red fluorescent autophagic punctae, indicative of increased autophagic flux. Conversely, inhibiting autophagy induces an increase of overall green fluorescence due to the reduction in fusion of autophagosomes with lysosomes. Although this reporter has been broadly used in adherent culture systems (Morisaki et al., 2009; Gump and Thorburn, 2014), we have adapted the generic reporter usage protocol to fit our spheroid model (Pampaloni et al., 2017). By placing OVCAR8 mCherry-eGFP-LC3B cells into ULA conditions and assessing overall fluorescence colour shift rather than individual autophagic punctae, we can characterize autophagic flux in clusters of spheroids in a rapid manner.

Knockdown of AMPK in OVCAR8 mCherry-eGFP-LC3B spheroids shows stark increases in overall green fluorescence relative to non-targeting or untreated control spheroids which have predominantly low levels of green fluorescence (Figure 2.4B). To validate the apparent block of autophagic flux, we treated spheroids with chloroquine (CQ), a well-characterized lysomotropic agent that inhibits lysosomal fusion to the autophagosome (Klionsky et al., 2015). Treatment of OVCAR8 mCherry-eGFP-LC3B spheroids with 50 µM CQ for 4 hours resulted in similar buildup of green fluorescence as seen with the AMPK knockdown (Figure 2.4B). These findings validate that the reporter cells within spheroids are sensitive to autophagy blockade and that AMPK is capable of reducing autophagic flux as well. However, based on our immunoblot data, AMPK mediated autophagy blockade may occur in an LC3- and p62-independent manner.
Figure 2.3. AMPK siRNA knockdown does not affect LC3/p62 processing or viability of iOvCa147-MA or OVCAR8 spheroids.

Double knockdown of AMPK catalytic subunits was achieved using DharmaFECT, non-targeting (NT) and PRKAA1/2 siRNA in adherent cells. Cells were subsequently trypsinized and seeded in ULA plates for 48 hours after which protein and cells were isolated for (A, D) immunoblotting and (C, F) viability assessment using the Trypan Blue Exclusion Method (N=3). (B, E) Densitometric analysis for AMPK, p62, LC3-II, LC3-II/LC3-I levels. Tubulin was used as loading control. Data presented are the mean (n=3) ±SEM. Significance tested by Student’s t-test. Asterisks indicate significant differences relative to control (****P<0.001).
Figure 2.4. AMPK knockdown results in inhibition of autophagic flux in OVCAR8 mCherry-eGFP-LC3B spheroids.

(A) General schematic depicting colour shift of fluorescent mCherry-eGFP-LC3B reporter following autophagy induction. (B) Adherent cells were transfected with non-targeting siRNA (NT) or siPRKAA1/2, and subsequently plated into ULA culture dishes for 48 hours. Spheroids treated with a chloroquine burst (50 µM) for 4 hours prior to imaging were used as a positive control for autophagic flux inhibition. All spheroids were imaged 48 hours post-seeding. Scale bar = 200 µm.
2.3.4 AMPK inhibition using Compound C inhibits autophagic flux and reduces LC3 clearance in EOC cells

In addition to siRNA-mediated knockdown of AMPK, we sought to examine the effect of a pharmacological inhibitor of AMPK on EOC spheroids. Currently, Compound C (also known as dorsomorphin) is the only known selective inhibitor of AMPK (Dasgupta and Seibel, 2018).

In both iOvCa147-MA and OVCAR8 cells, treatment with Compound C resulted in modest reduction of phosphorylated AMPK, which was not statistically significant. However, significant increases in LC3 processing as well as slight increases of p62 levels (Figure 2.5) suggest that Compound C at higher concentrations is capable of inhibiting autophagy. Fluorescent OVCAR8 mCherry-eGFP-LC3B spheroids treated with a Compound C (10 µM) for 24 hours exhibited a large buildup of green fluorescence relative to their untreated controls (Figure 2.6). This strengthens the notion that Compound C itself is capable of inhibiting autophagic flux in spheroids although this may occur in an AMPK-independent manner.
Figure 2.5. Pharmacologic inhibition of AMPK using Compound C results in significant increase in LC3 processing in iOvCa147-MA and OVCAR8 spheroids.

Spheroids were generated for 24 hours in 6-well ULA plates prior to the addition of Compound C for an additional 24 hours before protein was isolated for (A, C) immunoblotting. (B, D). Densitometric analysis for p-AMPK/AMPK, p62, LC3-II/LC3-I and LC3-II levels. Tubulin was used as loading control. Data presented are the mean (n=3) ±SEM. Significance tested by one-way ANOVA followed by Dunnett’s multiple comparison test. Asterisks indicate significant differences relative to control (*P<0.05).
Figure 2.6. Compound C decreases autophagic flux in OVCAR8 mCherry-eGFP-LC3B spheroids.

Representative fluorescent images of Compound C treated spheroids. 25,000 cells/well were plated into 24-well ULA dishes. Spheroids were generated over the course of 24 hours at which point 10 µM of Compound C was added to the well. Spheroids were left in culture for an additional 20 hours at which point certain wells received chloroquine (CQ) (50 µM) for 4 hours. All images were taken 48 hours post-seeding. Scale bars= 200 µm
2.3.5 Pharmacologic inhibition of CAMKKβ reduces AMPK phosphorylation and inhibits autophagic flux

Due to the limited availability of small molecule inhibitors of AMPK, we sought to attenuate AMPK phosphorylation by targeting upstream kinases that lead to AMPK activation. One of the best-characterized upstream kinases of AMPK is Liver Kinase B1 (LKB1) encoded by the gene STK11. A serine-threonine kinase, LKB1 is a highly conserved protein that functions as a regulator of cellular metabolism within the AMPK signaling axis (Neumann et al., 2003). Surprisingly, previous work in our laboratory identified that EOC cells lacking LKB1 retain high levels of p-AMPK, suggesting alternative kinases may target AMPK (Buensuceso & Shepherd, submitted). This finding led us to examine a secondary regulatory kinase of AMPK, calcium/calmodulin-dependent protein kinase kinase beta (CAMKKβ).

Previous literature has implicated CAMKKβ as an alternative AMPK activating kinase (Hawley et al., 2005). More recently, work examining the effects of cellular matrix deprivation on AMPK activity has identified CAMKKβ as main regulator of AMPK phosphorylation in breast cancer cell lines (Sundararaman et al., 2016). As such, we decided to use a selective inhibitor of CAMKKβ STO-609, as a complementary method of attenuating AMPK phosphorylation. To perform these experiments, we examined the effect of ST0609 in additional mCherry-eGFP-LC3B reporter cell lines. To this end, we expanded our work using the EOC line COV318, as well as a non-malignant human fallopian tube epithelial FT190 cell line. Treatment of FT190 and EOC spheroids with 10 µM ST0-609 resulted in significant reduction in p-AMPK status. We additionally observed a significant increase in p62 accumulation but with no change in LC3 processing (Figure 2.7). ST0-609 treated spheroids exhibited strong increases in green fluorescence relative to their DMSO control, again indicating an inhibition of autophagic flux. We observed a large buildup of green fluorescence in spheroids using the FT190 line, which suggests that CAMKKβ mediated regulation of AMPK and autophagic flux occur in EOC cells and their premalignant precursors too.
Figure 2.7. Continued on next page
Figure 2.7. STO-609 treated spheroids exhibit reduced levels of p-AMPK relative to their DMSO controls with significant increases in p62 accumulation and dramatic autophagic flux blockade.

Non-transfected iOvCa147-MA and transfected OVCAR8, COV318 and FT190 with the mCherry-eGFP-LC3B reporter were seeded as spheroids for 24 hours in 24-well ULA plates prior to the addition of 10 µM ST0-609 for an additional 24 hours before protein was isolated for (A) immunoblotting (B) Densitometric analysis for p-AMPK/AMPK, p62, LC3-II /LC3-I, LC3-II levels in three pooled EOC cell lines (iOvCa147-MA, OVCAR8, COV318). Tubulin was used as loading control. Data presented are the mean (n=3) ±SEM. Significance tested by one-way ANOVA followed by Student’s t-test. Asterisks indicate significant differences relative to control (*P<0.05). (C) Representative fluorescent images of STO-609 treated spheroids.100,000 cells/well were plated into 24-well ULA dishes and treated as described above. Images were taken 48 hours post-seeding. Scale bars= 200 µm
2.3.6 Treatment of EOC cells with either Compound C or STO-609 results in minor reduction in overall spheroid viability

We did not observe any change in spheroid viability due to AMPK knockdown EOC spheroids (Figure 2.3); thus we decided to examine whether these results were reproduced following treatment with Compound C and STO-609. OVCAR8 mCherry-eGFP-LC3B and iOvCa147-MA cells were seeded in 96-well flat-bottom ULA plates and allowed to aggregate as spheroids for 24 hours before the addition of either Compound C (10 µM) or STO-609 (10 µM) for an additional 24 hours. No significant differences were observed between control and drug treatment groups for the iOvCa147-MA cell line.

Spheroid viability was decreased in the OVCAR8 cell line due to Compound C and STO-609 drug treatments. However, such a small change (15%) within the treatment groups suggests that this observed phenomenon might not be biologically significant (Figure 2.8). Overall, multiple strategies to block AMPK in EOC spheroids can block autophagic flux yet have little to no effect on cell viability.
Figure 2.8. Compound C and ST0609 treatment reduces spheroid viability in OVCAR8 cells but not in iOvCa147-MA cells.

Cells were seeded at 20,000 cells/well for 24 hours in 96-well ULA plates prior to the addition of 10 µM Compound C or ST0-609 for an additional 24 hours. Alamar Blue reagent was subsequently added for an additional 6 hours at which point fluorescence intensity was measured (excitation 560 nm, emission 590nm). Data are normalized to spheroid treated with DMSO.
2.3.7 AMPK activation in adherent OVCAR8 cells is not sufficient to induce autophagy

It has been previously shown that adherent EOC cells have significantly lower levels of autophagy relative to their spheroid counterparts (Correa et al., 2014). However, all EOC cells seem to express variable levels of basal autophagy. As such, we deemed it important to investigate whether AMPK-independent autophagy regulation is an inherent characteristic of all EOC cells independent of their adherence status. Seeing as activation of AMPK is elicited by increased AMP/ATP ratio in cells (Hardie, 2014), adherent OVCAR8 and iOvCa147-MA cells were treated with the AMP mimetic, AICA ribonucleotide (AICAR) to increase p-AMPK levels. Following AICAR treatment for 24 hours, autophagy markers were examined by immunoblot analysis. Unfortunately, we were unable to elicit an activation of p-AMPK with AICAR despite numerous changes to the treatment protocol (data not shown). As such, we employed an alternative approach by using oligomycin (100 nM) and metformin (2 mM) for 24 hours. Both drugs are known activators of p-AMPK (Kim et al. 2016) and were able to increase p-AMPK levels in OVCAR8 cells, however no significant changes were observed with LC3 processing or p62 levels (Supp. Figure 2.2). No significant p-AMPK activation was observed in the iOvCa147-MA cell line; therefore we cannot attribute AMPK to regulate autophagy activation in this particular EOC line. These results suggest that AMPK activation on its own may not be sufficient to induce autophagy in adherent EOC cells.
2.4 Discussion
Aberrant dysregulation of autophagy has long been implicated in numerous pathologies (Dikic and Elazar, 2018). In the context of ovarian cancer, it appears that autophagy serves a tumour protective role. We have previously demonstrated that EOC spheroids display increased levels of autophagy and that subsequent autophagy blockade can reduce overall EOC spheroid viability (Correa et al., 2014). We have also shown previously how p-AMPK is increased in both primary and immortalized EOC spheroids relative to adherent counterparts (Peart et al., 2015). In this study we have bridged the two phenomena together and examined the role of AMPK in regulating autophagy induction.

In our current study, we have identified that RNAi-mediated AMPK inhibition strongly inhibits autophagic flux as visualized by fluorescence microscopy. Interestingly, this phenotype appears to occur in an LC3 and p62-independent manner. The discrepancy in our results between fluorescent and immunoblot assays raise certain questions as to what specific players mediate autophagy induction in ovarian cancer. Initially described in yeast as ATG8, several orthologs of the ubiquitin-like LC3 protein have been identified in mammals, although most work has been focused on LC3B. More recently, studies have implicated LC3B-independent forms of autophagy. One of the promising LC3 orthologs is Gamma-aminobutyric acid receptor-associated protein (GABARAP). GABARAP has been shown to possess separate functions from LC3, as it is involved in late stage autophagosome maturation (Weidberg et al., 2010). More recently, LC3-independent autophagy in rat hepatocytes was shown to be regulated primarily through the GABARAP complex with the autophagosome (Engedal and Seglen, 2016). Since we did not observe major effects on LC3, we are currently investigating what effect AMPK knockdown has on overall GABARAP levels.

An alternative explanation for the discrepancy in our results could be the issue of sensitivity of the assays used in our study. Immunoblotting may require larger changes in autophagic flux to exhibit a significant difference in protein levels of LC3 and p62. Our fluorescent reporter cell lines might be more sensitive to disruptions in autophagic pathway signaling as they measure the amount of autophagosome-lysosome fusion events.
rather than buildup of particular protein levels. To address this, future experiments may require longer treatment times with AMPK inhibitors prior to protein isolation for immunoblotting. Since our experiments were conducted at 48 hours, increasing drug exposure time might allow for a larger buildup of p62 protein levels.

Our findings with the CAMKKβ inhibitor (STO-609) corroborate our AMPK knockdown data, strengthening the notion that AMPK is required for autophagy induction under spheroid conditions. This phenotype holds true not only for EOC cell lines, but also in nonmalignant fallopian tube cells. This novel finding supports future directions investigating the role of CAMKKβ-AMPK signaling in modulating autophagy in EOC. Due to inherent limitations of using a small molecule inhibitor on CAMKKβ with multiple downstream effectors (Høyer-Hansen et al., 2007), future work will validate the effect of CAMKKβ knockdown on autophagy blockade. Nevertheless, understanding the CAMKKβ-AMPK signaling axis in the context of EOC spheroid biology will provide a better understanding of the downstream metabolic processes, including autophagy regulation.

Although Compound C does not seem to attenuate p-AMPK signalling nearly to the same extent as either RNA interference or STO-609 treatment, Compound C inhibition similarly inhibits autophagic flux in EOC spheroids. The observed ineffectiveness of p-AMPK attenuation using Compound C might be attributed to the potential off-target effects using this drug. Previous literature has identified multiple intersecting pathways that are potently affected by Compound C that can occur in an AMPK independent-manner (Harhaji-Trajkovic et al., 2010; Zhao et al., 2018). Compound C’s targeting of multiple kinases may explain the observed changes in LC3 processing in EOC spheroids that could be AMPK-independent.

Treatment of adherent OVCAR8 cells with several AMPK activators did not show any significant changes in LC3 or p62 processing. Using metformin and oligomycin, we were able to activate AMPK, however these drugs function indirectly on AMPK by inhibiting mitochondrial respiration (Gledhill et al., 2007; Galdieri et al., 2016). Due to the
potentially confounding variables associated with disrupting the electron-transport chain (ETC), care must be taken when interpreting AMPK activation results with respect to autophagy. Moreover, these agents did not elicit any changes in p-AMPK in the iOvCa147-MA line. Taken together, we consider it unlikely that AMPK activation on its own is able to induce autophagy in EOC cells.

Finally, our transcriptome analysis of adherent and spheroid OVCAR8 cells has indicated that very few autophagy related genes (only 2 out of 422 examined) are differentially expressed between adherent and spheroid EOC cells. As expected, the majority of changes in autophagy that we see during the early stages of EOC spheroid development can be attributed to post-translational processing, highlighting the importance of protein-based assays in evaluating autophagic flux.

Overall, it appears that AMPK is required in part to induce autophagy in EOC spheroids although this may occur in an LC3-and p62-independent manner. We also show that p-AMPK regulation is likely regulated through CAMKKβ in EOC spheroid and it can impact autophagic flux in these structures. These findings will help to further characterize signaling axes regulating autophagy induction in EOC, and may represent novel therapeutic targets for modulating autophagic response in metastatic disease.
2.5 Supplementary Tables and Figures

**Supplementary Table 2.1: Clariom™ S Transcriptome Analysis of Autophagy Related Genes in OVCAR8 48h adherent (ADH) cells vs. 4h spheroids (SPH).**

Table of differentially expressed autophagy related genes in 48h adherent and 4h spheroid OVCAR8 cells. False Discovery Rate (FDR) p value set at 0.05. Raw data processing was conducted using the Transcriptome Analysis Console Software.

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<th>OVCAR8 Adh 48h Avg (log2)</th>
<th>OVCAR8 4h Sph Avg (log2)</th>
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<th>Gene Symbol</th>
<th>FDR P-val</th>
<th>Description</th>
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Supplementary Table 2.2: Clariom<sup>TM</sup> S Transcriptome Analysis of Autophagy Related Genes in OVCAR8 48h adherent (ADH) cells vs. 24h spheroids (SPH).

Table of differentially expressed autophagy related genes in 48h adherent and 24h spheroid OVCAR8 cells. False Discovery Rate (FDR) p value set at 0.05. Raw data processing was conducted using the Transcriptome Analysis Console Software.

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<tr>
<th>OVCAR8 Adh 48h Avg (log2)</th>
<th>OVCAR8 Sph 24h Avg (log2)</th>
<th>Fold Change</th>
<th>P-val</th>
<th>Gene Symbol</th>
<th>FDR P-Val</th>
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Supplementary Figure 2.1 AMPK knockdown results in overall increased green fluorescence in OVCAR8 eGFP-LC3B spheroids.

Adherent cells were transfected with non-targeting siRNA (siNT) or siPRKAA1/2, and subsequently plated into ULA culture dishes for 48 hours. All spheroids were imaged 48 hours post-seeding. Scale bar = 200 µm
Supplementary Figure 2.2. AMPK activation does not alter LC3 and p62 processing in adherent iOvCa147-MA and OVCAR8 cells

EOC cells were plated at a density of 150,000 cells/well in 6-well adherent plates and let to attach overnight. Cells were subsequently treated with either Oligomycin (100nM) or Metformin (2mM iOvCa147-MA, 1mM OVCAR8) for 24 hours at which point cells were collected for (A,C) immunoblotting. (B,D). Densitometric analysis for p-AMPK/AMPK levels, p62 levels. LC3-II : LC3-I ratio, tested by one-way ANOVA followed by Dunnett’s multiple comparison test (n=3). Asterisks indicate significant differences relative to control (*P<0.05), NS (non significant).
2.6 References


Ichimura, Y., T. Kumanomidou, Y.S. Sou, T. Mizushima, J. Ezaki, T. Ueno, E.


Complications.


Ono, T., H. Nishijima, N. Adachi, S. Iiizumi, A. Morohoshi, H. Koyama, and K. ichi


3 Chapter 3
Discussion

3.1 Summary

The work presented in this thesis outlines the role of AMPK in mediating autophagy regulation in EOC spheroids, which to this date was uncharacterized. Transcriptome analysis of adherent and spheroid EOC cells showed few transcriptional changes following substrate detachment in autophagy markers, with only two genes (GABRA5, IFI16) being differentially expressed after 24 hours of suspension culture. Additionally, PRKAA1/2 expression remained unchanged throughout suspension conditions supporting the notion that both AMPK and conventional autophagy markers are post-translationally processed.

At the protein level, following substrate detachment, EOC cells increase p-AMPK, with highest levels of p-AMPK appearing during the later (48-72hr) time points. Knockdown of p-AMPK at the later time point (48 hr) markedly inhibits autophagic flux, yet this phenomenon appears to largely occur in an LC3/p62 independent manner. Pharmacologic inhibition of AMPK through either a direct AMPK inhibitor (Compound C) or an inhibitor of the AMPK upstream kinase CAMKKβ (STO-609) similarly reduces autophagic flux, although with buildup of p62. Interestingly, AMPK knockdown or pharmacologic inhibition had little to no effect on overall spheroid viability. As an additional observation, it appears that p-AMPK in EOC spheroids is most likely mediated by upstream CAMKKβ activity.

3.2 Findings and future directions

Aberrant dysregulation of autophagy has long been implicated in numerous pathologies (Dikic and Elazar, 2018). In the context of ovarian cancer, it appears that autophagy serves a tumour protective role. We have previously demonstrated that EOC spheroids display increased levels of autophagy and that subsequent autophagy blockade can reduce overall EOC spheroid viability (Correa et al., 2014). We have also shown previously how
p-AMPK is increased in both primary and immortalized EOC spheroids relative to adherent counterparts (Peart et al., 2015). In this study we have bridged the two phenomena together and examined the role of AMPK in regulating autophagy induction. In our current study, we have identified that RNAi-mediated AMPK inhibition strongly inhibits autophagic flux as visualized by fluorescence microscopy. Interestingly, this phenotype appears to occur in an LC3 and p62-independent manner. The discrepancy in our results between fluorescent and immunoblot assays raises certain questions as to what specific players mediate autophagy induction in ovarian cancer. A recent report identified that in certain contexts, macroautophagy does not require LC3, but exhibits a strong requirement for its ortholog, GABARAP (Engedal and Seglen, 2016). This validates our fluorescent AMPK knockdown findings, particularly as GABARAP is noted to be important during lysosomal fusion with the autophagosome (Weidberg et al., 2010). The observed buildup of green fluorescence in AMPK knockdown cells may be indicative of incomplete lysosomal fusion that could potentially be regulated through GABARAP. We are currently working on identifying GABARAP processing patterns in both siRNA and pharmacologically treated EOC spheroids.

An alternative explanation for the discrepancy in our results could be the issue of sensitivity of the assays used in our study. Immunoblotting may require larger changes in autophagic flux to exhibit a significant difference in protein levels of LC3 and p62. Our fluorescent reporter cell lines might be more sensitive to disruptions in autophagic pathway signaling as they measure the amount of autophagosome-lysosome fusion events rather than buildup of particular protein levels. To address this, future experiments may require longer treatment times with AMPK inhibitors prior to protein isolation for immunoblotting. Since our experiments were conducted at 48 hours, increasing drug exposure time might allow for a larger buildup of p62 protein levels. Furthermore, it has been noted that autophagic response is highly reliant on dosage of the autophagy inducers and inhibitors. A large autophagy activator/inhibitor drug screen identified conflicting results as to the effect of certain drugs on autophagic flux. Most notably, the authors identified that disaccharide trehalose, a previously reported autophagy activator actually inhibits autophagic flux in U343 spheroids. The authors attributed the discrepancy in
drug function to the different dosages used by others studies. Similarly, the authors identified that certain previously cited autophagy regulators such as clonidine and rileminidine had no effects on autophagic flux. Herein again, the authors hypothesized that varying dosage of the autophagy modulators could explain the differences in observed effects on autophagic flux (Pampaloni et al., 2017).

It has been previously reported that a rise in cytosolic calcium can induce autophagy through CAMKKβ in both MCF-7 and HeLa cell lines, highlighting an ATP-independent mechanism for autophagy induction (Høyer-Hansen et al., 2007). More recently, cellular matrix deprivation has been identified as an inducer of intracellular calcium spikes, which in turn can activate AMPK through CAMKKβ signaling (Sundararaman et al., 2016). Interestingly, our findings with the CAMKKβ inhibitor (STO-609) corroborate our AMPK knockdown data, strengthening the notion that calcium signaling may be central for autophagy induction in EOC spheroids. This phenotype holds true not only for EOC cell lines, but also in non-malignant fallopian tube epithelial cells. Furthermore, work in our laboratory has identified that LKB1-deficient cells still retain high levels of p-AMPK (Buensuceso et al., 2019), suggesting a central role for CAMKKβ in regulating p-AMPK levels in EOC cells (Fig 3.1). As such, it would be prudent to further characterize both the AMPK-dependent and independent roles of CAMKKβ in the context of ovarian cancer. Examination of the calcium-oxidant signaling network in EOC spheroids might highlight a unique characteristic of these cancer cells that would lend itself to therapeutic inhibition.
Fig 3.1 Proposed model for AMPK regulation of autophagy in EOC spheroids.

Metabolic stressors such as hypoxia, cell detachment, or ATP depletion can directly affect AMPK phosphorylation status leading to activation of critical downstream processes, such as autophagy. During the process of EOC spheroid formation, we postulate that CAMKKβ mediates the activation of AMPK signaling, likely triggered by detachment-induced calcium influx. As such, inhibition of either CAMKKβ or AMPK is able to potently block autophagic flux in EOC spheroids.

Although Compound C does not seem to attenuate p-AMPK signalling nearly to the same extent as either RNA interference or STO-609 treatment, Compound C inhibition similarly inhibits autophagic flux in EOC spheroids. The observed ineffectiveness of p-AMPK attenuation using Compound C might be attributed to the potential off-target effects using this drug that are further discussed in the limitations section. Furthermore, Compound C’s reported conflicting roles as both an activator and inhibitor of autophagy (Harhaji-Trajkovic et al., 2010; Lu et al., 2014) make establishing the connection of AMPK with autophagy challenging. As such, it would be ill-advised to extrapolate findings obtained with this inhibitor to a broad spheroid biology context.
Treatment of adherent EOC cells with an AMP-mimetic (AICAR) did not elicit an increase in p–AMPK in any EOC cell line. As such activation of AMPK was achieved using metformin and oligomycin. No changes in LC3 or p62 processing were observed following AMPK activation in adherent OVCAR8 cells. To further improve reliability and consistency of AMPK activation, subsequent work should examine alternate AMPK activation methods. It would be important to use activators that do not directly involve autophagy mediators (such as rapamycin or starvation), to further examine the specific role of AMPK activation in the adherent EOC context.

Finally, our transcriptome analysis of adherent and spheroid OVCAR8 cells has indicated that very few autophagy-related genes (only 2 out of 422 genes examined) are differentially expressed between adherent and spheroid EOC cells. As expected, the majority of changes in autophagy that we see during the early stages of EOC spheroid development can be attributed to post-translational processing, highlighting the importance of protein-based assays in evaluating autophagic flux. However, as a future direction for this work, it would be interesting to examine the autophagic transcriptional changes that occur after long periods (beyond one week) in suspension culture conditions. This might inform us of previously uncharacterized regulation of autophagic response in severally metabolically challenged spheroids.

3.3 Limitations and their mitigation strategies

When examining the function of a particular protein, it is important to be able to fully attenuate its activity to ensure that the basal protein levels are not contributing to the observed phenotype. Unfortunately, our group was unable to obtain CRISPR knockout cell lines that lacked functional AMPK, as no transfected EOC cell clones survived the selection process. Although I achieved adequate knockdown of AMPK (over 90%) using siRNA, a possibility exists that basal p-AMPK levels are still able to regulate autophagy induction in EOC spheroids. To circumvent this issue, conditional-knockout cell lines could be employed as described previously (Ono et al., 2009; Nishimura and Fukagawa, 2017). The inducible system would allow for greater flexibility and control for AMPK deletion in EOC cells. Additional benefits to this system include the lack of cellular
compensatory mechanisms that may occur with global AMPK knockout over time and selection pressures.

Although I developed a method for assessing autophagic flux in real time in EOC spheroids using the mCherry-eGFP-LC3B reporter, I was unable to accurately quantify the observed changes in autophagic flux. Part of the reason for this is the unpredictability of spheroid size, where large regions of fluorescent cells can oversaturate a particular image, thus skewing the quantification. To circumvent this issue, I could adapt a protocol described by Gump and colleagues, where fluorescent cells are sorted through flow cytometry and quantified based on the ratio of green-to-red fluorescence (Gump and Thorburn, 2014). Although this protocol is described for use in an adherent culture system, dissociating spheroids into single cell suspensions prior to sorting could improve the sensitivity of the system. Alternatively, confocal microscopy of individual uniform spheroids generated in round bottom ULA dishes could control for spheroid size and allow for better resolution of the spheroid itself. However, care would have to be taken to avoid overexposing the entirety of the spheroid, a difficult task when tracking the cells over long periods of time with preset exposure times. Recent work published by Pampolni et al. describes a protocol that helps track and quantify autophagic flux in spheroids formed with the liquid overlay method, supporting the feasibility of tracking autophagic spheroids with various autophagy modulators (Pampaloni et al., 2017).

The use of pharmacologic inhibitors to elucidate molecular mechanisms is problematic as there may be observable off-target effects. Particularly with Compound C, a drug known to affect BMP and mTOR signaling (Dasgupta and Seibel, 2018; Harhaji-Trajkovic et al., 2010), isolating AMPK’s contribution to autophagy regulation is difficult. Similarly, pharmacologic inhibition of CAMKKβ may affect multiple downstream effectors beyond just AMPK. As such, siRNA knockdowns remain as the preferred method of attenuating protein levels in our system. However, validating the phenotype with pharmacologic inhibitors will help strengthen the biological validity of the observed phenomenon.

Finally, a significant limitation in my project was the method for activating AMPK in adherent EOC cells. I was unable to increase levels of p-AMPK using the conventional AMPK activator AICAR in any EOC cell line. As such, I resorted to using metformin
and oligomycin, and was able to activate AMPK. These drugs however function indirectly on AMPK by inhibiting mitochondrial respiration (Gledhill et al., 2007; Galdieri et al., 2016). Due to the potentially confounding variables associated with disrupting the electron-transport chain (ETC), care must be taken when interpreting AMPK activation results with respect to autophagy. Moreover, these agents did not elicit any changes in p-AMPK in the iOvCa147-MA line. An alternative approach to directly activating AMPK in adherent EOC cell lines would be generate clones stably expressing constitutively active AMPK as shown by Nagata et al (2009). By removing AMPK α1’s auto inhibitory domain and replacing the threonine-172 residue with aspartate, Nagata and colleagues were able to achieve a constitutively active form of AMPK (Nagata et al., 2009). Using the abovementioned inducible transgenic method circumvents the confounding issues observed with AMPK’s molecular activators and would provide a more elegant approach to elucidating the autophagic response in adherent EOC cells.

3.4 Conclusion

This is the first study to show that in EOC spheroids, AMPK can function as a modulator of autophagic flux. However, the lack of observed viability changes in spheroids lacking p-AMPK, coupled with AMPK’s role as a master regulator of multiple downstream pathways makes it difficult to fully establish the importance of AMPK mediating ovarian cancer progression. As such, I do not believe there is currently enough evidence to justify pharmacologic inhibition of AMPK in ovarian cancer as a therapeutic modality. Nevertheless, the work presented here identifies the CAMKKβ signaling pathway as a previously unexplored modulator for autophagy in ovarian cancer spheroids.

The results of this investigation may shed light on a novel mechanism in which autophagy is regulated in HGSOC, furthering our understanding of spheroid biology and provide novel therapeutic avenues to explore.
3.5 References


Mao, Y., J. Xu, Z. Li, N. Zhang, H. Yin, and Z. Liu. 2013. The role of nuclear β-catenin


## 4 Appendix A – Clariom™ Autophagy Gene List

List of Autophagy Related Genes used for Clariom™ Transcriptome Analysis Gene list obtained from Geneontology and Qiagen Autopahy-Specific PCR array

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<td>SLC1A4</td>
<td>NTHL1</td>
<td>NHLRC1</td>
</tr>
<tr>
<td>SLC25A19</td>
<td>GABRA5</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
5 Academic CV

Education
Master of Science (MSc) Candidate, Research-Based, Anatomy & Cell Biology, Western University Sep 2017 – Sep 2019
Bachelor of Science (BSc) Honours Specialization in Biology, Western University Sep 2013 – Apr 2017

Scholarships
Obstetrics & Gynaecology Graduate Scholarship (OGGS) Sep 2018 – Sep 2019
• For excellence in research at the graduate level with the Department of Obstetrics and Gynaecology, Schulich Medicine & Dentistry, Western University
• Scholarship valued at $15,500 for one academic year

Master’s Fellowship Stipend: Cancer Research and Technology Transfer (CaRTT) Strategic Training Program Sep 2017 – Sep 2018
• Received training in cancer biology and on developing interdisciplinary partnerships between basic science researchers, clinicians and industry partners
• Stipend valued at $17,000 for one academic year

Western Graduate Research Scholarship Sep 2017 – Sep 2019
• Scholarship valued at $3000 per academic year for two years

Abstracts and Presentations at Scientific Meetings, Peer Reviewed
  *Investigating AMPK Signalling Regulation of Autophagy in a Model of Ovarian Tumour Dormancy*. Western Research Forum, March 16th, 2018, London, ON

**Teaching experience**

Teaching Assistant for Mammalian Histology (ACB3309)  
Sep 2018 – Apr 2019
- Assists students with microscopy techniques and slide interpretation
- Marks lab reports, quizzes and proctors exams

Undergraduate Student Mentor  
Aug 2018 – Apr 2019
- Mentored undergraduate fourth year thesis student
- Assists student with mammalian cell culture, microscopy and biochemical assays.

Guest Lecturer for Undergraduate Class Biology of Aging (BIO 4355F)  
Oct 2018
- Gave 50 minute lecture on autophagy and its implications in aging and neurodegeneration