Distinct roles of BMP and LKB1/AMPK signalling impacting ovarian cancer spheroid biology

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Anatomy and Cell Biology
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DISTINCT ROLES OF BONE MORPHOGENETIC PROTEIN AND LIVER KINASE B1/AMP-ACTIVATED PROTEIN KINASE SIGNALLING IMPACTING OVARIAN CANCER SPHEROID BIOLOGY

(Thesis format: Integrated Article)

by

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Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

High-grade serous (HGS) carcinoma, the most prevalent and most deadly subtype of epithelial ovarian cancer (EOC), presents unique therapeutic challenges since the majority of cases are diagnosed at advanced, metastatic stage. At this point widespread intraperitoneal metastatic lesions are numerous, which is why models that recapitulate disease dissemination are critical to uncover novel therapeutic targets. One of the initiating events in ovarian cancer metastasis is shedding from the primary tumour into the peritoneal cavity where cells must survive in suspension in order to seed secondary tumours. This non-adherent population of cells exists as multicellular aggregates, or spheroids; data from our lab has demonstrated that cells within spheroids are dormant, yet are readily alter their phenotype upon reattachment to an adherent substratum. To further explore the pathobiology of ovarian cancer spheroids, my thesis work describes the functional characterization of two different signalling pathways—bone morphogenetic protein (BMP), and the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK)—which mediate distinct and important aspects of spheroid formation and reattachment. Activated BMP signalling resulted in smaller, loosely-aggregated spheroids, which were more readily able to reattach and disperse. These phenotypic alterations observed as a result of active BMP signalling were mediated, at least in part, by cooperation with the AKT signalling pathway. These studies implicate inhibition of BMP and AKT signalling as potential strategies for therapeutic targeting of reattaching spheroids, which is critical for the formation of secondary metastatic lesions. Other work in our lab implicated the downregulation of AKT signalling in spheroid formation-induced dormancy. In an attempt to uncover additional pathways promoting the dormant phenotype of ovarian cancer spheroids, I investigated the LKB1/AMPK signalling cascade given its ability to alter cellular metabolism in response to nutrient and energy availability. Despite a dramatic enhancement in AMPK activity observed in ovarian cancer spheroids, targeted knockdown had no effect on viability of cells in this context. However, knockdown of its upstream kinase, LKB1, revealed a dramatic decrease in ovarian cancer spheroid viability, suggesting a role for this kinase in mediating anoikis-resistance in an AMPK-independent manner. Taken together, my results have uncovered two distinct and important signalling pathways that regulate unique aspects of spheroid formation, cell survival, and reattachment. By understanding the molecular mechanisms used by ovarian cancer spheroids to survive during dissemination and promote
secondary metastasis, my work has uncovered additional therapeutic targets for the potential treatment of advanced-stage ovarian cancer.
Keywords

Ovarian cancer, high-grade serous ovarian carcinoma, patient samples, spheroids, BMP, LKB1, AMPK
Co-Authorship Statement

All chapters were written by Teresa Peart and edited by Dr. Trevor Shepherd and Dr. Gabriel DiMattia.

The data presented in Chapter 2 appeared in the published manuscript “BMP signalling controls the malignant potential of ascites-derived human epithelial ovarian cancer spheroids via AKT kinase activation.” Teresa Peart, Rohann Correa, Yudith Ramos-Valdes, Gabriel DiMattia, Trevor Shepherd. *Clin Exp Metastasis*. 2012. 29: 293-313. YRV and RC contributed to flow cytometry and reattachment assays respectively. All other data was generated and analyzed by TP. The manuscript was written by TP and edited by TS and GD.

In Chapter 3 analysis of TCGA dataset was performed by RC, immunofluorescence staining was performed by Dr. Elena Fazio, and the immunoblot in Figure 3.7 was performed by YRV. All other data appearing in this Chapter was generated and analyzed by TP.
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Next I would like to thank the members of the Translational Ovarian Cancer Research laboratory, past and present, for making coming into work fun and for putting up with me all these years! Specifically, I would like to thank Yudith Ramos-Valdes who is not only a technician but also a dear friend.

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<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>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>Alk</td>
<td>Activin receptor-like kinases</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ARID1A</td>
<td>AT-rich interactive domain-containing protein 1A</td>
</tr>
<tr>
<td>ARKs</td>
<td>AMPK-related kinases</td>
</tr>
<tr>
<td>ATG 13</td>
<td>autophagy-related 13</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAMBI</td>
<td>BMP and activin membrane-bound inhibitor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-Raf murine sarcoma viral oncogene homolog B1</td>
</tr>
<tr>
<td>CAMKKβ</td>
<td>Calmodulin-dependent protein kinase kinase β</td>
</tr>
<tr>
<td>CICs</td>
<td>Cortical inclusion cysts</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>catenin (cadherin-associated protein), beta 1</td>
</tr>
<tr>
<td>Dan</td>
<td>Differential screening-selected gene aberrative in neuroblastoma</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>Epithelial Cadherin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
</tbody>
</table>
EOC  Epithelial Ovarian Cancer
GS domain  Glycine and serine rich domain
HGSCs  High-grade serous carcinomas
HMGR  3-hydroxy-3-methylglutaryl-CoA reductase
KRAS  v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LKB1  Liver kinase B1
MAPK  Mitogen-activated protein kinase
MARK4  microtubule affinity-regulating kinase 4
MEK  MAPK and ERK kinase
MO25  Mouse protein 25
mTORC1  mechanistic Target of Rapamycin 1
N-cadherin  Neural cadherin
NLS  Nuclear localization signal
NSCLC  Non-small-cell lung carcinoma
OSE  Ovarian surface epithelium
P-cadherin  Placental cadherin
PERK  protein kinase (PKR)-like endoplasmic reticulum kinase
PIK3CA  Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PJS  Peutz-Jeghers syndrome
PKA  Protein kinase A
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RAPTOR</td>
<td>regulatory-associated protein of mTOR</td>
</tr>
<tr>
<td>RSK</td>
<td>p90 ribosomal S6 protein kinase</td>
</tr>
<tr>
<td>Smurf</td>
<td>Smad ubiquitination regulatory factors</td>
</tr>
<tr>
<td>STICs</td>
<td>Serous tubal intraepithelial carcinomas</td>
</tr>
<tr>
<td>STK11</td>
<td>Serine threonine kinase 11</td>
</tr>
<tr>
<td>STRAD</td>
<td>STE20-related adaptor</td>
</tr>
<tr>
<td>TCGA</td>
<td>The cancer genome atlas</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein 53</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous sclerosis complex 2</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51 like autophagy activating kinase 1</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
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Chapter 1

1 Introduction

1.1 Overview of Chapter 1

This thesis focuses on examining signalling pathways, which we believe mediate important aspects of ovarian cancer spheroid formation and survival. This chapter begins with a description of ovarian cancer (Section 1.2) specifically focusing on the origins, classification, and mortality associated with this very complex disease. The next section (Section 1.3) focuses on the multicellular spheroid as an \textit{in vitro} model of ovarian cancer metastasis and the unique properties that spheroid cells acquire to avoid anoikis, including induction of cellular quiescence, altered cellular metabolism, and altered adhesion characteristics. The bone morphogenetic (BMP) (Section 1.4) and adenosine monophosphate-activated protein kinase (AMPK) (Section 1.5) signalling pathways will be described, and their relevance to ovarian cancer given my data in Chapters 2 and 3 showing that these pathways are important to the formation and survival of ovarian cancer spheroids. The final section provides rationale for our studies (Section 1.6) and outlines the studies presented in this thesis.

1.2 Ovarian Cancer

1.2.1 Ovarian Cancer Classification and Genetics

Ovarian cancers can be broadly characterized as epithelial and non-epithelial. Non-epithelial ovarian cancers, which are not the subject of my research, include granulosa cell tumours, fibrothecomas, teratomas and yolk sac tumours\textsuperscript{1}. The most common form of ovarian cancer however is epithelial, comprising over 90\% of cases\textsuperscript{2}.

Epithelial Ovarian Cancer (EOC) is not a single entity but rather consists of several subtypes that are distinguishable by unique histology and molecular aberrations\textsuperscript{3,4}. The four main subtypes of ovarian cancer (mucinous, endometrioid, clear-cell and serous) can be further characterized as benign, malignant or borderline and classified as low or high-grade\textsuperscript{5}. Each of these histologic subtypes has distinct clinical
characteristics and rates of occurrence. Serous carcinomas are the most common subtype and are typically high-grade neoplasms, which initially respond well to treatment with platinum/taxane-based chemotherapy but recur in the majority of cases\textsuperscript{6,7}. Endometrioid and mucinous carcinomas are much less common (10\% and 3-4\% respectively) and are typically low-grade lesions with a relatively indolent course of progression, allowing them to be diagnosed at early stage\textsuperscript{8,9}. Clear-cell carcinomas account for 10\% of all cases of ovarian cancer and typically do not respond to conventional chemotherapeutics, resulting in a poor outcome for most patients\textsuperscript{10,11}.

In 2004, a dualistic model for the classification of ovarian cancer was proposed, which incorporated histopathological discoveries, clinical and molecular genetic findings\textsuperscript{12}. In this model, the various types of ovarian cancer are broadly separated into two categories. Type I tumours include all of the major histotypes (serous, endometrioid, mucinous and clear-cell) but are low-grade and typically slow growing\textsuperscript{10,13}. These tumours are associated with mutations in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), phosphatase and tensin homolog (PTEN), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA), catenin (cadherin-associated protein), beta 1 (CTNNB1), and AT-rich interactive domain-containing protein 1A (ARID1A)\textsuperscript{12,14}. Type II tumours, on the other hand, are comprised almost exclusively of high-grade serous carcinomas but also include high-grade endometrioid, undifferentiated carcinomas and carcinosarcomas\textsuperscript{13}. These tumours are aggressive in nature and often present at an advanced, metastatic stage, owing to their relatively poor prognosis\textsuperscript{15}. Type II tumours display a high degree of chromosomal aberrations and genomic instability unlike type I tumours which are relatively genetically stable\textsuperscript{13}. An overwhelming proportion of these type II tumours (~95\%) have mutated TP53\textsuperscript{16-19}(Table 1.1). This new classification takes into account the idea that low and high-grade ovarian tumours of the same subtype are not a spectrum of disease, but rather, two distinct entities with different origins, mutations and clinical course\textsuperscript{1,10,20,21}. 
### Table 1.1: Classification of type I and type II ovarian carcinomas

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Precursor</th>
<th>Frequent Mutation(s)</th>
<th>Level of genomic instability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-grade serous</td>
<td>Serous borderline tumour</td>
<td>KRAS, BRAF</td>
<td>low</td>
</tr>
<tr>
<td>Low-grade endometrioid</td>
<td>Endometriosis</td>
<td>CTNNB1, PTEN, ARID1A</td>
<td>low</td>
</tr>
<tr>
<td>Clear-cell</td>
<td>Endometriosis</td>
<td>PIK3CA, ARID1A, FBXW74</td>
<td>low</td>
</tr>
<tr>
<td>Mucinous</td>
<td>Mucinous borderline tumour (Gastrointestinal)</td>
<td>KRAS</td>
<td>low</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-grade serous</td>
<td>Fallopian tube</td>
<td>TP53, BRCA1/2</td>
<td>High</td>
</tr>
<tr>
<td>High-grade endometrioid</td>
<td>Unknown</td>
<td>TP53</td>
<td>High</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Carcinosarcoma</td>
<td>Unknown</td>
<td>TP53</td>
<td>Unknown</td>
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</table>

*adapted from Nik et al. (2013) and references therein*
1.2.2 Origins of Ovarian Cancer

Many epithelial malignancies have well-defined precursor lesions and cells of origin\(^1\). This is not the case for EOC where until recently the origin and pathogenesis of this disease remained elusive\(^13\). The traditional view assumed that all ovarian cancer subtypes share a common site of origin within the ovarian surface epithelium (OSE). This is interesting, given the fact that the OSE is not a well-differentiated epithelium, but rather a mesothelial layer that originates embryonically from the mesodermally-derived coelomic epithelium\(^2\). This theory postulates that the process of damage and repair of the ovarian surface that occurs as a result of multiple ovulations throughout a woman’s reproductive life increases the susceptibility of the OSE to transformation. In addition, multiple invaginations of the ovarian surface are also common as women age. These invaginations can pinch off over time and become entrapped within the ovarian stroma where they form cortical inclusion cysts (CICs). It is hypothesized that the epithelial cells lining these cysts undergo metaplasia in response to the hormone-rich environment within the ovary, differentiating into a Müllerian-like epithelium that eventually becomes dysplastic leading to ovarian carcinoma\(^2,5\). Although this model is consistent with epidemiologic evidence demonstrating that decreased ovulation is significantly correlated with a decreased risk of developing ovarian cancer, it does have many limitations\(^22\). One of the major drawbacks to this model is it does not address the significantly divergent phenotypes and genotypes that exist between tumour subtypes\(^5\).

As technology has improved, so has our understanding of ovarian cancer where it is now established that it is a complex and heterogeneous disease without a single cell of origin\(^1\). Many studies have provided strong evidence indicating that endometriosis is the precursor lesion for clear-cell and endometrioid carcinomas\(^23-30\). Additionally, mucinous carcinomas have been shown to originate from appendiceal and other gastrointestinal origins\(^1\). In the late 1990s to early 2000s, pathologists identified occult non-invasive and invasive carcinomas in the fimbria of fallopian tubes collected from prophylactic salpingo-oophorectomy specimens in \(BRCA1/2\) mutation carriers\(^31-36\). Based on this, Piek and colleagues\(^37\) proposed a model whereby occult tubal carcinomas shed malignant cells that implant and grow on the ovary, mimicking primary ovarian cancer. The hypothesis
that the fallopian tube is the primary site of high-grade serous carcinomas has since been
supported by multiple studies\textsuperscript{38-41}. In 2007, for example, a study performed on women
with high-grade serous carcinomas (HGSCs) who did not harbor a \textit{BRCA} mutation
reported the presence of serous tubal intraepithelial carcinomas (STICs) in 48% of
patients\textsuperscript{41}. Additionally, studies matching STICs and HGSCs from the same patient not
only reveal TP53 mutations in 92% of STICs but also show that these mutations match
the mutation found in the ovarian carcinoma\textsuperscript{13}. Most recently, the Drapkin and Dinulescu
labs reported the development of an HGSC murine tumour model emanating specifically
from the murine fallopian tube (oviduct) even after hysterectomy and oophorectomy\textsuperscript{42}. This study provides additional support for the fallopian tube and STICs as the origin of
HGSC. This new model defining the origin of high-grade serous ovarian cancer will open
up new avenues for early detection and intervention as we gain a better understanding of
STICs and their role in carcinogenesis. In fact, it may no longer be appropriate to
categorize HGSCs as ‘ovarian cancer’ since it seems as though the ovary is simply a
favourable microenvironment for these cancer cells to spread and grow.

\subsection{1.2.3 Ovarian cancer treatment and prognosis}

Ovarian cancer is the most lethal gynecologic malignancy in the western world,
the overall survival of which has remained unchanged for more than 50 years\textsuperscript{13,43}. Ovarian cancers that are diagnosed at an early-stage, before they have spread beyond the
ovary (stage I) have a 90% cure rate through surgical resection. Unfortunately, the
majority of cases (>75%) are diagnosed once the disease has metastasized to the pelvic
organs, abdomen (stage III) or to distant sites (stage IV), at which point the chance of
cure decreases substantially\textsuperscript{44}.

The high mortality rate associated with this disease is not only due to the lack of
screening methods for early detection but also to the lack of effective therapies for
advanced stage disease. Despite the high degree of heterogeneity associated with ovarian
tumours, the majority of ovarian cancer patients are treated with cytoreductive surgery
followed by platinum and taxane-based chemotherapy\textsuperscript{5}. Although most tumours initially
respond to chemotherapeutics, approximately 70% will develop platinum resistance and
succumb to recurrent disease\textsuperscript{45}. This results in a dismal five-year survival rate for
advanced-stage ovarian cancer patients of only 30\%\textsuperscript{46}. It is becoming obvious as we gain a better understanding of the molecular underpinnings of this complex disease that a “blanket approach” to treatment is not going be enough. Rather, we must use our knowledge of the molecular genetic characteristics of individual tumours to focus our efforts into developing more targeted therapeutics\textsuperscript{5}.

1.2.4 Ovarian cancer metastasis

Ovarian cancer metastasis is unique in that it rarely occurs through the bloodstream as is common for other solid tumours\textsuperscript{3}. Instead, single cells or small clusters of cells are shed into the peritoneal cavity where they subsequently adhere to mesothelial cells of various abdominal organs to establish secondary lesions\textsuperscript{47-49}. Since there is no anatomical barrier to prevent metastasis, tumour implants become widespread, blocking lymphatic vessels, and allowing ascites fluid to accumulate from leaky vasculature \textsuperscript{44}. This peritoneal ascites fluid is a relatively unique environment in which tumour cells must survive in suspension\textsuperscript{4}. The composition of ascites fluid from ovarian cancer patients has been shown to vary considerably; in fact, one study showed higher proportions of red blood cells when the fluid had rapidly accumulated\textsuperscript{50}. A typical distribution of the cellular components of ascites fluid consists of 37\% lymphocytes, 29\% mesothelial cells, 32\% macrophages, and <0.1\% adenocarcinoma cells\textsuperscript{51}. This fluid is a convenient source of tumour cells because it is routinely removed by paracentesis and is often of high volume facilitating isolation of tumour cells for study into the unique biological characteristics of cancer cells from different patients.

1.3 Multicellular spheroids

1.3.1 Spheroids as an \textit{in vitro} model of metastasis

Multicellular spheroids have been recognized as a valuable tool in the fields of cell and developmental biology for over 50 years\textsuperscript{52-55}. It wasn’t until the 1970s, however, that Sutherland and colleagues established multicellular spheroids as a valuable \textit{in vitro} model with which to study tumour biology\textsuperscript{56-58}. Since then, tumour spheroids have been widely used to recapitulate the functional and microenvironmental features of human tumour tissue in order to study biological processes such as proliferation, metabolism,
differentiation, cell death, invasion, angiogenesis and immune response in an *in vitro* setting\textsuperscript{59-66}. Spheroids exhibit many histologic similarities to their solid tumour counterparts including areas of necrosis as well as expression of ECM components\textsuperscript{67}.

### 1.3.1.1 ECM and cell adhesion

The ECM is a complex network made up of several proteins and polysaccharides such as fibronectin, collagen, laminin, hyaluronate, heparin sulfate, and elastin. These components are produced and secreted by cells, the combination of which depends on the functional requirements of a particular tissue\textsuperscript{68}.

The link between cell survival and adhesion to the extracellular matrix (ECM) has been well-established in the literature\textsuperscript{69-72}. Anoikis, from the Greek word meaning “homelessness”, refers to apoptosis induced by loss of cell adhesion to ECM\textsuperscript{73}. This is an important physiological process as it prevents cells from reattaching to new matrices and growing in a dysplastic manner\textsuperscript{74}. The ability to overcome anoikis has important implications for metastatic cancer. In fact, cancer cell lines are significantly less sensitive to anoikis than normal epithelial cells and in many cases have developed anchorage-independence, meaning they are able to survive and proliferate without attachment to ECM\textsuperscript{75-78}. Integrins are important mediators of anchorage-independent survival that through their interaction with the ECM, stimulate numerous signalling pathways capable of modulating organization of the cytoskeleton, cell motility, and cell growth\textsuperscript{79-81}. In addition to integrin-associated signalling molecules, many cancer cells also have alterations in cell-cell adhesion molecules, protein kinases, and cell cycle regulators. This also contributes to anoikis-resistance, allowing these cells to disseminate and become metastatic\textsuperscript{77,78,82-84}. Epithelial cadherin (E-Cadherin), for example, has been shown to be a crucial mediator of cell-cell adhesion in multicellular spheroids. Oral squamous carcinoma cells, as well as mammary and prostate epithelial cells require E-cadherin in order to avoid anoikis in suspension\textsuperscript{85,86}. These studies as well as others have shown that part of the pro-survival function of E-cadherin involves the induction of quiescence, or reversible exit from the cell cycle. When E-cadherin is overexpressed in the EMT/6 breast cancer cell line, which lacks endogenous E-cadherin expression, cells form
compact spheroids and the proportion of dividing cells is greatly reduced. This exit from the cell cycle is mediated by induction of $p27^{kip1}$ activity in E-cadherin expressing cells$^{87}$.

Many tumour cells are not able to survive under anchorage-independent conditions if they remain as single cells. Rather, single cells must aggregate in order to avoid anoikis. In this context, survival signals arising from cell-cell contact substitute signals that normally come from matrix adhesion. Understanding the complex relationship between ECM components and cell-cell contact in multicellular spheroids is relevant in the field of tumour biology as they may more closely recapitulate the in vivo situation when tumour cells are detached from their tissue of origin$^{68}$. In fact, the ECM profile and organization of glioma, osteosarcoma and melanoma spheroids have been shown to more closely resemble in vivo tumours than that of conventional monolayer cultures$^{88-90}$. Additional studies in human epidermoid and colorectal carcinoma spheroids revealed a similar pattern with respect to integrin expression, whereby the expression pattern of various integrins observed in multicellular spheroids closely resembled that of solid tumours$^{91,92}$. These results provide support for the use of multicellular spheroids as in vitro models with which to study the contribution of cell-matrix and cell-cell contacts in anoikis resistance.

1.3.1.2 Response to cytotoxic drugs

Many studies on multicellular spheroids have focused on the response of these structures to various tumour therapies$^{93-108}$. The most extensively studied phenomenon is the response of spheroids to ionizing radiation. One of the most interesting findings from these studies was the observation that cells within multicellular spheroids are more resistant to ionizing radiation than monolayer cultures$^{109-111}$. This was some of the first evidence to support the idea that spheroids mimic the in vivo response of cancer cells to treatment more closely than conventional monolayer cell cultures$^{67}$.

Multicellular spheroids remain an attractive model with which to examine the role of the tumour microenvironment on response to various therapeutic strategies. These structures maintain many of the metabolic and proliferative gradients that occur as a result of cellular interactions in a 3D context$^{112}$. In fact, spatial variations in cellular
proliferation are quite common in solid tumours, where cellular proliferation is highest in areas adjacent to microvessels. Decreased proliferation in areas with lower oxygen and nutrient concentrations is often associated with quiescence, reversible exit from the cell cycle. These proliferation gradients common to solid tumours have not been demonstrated in monolayer cultures, but have been well-documented in multicellular spheroids. In fact, cells toward the center of the spheroid exhibit prolonged cell-cycle times and often enter a non-proliferating or quiescent state. Given the fact that the vast majority of therapeutics aimed at cancer cells target rapidly dividing cells, it is not surprising that multicellular spheroids are generally more resistant to cytotoxic drugs than the same cells in monolayer culture.

### 1.3.2 Multicellular spheroids in ovarian cancer

Multicellular spheroids are valuable tools for the study of ovarian cancer because, as described above, they more closely mimic the characteristics of solid tumours, but also because of the unique way ovarian cancer metastasizes. One of the early events in ovarian cancer metastasis is the proteinase-mediated shedding of cells from the primary tumour into the peritoneal cavity, which has now been elegantly demonstrated in a murine model of HGSC. It is here, suspended within peritoneal ascites fluid, that this unique non-adherent population of ovarian cancer cells must respond to a series of unique environmental cues in order to survive and metastasize. It is believed that in order to maintain cell-cell contact and avoid anoikis, cells under these conditions aggregate to form multicellular spheroids (Figure 1.1). When forced into suspension, cells spontaneously aggregate as part of their natural survival response. Spheroid compaction is mediated by the interaction of key cell adhesion molecules such as integrins and cadherins. E-cadherin expression, for example, has been shown to be lower in cells suspended within ascites fluid as compared to the primary tumour. This loss of E-cadherin is part of a global “cadherin switch” whereby Neural Cadherin (N-Cadherin) and Placental cadherin (P-cadherin) are upregulated to compensate. This switch in cadherin expression is indicative of an epithelial-to-mesenchymal transition (EMT), which has been shown to allow cells to survive under hypoxic conditions when cells are crowded together. Integrins have also been shown to be important mediators
of survival when cells are in suspension. Ovarian cancer spheroid formation is greatly inhibited, for example, when cells are treated with a blocking antibody against β1 integrin\textsuperscript{128}. Another important attribute of ovarian cancer spheroids is their ability to implant on mesothelial-lined peritoneal surfaces such as the peritoneum, omentum and pleural surface\textsuperscript{49}. Skubitz and colleagues were the first to model this \textit{in vitro}, demonstrating that ovarian cancer spheroids had the ability to reattach and invade live mesothelial cell monolayers\textsuperscript{48}. More recently, the Brugge lab has shown that ovarian cancer spheroids use myosin-generated force in order to displace the mesothelial layer of cells and gain access to the underlying ECM to promote invasion\textsuperscript{129}.

These studies have taken the first steps towards gaining a better understanding of ovarian cancer spheroid biology, however, the stresses associated with ECM-detachment puts cells under a significant selection pressure. The cells that are able to survive within the peritoneal cavity and subsequently metastasize have likely altered many key signalling pathways. We have just begun to scratch the surface when it comes to understanding the adaptations of cells in this unique environment where they must exist in suspension. Since ovarian cancer mortality can be directly attributed to disseminated peritoneal mestastasis, it is critical that we identify signalling pathways which are important for spheroid formation, survival and reattachment\textsuperscript{\textsuperscript{4}}.
Figure 1.1: Mechanism of high-grade serous ovarian cancer metastasis. During the process of ovarian cancer metastasis, malignant cells are shed from the primary tumour into the peritoneal cavity. It is here, suspended within ascites fluid, that single-cells and multicellular aggregates (spheroids) disperse throughout the peritoneal cavity. Widespread secondary metastatic lesions are formed when cells re-attach to mesothelial surfaces throughout the peritoneal cavity.
1.4 BMP/TGF-β signalling

1.4.1 Overview

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and, as their name suggests, were originally identified based on their ability to induce bone and cartilage formation at extraskeletal sites\textsuperscript{130-132}. These powerful cytokines have since become recognized for their role in other cellular processes such as, differentiation, apoptosis and migration\textsuperscript{133-180}. Given the importance of BMP signalling in controlling proliferation and differentiation during development and in maintaining and regenerating tissue during adulthood, it is not surprising that this pathway has also been shown to play an important role in many types of cancer\textsuperscript{157,180-187}. This section will discuss TGF-β/BMP signal transduction, how this pathway is regulated as well as its role in various different cancers, including ovarian cancer.

1.4.2 Pathway activation

BMP dimers are secreted to the extracellular environment, where they initiate signalling by cooperatively binding to two types of serine/threonine kinase receptors (type I and type II)\textsuperscript{188,189}. These receptors are separated in the plasma membrane until a ligand binds and increases oligomerization, essentially acting as a bridge between the two receptors. Both types of receptors are structurally similar consisting of an extracellular domain, a single transmembrane-spanning domain, and an intracellular domain with serine/threonine kinase activity\textsuperscript{190}. The type II receptors are constitutively active and are responsible for transphosphorylating the type I receptors when a signal is present\textsuperscript{191}. This phosphorylation occurs on the GS domain (glycine and serine rich domain) which is located N-terminal to the serine/threonine kinase domain on the type I receptor (Figure 1.2). The activated type I receptor is responsible for binding and activating the downstream signalling mediators for this pathway, the Smads\textsuperscript{190,191}.
Figure 1.2: Activation of Bone Morphogenetic Protein (BMP) signalling. Dimeric BMP ligands transduce their signal through a heterotetrameric complex composed of type I and type II transmembrane receptors. Following phosphorylation by the type II receptor, the type I receptor phosphorylates and activates Smads 1,5,8, allowing them to complex with Smad 4 and translocate to the nucleus to regulate target gene expression. Negative regulation of this pathway can occur through extracellular antagonists as well as intracellular inhibitory Smads.
1.4.2.1 \textit{Smad protein family}

Eight different Smad proteins have been identified in mammals, each of which can be separated into three categories: Receptor-regulated Smads (R-Smads), Common-mediator Smad, or Inhibitory Smads (I-Smads)\textsuperscript{192-194}.

The Receptor-regulated Smads or R-Smads are those that interact with the type I receptor and are activated by phosphorylation. This interaction with the receptor is transient and once phosphorylation occurs, the R-Smad is released to the cytoplasm where it is free to form a complex with co-Smad\textsuperscript{190}. The particular Smad that interacts with the receptor depends on the type of receptor as well as the ligand that triggers the signal. Smad2 and Smad3 transmit the signal from TGF-β, Nodal and activin ligands\textsuperscript{195,196}, whereas Smad1, 5 and 8 transduce the signal from BMP ligands\textsuperscript{197-199}.

The common-mediator Smad, of which there is only one, Smad4, has the ability to interact with any R-Smad, forming a heteromeric complex that can translocate to the nucleus to affect the expression of target genes\textsuperscript{191}. This complex can be composed of one R-Smad bound to Smad4 or two R-Smads, depending on the target gene\textsuperscript{200}.

The third group of Smads, the inhibitory Smads (I-Smads), is comprised of Smad6 and Smad7. These Smads have the ability to modulate signalling by competing with R-Smads for binding to either the receptor or Smad4\textsuperscript{201,202}.

1.4.2.2 \textit{TGF-β/BMP receptors}

Seven different type I receptors for the TGF-β family have been identified in mammals (activin receptor-like kinases 1-7; Alk 1-7). Of these, type I BMP receptors BMPR1A (Alk3) and BMPR1B (Alk6), as well as, type I activin receptor Acvr1 (Alk2) activate Smad 1,5, and 8 in response to BMP ligands. The remaining type I receptors activate Smad 2 or 3 and are responsible for transducing signal from TGF-β, Activin, or Nodal ligands\textsuperscript{190}. Table 1.2 describes the various type I receptors and summarizes their expression patterns, their ligand-affinity and downstream signalling targets.
The binding preferences of a particular ligand for a type I receptor is determined by the presence of the type II receptor\textsuperscript{203}. There are three type II receptors used by BMPs in mammals. BMPR-II is specific for BMPs, whereas, ActR-II and ActR-IIIB are also used by activins and myostatin\textsuperscript{190}. 
Table 1.2: Type I BMP receptors.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Cell type</th>
<th>Ligands</th>
<th>Smads</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPR-1A (Alk-3)</td>
<td>Ubiquitously expressed</td>
<td>BMP2,4</td>
<td>1,5,8</td>
<td>(ten Dijke, Yamashita et al. 1994; Dewulf, Verschueren et al. 1995; Miyazono, Kamiya et al. 2010)</td>
</tr>
<tr>
<td>BMPR-1B (Alk-6)</td>
<td>Brain</td>
<td>BMP2,4,6,7</td>
<td>1,5,8</td>
<td>(ten Dijke, Yamashita et al. 1994; Dewulf, Verschueren et al. 1995; Miyazono, Kamiya et al. 2010)</td>
</tr>
<tr>
<td>Alk-1</td>
<td>Endothelial cells, chondrocytes</td>
<td>TGF-β, BMP9</td>
<td>2,3</td>
<td>1,5,8 (Goumans and Mummery 2000; Oh, Seki et al. 2000; Seki, Hong et al. 2006; Finnson, Parker et al. 2008; Luo, Tang et al. 2010; Miyazono, Kamiya et al. 2010)</td>
</tr>
<tr>
<td>Alk-2</td>
<td>Ubiquitously expressed</td>
<td>BMP6,7,9</td>
<td>1,5,8</td>
<td>(Zhang, Schwarz et al. 2003; Luo, Tang et al. 2010; Miyazono, Kamiya et al. 2010)</td>
</tr>
<tr>
<td>ActR-1B (Alk-4)</td>
<td>Blood</td>
<td>TGF-β, Nodal</td>
<td>2,3</td>
<td>(Reissmann, Jornvall et al. 2001; Bianco, Adkins et al. 2002; Miyazono, Kamiya et al. 2010)</td>
</tr>
<tr>
<td>TßR-1 (Alk-5)</td>
<td>Endothelial cells, chondrocytes</td>
<td>TGF-β</td>
<td>2,3</td>
<td>(Seki, Hong et al. 2006; Finnson, Parker et al. 2008; Miyazono, Kamiya et al. 2010)</td>
</tr>
<tr>
<td>ActR-IC (Alk-7)</td>
<td>Adipose tissue</td>
<td>Nodal</td>
<td>2,3</td>
<td>(Carlsson, Jacobson et al. 2009; Miyazono, Kamiya et al. 2010)</td>
</tr>
</tbody>
</table>
1.4.3 Pathway attenuation

Both BMP and TGF-β signalling are modulated at many different levels: outside the cell, inside the cell as well as at the membrane. In many instances, the expression of these inhibitory signals is controlled by the TGF-β/BMP signalling cascade, which creates a negative-feedback loop\textsuperscript{204}.

1.4.3.1 Extracellular modulation

At the extracellular level, secreted antagonists are capable of sequestering BMP ligands and preventing them from binding to the receptor. In vertebrates, more than seven of these antagonists have been identified\textsuperscript{191,204}. These proteins are not redundant inhibitory signals as each antagonist displays a unique affinity for different ligands. Table 1.3 describes a number of BMP ligands and their antagonists in addition to knockout mouse models demonstrating their important embryonic functions.
Table 1.3: Knockout mouse models of BMP ligands and antagonists.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Embryonic lethal?</th>
<th>Phenotype</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMP ligands</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP2</td>
<td>Yes</td>
<td>Amnion/chorion malformation Defects in cardiac development</td>
<td>(Zhang and Bradley 1996)</td>
</tr>
<tr>
<td>BMP3</td>
<td>No</td>
<td>Increased tranecular bone density</td>
<td>(Daluiski, Engstrand et al. 2001)</td>
</tr>
<tr>
<td>BMP4</td>
<td>Yes</td>
<td>Defects in extraembryonic and posterior/ventral mesoderm formation</td>
<td>(Winnier, Blessing et al. 1995)</td>
</tr>
<tr>
<td>BMP5</td>
<td>No</td>
<td>Abnormal skull and axial part of skeleton</td>
<td>(Green 1958; Kingsley, Bland et al. 1992)</td>
</tr>
<tr>
<td>BMP6</td>
<td>No</td>
<td>Mild delay of sternum ossification in late gestation</td>
<td>(Solloway, Dudley et al. 1998)</td>
</tr>
<tr>
<td>BMP7</td>
<td>Postnatal lethal</td>
<td>Holes in the basisphenoid bone and the xyphoid cartilage, retarded ossification of bones, fused ribs and vertebrae, underdeveloped neural arches of the lumbar and sacral vertebrae</td>
<td>(Jena, Martin-Seisdedos et al. 1997)</td>
</tr>
<tr>
<td><strong>BMP Antagonists</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noggin (highest affinity for BMP2,4)</td>
<td>Yes</td>
<td>Failure of neural tube closure, broad club-shaped limbs, loss of caudal vertebrae, shortened body axis and retention of small vestigial tail</td>
<td>(Brunet, McMahon et al. 1998; McMahon, Takada et al. 1998; Choi, Stottmann et al. 2007)</td>
</tr>
<tr>
<td>Chordin (highest affinity for BMP2,4)</td>
<td>Still born</td>
<td>Normal early development and Neural induction, defects in inner and outer ear development, pharyngeal and cardiovascular organization at later stages of embryogenesis</td>
<td>(Bachiller, Klingensmith et al. 2000)</td>
</tr>
<tr>
<td>Follistatin (highest affinity for BMP7)</td>
<td>Postnatal lethal</td>
<td>Smaller than heterozygotes, less muscle, fail to breath after birth</td>
<td>(Matzuk, Lu et al. 1995)</td>
</tr>
<tr>
<td>DAN (highest affinity for BMP2)</td>
<td>No</td>
<td>No defects in head, mesoderm, somites, facial structures and limbs, normal neural tube development, viable and fertile</td>
<td>(Dionne, Skarnes et al. 2001)</td>
</tr>
</tbody>
</table>

All knockout mouse models described above are homozygous for gene of interest.
1.4.3.2 Intracellular modulation

As mentioned above, inhibitory Smads or I-Smads (Smad 6 & 7) function within the cell to antagonize TGF-β/BMP signalling. These Smads have the ability to interact with type I receptors but are never released and thus prevent R-Smads from interacting with these same receptors. Smad7 has the ability to inhibit TGF-β and BMP signalling, whereas Smad6 has been shown to preferentially inhibit BMP signalling. I-Smads have also been shown to have activity within the nucleus. Smad7, for example, is able to bind to Smad-responsive DNA elements and disrupt the formation of a functional Smad-DNA complex. On the other hand, Smad6, functions by recruiting transcriptional corepressors, such as histone deacetylases and C-terminal binding proteins (CtBP).

Another way that Smad activity is regulated is through ubiquitin-mediated degradation. Smad ubiquitin regulatory factors 1 & 2 (Smurfs 1 & 2) are E3 ubiquitin ligases that selectively target R-Smads as well as activated type I receptors for degradation. Smurf1 specifically interacts with Smads 1 and 5 to inhibit BMP signalling, whereas, Smurf2 acts more broadly to inhibit Smads 1 and 2 in order to repress both BMP and TGF-β signalling. Smurf1 is also able to enhance the interaction between I-Smads and type I receptors in order to inhibit BMP signalling.

1.4.4 Smad-independent signalling

Smads are not only phosphorylated at the C-terminus by type I receptors in a ligand-dependent manner, but can also be phosphorylated within their linker region by kinases from other pathways (ie: MAPKs, ERKs, JNK, p38). The Smad linker region is easily accessed by a number of kinases since it is loosely organized and highly flexible. Specifically, epidermal growth factor (EGF) treatment, which activates Ras/MAPK signalling, results in phosphorylation of the Smad1 linker region. This phosphorylation blocks the nuclear translocation of Smad1, inhibiting BMP signalling. Additionally, expression of a dominant negative mutant of Ras or treatment of intestinal epithelial cells with a MAP and ERK kinase (MEK) inhibitor decreased the ability of the BMP pathway to induce Smad1 phosphorylation. From this it was proposed that these two pathways converge on Smad1 by phosphorylation of the C-terminus (BMP pathway) and the linker
region (Ras/MAPK pathway). It is the balance of these two inputs that determines Smad1 activation and nuclear translocation\textsuperscript{214}. The crosstalk between BMP signalling and other signalling pathways could have important implications not only in development but also in cancer.

1.4.5 BMP signalling in cancer

The BMP signalling pathway can exhibit both tumour suppressive and oncogenic functions depending not only on the type of cancer but also the stage. In some cancers, BMP signaling is growth inhibitory and induces apoptosis\textsuperscript{153,215-220} via activation of downstream Smad-dependent pathways that promote apoptosis or inhibition of pathways that prevent apoptosis. Alternatively, the BMP signaling pathway can also increase metastatic potential\textsuperscript{221,222} and tumour angiogenesis\textsuperscript{223}. In fact, it has been reported in different cancers that BMPs serve a dual role, acting as a tumour suppressor at early stages of carcinogenesis and as a promoter of tumour metastasis at later stages\textsuperscript{224,225}.

1.4.5.1 Cancer promoting activities

The BMP signalling pathway has been shown to increase metastatic potential\textsuperscript{221,222} and tumour angiogenesis\textsuperscript{223} in a number of different cancers. For example, BMP2 is expressed in non-small-cell lung carcinoma (NSCLC) and has the ability to enhance the growth of lung cancer cell lines \textit{in vitro} and \textit{in vivo}\textsuperscript{226}. In addition to enhancing tumour growth, BMP2 has also been shown to play an important role in angiogenesis. Four days following injection of recombinant BMP2 a large increase in the size and number of blood vessels was observed in a NSCLC tumour xenograft model\textsuperscript{223}. In addition to this, BMP7 has been shown to enhance vascular endothelial growth factor (VEGF) expression in metastatic prostate cancer cells\textsuperscript{227}. The tumour-promoting properties of various components of the BMP signalling pathway have been illustrated in a number of other cancer sites including osteosarcoma, prostate, breast and colorectal\textsuperscript{228-231}. 
1.4.5.2 Anti-cancer activities

The potential tumour suppressive function of the BMP signalling pathway was highlighted in the early 2000s with the discovery of germline mutations in the type I BMP receptor, BMPR1A (Alk3), and Smad4 in up to 40% of juvenile polyposis patients\(^{232-237}\). This is an autosomal dominant syndrome characterized by multiple hamartomatous polyps and predisposition for gastrointestinal cancers\(^{233}\). A role for the BMP signaling pathway in this inherited syndrome was further supported by a transgenic mouse model expressing the BMP inhibitor noggin. At two to three months of age, these mice displayed a phenotype similar to that observed in juvenile polyposis patients. At a later age (6 to 8 months), adenomatous polyps could be observed in these mice, resembling the syndrome in humans\(^{238}\). In addition to this, recent studies have suggested that the BMP signalling pathway may in fact be inactivated in a number of cases of sporadic colorectal cancer\(^{239}\).

1.4.6 BMP signalling in ovarian cancer

BMPs serve critical functions in the normal ovary, controlling processes such as steroidogenesis, follicle formation, and apoptosis\(^{240-244}\). In addition to this, double knockout mouse models for either Smad 1 & 5 or BMPRIA & BMPRIB develop granulosa cell tumours by three and eight months of age respectively\(^{245,246}\). Given the importance of BMP signalling pathway in maintaining normal ovarian function, it is not surprising to that it may play a role in the development of ovarian cancer.

Human EOC cells have been reported to possess an autocrine BMP4 signalling loop\(^{247}\) and treatment of EOC cells with exogenous BMP4 or constitutively-active type I BMP receptor (Alk3\(^{QD}\)) resulted in an increase in cell adhesion and invasion, as well as a cell spreading response indicative of enhanced cell motility\(^{187,247}\). Additionally, BMP2 expression has been shown to be elevated in malignant ascites cells and solid tumour samples with expression positively correlating with tumour grade\(^{184}\). Perhaps some of the most convincing evidence for the cancer promoting functions of the BMP signalling pathway in ovarian cancer came from the Buckanovich lab in 2011. They demonstrated that activation of BMP signalling significantly increased the proportion of ovarian cancer
stem cells. Additionally, inhibiting BMP signalling *in vivo* resulted in a decreased proportion of ovarian cancer stem cells and decreased tumour growth\(^{248}\). Taken together these results indicate a cellular response which could contribute to EOC progression in response to BMP signalling, however, given the complexity of this pathway, it is likely that it serves different functions throughout ovarian cancer pathogenesis. The use of relevant models to dissect the role of this pathway during ovarian cancer metastasis could provide additional insight into the therapeutic potential for targeting this pathway for treatment of ovarian cancer.

### 1.5 LKB1/AMPK signalling

#### 1.5.1 Overview

As mentioned previously, the objective of my research was to identify signalling pathways that potentially interact and have a pro-survival effect on spheroid cells in ovarian cancer. In this vein, recent work uncovered a unique connection between the TGF-\(\beta\)/BMP signalling pathway and a tumour suppressor protein that plays an important role in the metabolic reprogramming of cancer cells, liver kinase B1 (LKB1). In this study they found that LKB1 is able to phosphorylate Smad4 and prevent it from binding to DNA, thus inhibiting both TGF-\(\beta\)/BMP signalling pathways\(^{249}\).

Metabolic reprogramming of tumour cells is an important disease driver that allows cells to survive in unfavourable conditions where oxygen and nutrients are scarce\(^{250-253}\). This is especially relevant to the ovarian cancer environment where cancer cells are released into the ascites fluid an environment where oxygen and nutrient access is severely compromised which necessitates a unique metabolic response for survival. In this context, Adenosine monophosphate-activated protein kinase (AMPK) is important in that it functions as a sensor of cellular energy and allows cells to cope with various forms of metabolic stress, such as nutrient and energy deprivation\(^{254}\). AMPK is the only kinase that has the ability to respond to adenosine nucleotide levels within a cell and is thus one of the most important mediators of metabolic reprogramming. This section will discuss how the LKB1/AMPK signalling pathway is activated, how each of these kinases
contributes to oncogenesis, and the potential contribution of this pathway to ovarian cancer pathogenesis.

1.5.2 Pathway activation and attenuation

1.5.2.1 AMPK structure and activity

AMPK was originally discovered in 1987 as the protein kinase responsible for phosphorylating and inactivating acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), enzymes crucial for fatty acid and sterol biosynthesis, respectively.\(^ {255} \) In response to cellular stress that results in ATP depletion either by inhibiting its production or accelerating its consumption, AMPK switches off ATP-consuming anabolic processes and turns on ATP-producing catabolic pathways in order to restore energy homeostasis.\(^ {256} \) This prevents cells from proliferating in situations where nutrients are scarce and allows them to survive periods of stress, an important attribute that many cancer cells have adapted especially ovarian cancer cells in ascites fluid.

AMPK is a highly conserved sensor of intracellular adenosine nucleotide levels that exists as a heterotrimeric complex consisting of catalytic α subunits and regulatory β and γ subunits.\(^ {257} \) In mammals, there are two isoforms of the α subunit (α1 and α2), two of the β (β1 and β2), and three of the γ subunit (γ1, γ2, and γ3), each of which is encoded by a distinct gene.\(^ {258} \) The α subunits contain a serine/threonine kinase domain in the N-terminus and are activated by phosphorylation of Threonine (Thr) 172 within the activation loop of this domain.\(^ {259,260} \) The γ subunits contain four tandem repeats known as CBS motifs. These repeats are arranged in a pseudo-symmetrical manner, yielding four potential adenosine nucleotide-binding clefts.\(^ {256} \) Site 4 binds only to AMP, site 2 appears to remain unoccupied while sites 1 and 3 competitively bind ADP, ATP or AMP.\(^ {261,262} \) The β subunits link the C-terminus of the α subunit to the N-terminal domain of the γ subunit.\(^ {262-264} \) In a cell that is not stressed and ATP:ADP ratios are high, sites 1 and 3 of the γ subunit are occupied primarily by ATP. However, when cells are exposed to metabolic stress and levels of ADP and AMP increase, ATP is gradually replaced at these sites.\(^ {256} \) When AMP or ADP is bound to the γ subunit a conformational change occurs in
the complex that promotes phosphorylation of the α subunit, which is required for its activation\textsuperscript{257}.

For many years the upstream kinase responsible for activating AMPK remained elusive. In 2003, after over 20 years of work and the combination of studies in yeast and mammals, three different groups published consecutive papers identifying liver kinase B1 (LKB1) as the primary kinase responsible for phosphorylating AMPK\textsuperscript{265-267}. Since this time, calmodulin-dependent protein kinase β (CAMKKβ) has also been shown to phosphorylate AMPK at Thr172 in response to calcium flux\textsuperscript{268-270} (Figure 1.3).
Figure 1.3: Activation of the LKB1/AMPK signalling cascade.
AMPK is activated when AMP or ADP levels increase due to a number of physiological stresses. It can also be activated pharmacologically (AICAR, A-769662). LKB1, in complex with STRAD and MO25, is the major upstream kinase that phosphorylates Thr172 on the α-subunit of AMPK in response to a rise in AMP or ADP. AMPK can also be phosphorylated by CAMKK2 in response to calcium flux. Activated AMPK directly phosphorylates a number of substrates to affect cellular metabolism and growth.
1.5.2.2  **LKB1 structure and activity**

The human LKB1 gene also referred to as serine threonine kinase 11 (STK11), spans 23kb with ten exons, nine of which are coding. LKB1 is phosphorylated on at least eight different residues, 4 of which are phosphorylated by upstream kinases (Ser31, Ser325, Thr366 and Ser428), while the other 4 are autophosphorylation sites (Thr185, Thr189, Thr336, and Ser 404). Mutation of these sites to an alanine (Ala) or glutamic acid (Glu) does not appear to have any effect on the catalytic activity or subcellular localization of LKB1. However, mutation of Ser428 rendered LKB1 unable to suppress cell growth *in vitro*, suggesting that phosphorylation of this residue may contribute to LKB1 tumour suppressor function. This particular site can be phosphorylated by the p90 ribosomal S6 protein kinase (RSK) as well as Protein kinase A (PKA), suggesting that phosphorylation of LKB1 may be an avenue through which these kinases can regulate cell growth.

LKB1 exists in mammalian cells in a complex with STE20-related adaptor (STRAD) and mouse protein 25 (MO25). STRAD\(\alpha\) and STRAD\(\beta\) are referred to as pseudokinases because although they exhibit high sequence homology to the STE20 family of protein kinases, they lack several key catalytic residues, rendering them inactive. MO25\(\alpha\) and MO25\(\beta\) are closely related to each other but don’t appear to resemble any other protein. They were originally identified as genes expressed at the early cleavage stage during mouse embryogenesis. STRAD and MO25 are required not only to enhance the activity of LKB1 but also to ensure proper localization within the cell.

LKB1 on its own is located predominantly in the nucleus, with only a small proportion in the cytoplasm. This nuclear retention is mediated by a nuclear localization signal (NLS) located within the N-terminal non-catalytic region of LKB1. When the NLS is mutated, LKB1 becomes distributed throughout the cell but retains its ability to suppress cell growth. This suggests that the cytosolic pool of LKB1 may be the primary mediator of its tumour suppressive function. When STRAD and MO25 are present LKB1 is relocalized to the cytoplasm where the catalytic
activity towards its substrates is 10-fold higher. When a mutant of LKB1 that is unable to bind STRAD is introduced into the G361 melanoma cell line, it is unable to induce cell cycle arrest. Thus, MO25/STRAD/LKB1 complex is essential for the tumour suppressive function of LKB1\textsuperscript{272,276,282}.

### 1.5.2.3 LKB1-mediated activation of AMPK

LKB1 does not respond to changes in AMP concentration, rather, it is constitutively active, constantly phosphorylating the Thr172 residue within the activation loop of AMPK\textsuperscript{286,287}. When cells have an adequate supply of energy and nutrients, this site is immediately dephosphorylated, a process mediated by the conformational change induced in the \(\gamma\) subunit when adenosine nucleotides bind. This allows the phosphorylation state and activity of AMPK to change according to cellular energy status\textsuperscript{288}. Specifically, in the presence of ADP or AMP, dephosphorylation of Thr172 by protein phosphatase 2C (PP2C) is inhibited\textsuperscript{289,290}, LKB1-mediated phosphorylation of Thr172 is promoted\textsuperscript{291,292}, and further activation is achieved by allostERIC binding of AMP\textsuperscript{288,293}.

One of the initial papers identifying LKB1 as the upstream kinase for AMPK also highlighted the importance of this kinase in mediating AMPK stress responses. They showed that LKB1/\textit{STK11}-null cells failed to activate AMPK in response to metabolic stress and that this response could be rescued by re-expression of LKB1\textsuperscript{265}. This has more recently also been demonstrated \textit{in vivo} where mice lacking LKB1 expression in skeletal muscle have significantly lower AMPK activity, fatty acid oxidation and glucose uptake in response to muscle contraction, a process which normally significantly enhances AMPK activation\textsuperscript{294}. This was the first genetic evidence demonstrating the ability of the LKB1/AMPK pathway to regulate and maintain cellular energy levels.

### 1.5.2.4 LKB1-mediated activation of AMPK-related kinases (ARKs)

In the early 2000s, as we became more aware of various protein kinases and how they interact functionally as well as their sequence similarities, came the development of the human kinome dendogram\textsuperscript{295}. It is from this that a group of 12 protein kinases closely related to AMPK were discovered and are now referred to as AMPK-related kinases.
(ARKs; BRSK1/SAD-A, BRSK2/SAD-B, NUAK1/ARK5, NUAK2/SNARK, SIK1, OIK/SIK2, SIK3, SNRK, MARK1, MARK2, MARK3 and MARK4)\textsuperscript{271}. LKB1 is the master kinase of this entire subfamily of protein kinases, phosphorylating and activating the residue equivalent to Thr172 within each AMPK-related kinase. Correspondingly, the activity of these kinases is decreased significantly in LKB1-deficient cells\textsuperscript{286,296}. The catalytic subunits of the ARK subfamily do not interact with the \( \gamma \) subunits that provide AMPK with its ability to respond to changes in AMP/ADP concentrations, therefore, these kinases do not appear to be regulated by energy stress\textsuperscript{256,297}. ARKs have been shown to play roles in cell polarity (MARK, BRSK/SAD)\textsuperscript{298-301}, cell proliferation (NUAKs)\textsuperscript{271,302} and CREB-regulated gene transcription (SIKs)\textsuperscript{303,304}, although their regulation and function is poorly understood in comparison to AMPK\textsuperscript{256}. Further study of the ARKs is important in cancer because they may mediate some of the tumour suppressor effects previously ascribed to LKB1 (Figure 1.4).
Figure 1.4: LKB1 is a master kinase, phosphorylating a number of AMPK-related kinases (ARKs).
LKB1, in a complex with STRAD and MO25, phosphorylates 12 AMPK-related kinases (ARKs) in addition to AMPK itself. These ARKs play roles in many important cellular processes, including microtubule stability, protein translation, CREB transcription and apoptosis. Upstream, LKB1 is phosphorylated by RSK, PKCζ or PKA at Ser428 and ATM at Thr 363.
1.5.3 LKB1/AMPK signalling in cancer

1.5.3.1 Peutz-Jeghers syndrome and LKB1

In 1922, Dr. Johannes Peutz was the first to describe Peutz-Jeghers Syndrome (PJS). This was followed by additional characterization provided by Dr. Harold Jeghers in the 1940s. PJS is an autosomal dominant disorder characterized by development of benign hamartomatous polyps (benign tumour-like growths) within the gastrointestinal tract and marked cutaneous pigmentation (discolouration) of mucous membranes. PJS patients also have a significantly greater chance of developing malignant tumours in a number of different tissues, including the breast, ovary, and pancreas. In fact, 93% of PJS patients develop cancer by the age of 43 with 8% of patients developing a gynaecological cancer.

In 1998, parallel studies from two laboratories identified a number of mutations in the LKB1/STK11 gene in PJS families, providing the first evidence for LKB1’s function as a tumour suppressor. Since that time, 144 different mutations in the LKB1 gene have been identified in PJS families and a limited number of sporadic cancers. The majority of which result in significant truncation of the catalytic domain, impairing catalytic activity. These findings indicate that the tumour suppressive functions of LKB1 are mediated by its downstream targets (AMPK and ARKs).

1.5.3.2 LKB1/AMPK function as tumour suppressors

Given the tumour suppressive function LKB1 plays in PJS, its mutation status in a number of sporadic cancers has also been examined. Surprisingly, the occurrence of somatic LKB1/STK11 mutations in sporadic cancers is relatively rare, except in the case of NSCLC and cervical cancers where mutations in this gene have been identified in 30% and 20% of tumours, respectively. Additional evidence to support the tumour suppressive function of LKB1 was provided by overexpression studies whereby wild-type LKB1 induced a G1 cell-cycle arrest in the HeLa cervical cancer and G361 melanoma cell lines. Mice heterozygous for LKB1 are viable with no overt phenotype until 45 weeks of age, at which point most animals develop polyps in the...
gastrointestinal tract. Histology performed on these polyps revealed that they are remarkably similar to those found in PJS patients\textsuperscript{271,331,332}. When LKB1/STK11\textsuperscript{+/−} mice are aged beyond 50 weeks of age, the majority of animals will develop hepatocellular carcinomas. These tumours have no LKB1 mRNA or protein expression, indicating that complete loss of LKB1 may be required for carcinogenesis\textsuperscript{333}.

Prior to the discovery that AMPK activation involves a tumour suppressor (LKB1), AMPK had solely been viewed as kinase with important roles in metabolism and was not on the radar of many cancer biologists. Although AMPK mediates some of the tumour suppressor functions of LKB1, some of these effects are also likely mediated by AMPK-related kinases\textsuperscript{256}. A recent publication in which whole-animal knockout of AMPKα1 accelerates the development of B cell lymphomas in mice overexpressing c-myc supports the idea that AMPK may function as a tumour suppressor\textsuperscript{334}.

As part of its energy sensing capabilities, AMPK has a unique ability to control the cell-cycle under conditions of metabolic stress. This can occur through AMPK phosphorylation and stabilization of p53, which causes cells to arrest in the G1/S phase of the cell-cycle\textsuperscript{335,336}. This effect was mediated by upregulation of cyclin-dependent kinase inhibitors p21\textsuperscript{WAF1/CIP1}, which is a transcriptional target of p53, and p27\textsuperscript{KIP1}, which is phosphorylated by AMPK\textsuperscript{337}.

Perhaps the best characterized mechanism through which AMPK controls cell growth is by suppressing the mechanistic Target of Rapamycin 1 (mTORC1) pathway. mTOR is a crucial hub through which a number of kinases signal, integrating signals from nutrient and energy sensors in order to ensure that growth and proliferation are only triggered when conditions are favourable\textsuperscript{338}. AMPK is able to directly phosphorylate two crucial components of the mTORC1 signalling cascade, Tuberous sclerosis complex2 (TSC2/Tuberin)\textsuperscript{339} and regulatory-associated protein of mTOR (RAPTOR)\textsuperscript{340}. AMPK phosphorylation of both TSC2 and RAPTOR results in inhibition of mTORC1 signalling, thereby decreasing protein translation and inducing prosurvival process such as autophagy.


1.5.3.3  **LKB1/AMPK pathway is tumour-promoting**

Although many of the functions of the LKB1/AMPK pathway discussed above seem to support the idea that these kinases function as tumour suppressors, it has also been suggested that this may be highly context-dependent, based not only on the type of cancer but also the stage of metastasis. In fact, LKB1/AMPK may act as conditional tumour suppressors or oncogenes, depending on the magnitude or duration of stress. It has also been suggested that LKB1 and AMPK may not always act in concert. Interestingly, unlike LKB1, AMPK subunits are more frequently amplified than mutated in human cancer and there is no evidence of a germline cancer predisposition syndrome involving AMPK subunits. In addition to this, high levels of AMPK activity are observed in NSCLCs, where loss of LKB1 is common. Given the lack of genetic evidence to support loss of AMPK function in cancer, AMPK may in fact be required for cancer cell survival in some instances. In solid tumours, for example, AMPK is activated in areas of hypoxia allowing cells to tolerate nutrient starvation. In this context, some of the aforementioned ‘tumour suppressive’ functions of AMPK can actually contribute to cell survival during periods of energetic stress. The prosurvival role of AMPK is likely mediated at least in part by its ability to inhibit mTORC1 signalling, thereby inducing proliferative quiescence and autophagy.

AMPK is able to induce autophagy, a highly conserved cellular process through which cellular content is degraded and recycled through lysosomal machinery. This process has been shown to be upregulated during periods of starvation in order to generate nutrients essential to maintain basic cellular function. mTORC1 activity suppresses autophagy by phosphorylating autophagy-related 13 (ATG 13) and Unc-51 like autophagy activating kinase 1 (ULK1) and preventing autophagosome initiation. AMPK has the ability to indirectly induce autophagy when nutrients are scarce through its inhibition of mTORC1 signalling. It has also been reported that AMPK has the ability to directly phosphorylate ULK1, another mechanism through which AMPK directly promotes autophagy. The protective nature of AMPK activation and autophagy induction was illustrated in a recent study by Avivar et al. In this study autophagy-induction mediated by the LKB1/AMPK/mTORC1 signalling axis promoted
anoikis-resistance in mammary epithelial cells. This effect was also found to be mediated by suspension-induced PERK activation, a member of the unfolded protein response (UPR) pathway\textsuperscript{347}.

1.5.3.4 Therapeutic manipulation of LKB1/AMPK signalling in cancer

Based on the proposed tumour suppressive function of the LKB1/AMPK pathway and its ability to suppress mTORC1 activity, it has been proposed that AMPK-activating drugs may be useful as cancer therapeutics\textsuperscript{348}. One of the most commonly used AMPK agonists is the drug metformin, which is taken by approximately 120 million type 2 diabetics daily\textsuperscript{349}. This activation, however, is not direct as metformin fails to activate AMPK in cell-free assays, rather it has been hypothesized to be through metformin’s inhibition of the mitochondrial respiratory chain complex I\textsuperscript{350}. Retrospective studies have demonstrated a strong correlation between metformin use and a reduction in cancer risk of up to 30\%\textsuperscript{351}-\textsuperscript{353}. The most significant risk-reduction was observed for pancreatic and hepatocellular carcinomas\textsuperscript{354}. It has been suggested that these associations between cancer incidence and metformin use may be due to other effects that metformin has on the tumour cells themselves, rather than AMPK activation alone. This provoked studies in tumour-prone PTEN\textsuperscript{+/-} mice crossed to mice with decreased LKB1 expression (hypomorphic LKB1), in which development of lymphomas was delayed by administration of metformin or A-769662 (an allosteric AMPK agonist)\textsuperscript{355}. Since metformin and A-769662 have completely different mechanisms through which they activate AMPK, it is unlikely that the effects of either of these compounds, is AMPK-independent\textsuperscript{356}. Therefore, these data strongly suggest that metformin may be used as an AMPK-agonist in a therapeutic setting for cancer treatment.

As discussed above, the role of the LKB1/AMPK pathway largely depends on the stage of the tumour in question. In pre-neoplastic lesions, LKB1/AMPK may in fact function as a tumour suppressor through its ability to inhibit cell proliferation. Once a tumour is established, however, LKB1/AMPK may be needed to allow cells to survive periods of metabolic stress\textsuperscript{256}. Before we use AMPK-activators as a cancer therapeutic, we need to better understand the unique metabolic requirements of cancer cells during
different stages of the carcinogenic process. In some cases, like when cells lose ECM-attachment or become hypoxic, specific inhibitors of LKB1 or AMPK may in fact be more appropriate.

1.5.4 LKB1/AMPK signalling in ovarian cancer

The observation that females with PJS have a significantly higher risk of developing gynecological cancers lead researchers to suspect that LKB1 may play an important role in the female reproductive tract. In fact, 61% (176/288) of high-grade serous ovarian tumours analyzed within The Cancer Genome Atlas (TCGA) exhibit deletion of one or more of the alleles of the LKB1/STK11 gene. Correspondingly, immunofluorescence performed on 92 human high-grade serous ovarian carcinomas revealed complete loss of protein expression in 54% of samples and partial/scattered or no loss in the remaining specimens. The consequences of LKB1 loss in high-grade serous ovarian cancer was examined using a conditional knockout mouse model in which LKB1 is lost in the OSE and stromal cells of the ovary using the Amhr2-cre driver mouse strain. Although adult LKB1cko mice exhibit a high degree of surface papillary hyperplasia of the ovary, tumour formation was not observed. Perhaps this was due to the fact that it is now well-recognized that the cell of origin of high grade serous cancer is the secretory cell of the fimbria in the fallopian tube. When LKB1cko mice are crossed to conditional knockout PTEN mice under control of the same promotor, however, adnexal (adnexa of uterus; i.e. fallopian tubes or ovaries) tumours were observed with 100% penetrance. When these tumours are examined histologically and compared to human ovarian cancer specimens, they strongly resemble that of high-grade serous ovarian carcinomas. These studies provided the first evidence that LKB1 loss and its synergy with other tumour suppressors may be important for the initiation of high-grade serous ovarian cancer.

Contrary to LKB1, when tumour specimens from ovarian cancer patients were examined for expression of the various AMPK subunits, higher levels were observed in ovarian carcinomas compared to normal ovarian controls. Additional studies have demonstrated that activation of AMPK by metformin or AMP mimetic, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) inhibits cell growth and
induces apoptosis\textsuperscript{360,361}. The functional consequences of AMPK subunit overexpression in ovarian carcinoma specimens have yet to be determined with targeted knockdown experiments.

1.5.5 Summary

The LKB1/AMPK signalling pathway is unique in that it allows cells to respond to various forms of metabolic stress, such as nutrient deprivation, hypoxia, and energy depletion. The importance of metabolic reprogramming in allowing cancer cells to survive adverse microenvironments has become apparent over the last few years. It is clear that LKB1/AMPK may in fact play many context-specific roles throughout tumorigenesis and may not simply be classified as oncogenes or tumour suppressors. For example, loss of LKB1/AMPK signalling may be necessary during the initial stages of tumour development where there is need for rapid proliferation to support tumour growth. Subsequent to this, when this growth has stripped many of the available nutrient supplies, reactivation of LKB1/AMPK signalling may allow cells to survive until nutrients are replenished. Given the studies that have begun to implicate this pathway in ovarian carcinogenesis, there is a critical need for a more thorough molecular analysis and functional studies to determine the role of the LKB1/AMPK signalling pathway in ovarian cancer pathogenesis. This is particularly important given the unique way that ovarian cancer metastasizes and the crucial role that LKB1/AMPK signalling plays in mediating anoikis-resistance\textsuperscript{347,362}.

1.6 Scope of Thesis

Mechanisms of anoikis-resistance and spheroid formation are of particular importance when studying ovarian cancer given the way this disease metastasizes. Cells shed into the peritoneal cavity from the primary tumour must survive under non-adherent conditions until they reach the serosal surface of various abdominal organs at which point they are able to reattach and form secondary metastatic lesions. We developed an \textit{in vitro} system with which to model suspension-induced spheroid formation and re-implantation to an adherent substratum. This allowed us to examine two signalling pathways we
hypothesized to play important, although not necessarily overlapping roles, in the process of ovarian cancer spheroid formation and re-implantation.

Our investigations began with the BMP signalling pathway, which we demonstrated is autonomously down-regulated during spheroid formation (Chapter 2). Correspondingly, over-activation of this pathway has a detrimental effect on the ability of cells to aggregate in suspension, resulting in much smaller spheroids. Further to this, we also demonstrate that when cells are reattached to an adherent substratum, BMP signalling is activated and enhances cell dispersion. Global gene expression analyses revealed a number of molecular aberrations associated with activated BMP signalling in ovarian cancer cells under both adherent and suspension conditions. Of these, Akt, which is also autonomously down-regulated when cells are in suspension, was shown to be enhanced in spheroids with activated BMP signalling. We also demonstrate that the BMP and Akt signalling pathways have the ability to act in concert to mediate suspension-induced cell aggregation and subsequent reattachment to an adherent surface. This study highlights the context-dependent role for the BMP signalling pathway throughout the various stages of ovarian cancer metastasis and provides a crucial link between this pathway and the PI3K/AKT signalling cascade. Given the important role that AKT plays in mediating the phenotypic alterations associated with activated BMP signalling in spheroids and other studies in our laboratory demonstrating that AKT signalling is a crucial mediator of reversible spheroid formation-induced dormancy, we became interested in other pathways that may cooperate with the PI3K/AKT pathway in our system.

AMPK is an important sensor of cellular energy status that converges with the PI3K/AKT signalling cascade on mTOR. These kinases have opposing regulatory effects on mTOR and therefore, may act in concert to allow ovarian cancer cells to survive in suspension. Indeed, in contrast to AKT, the activity of AMPK and its upstream kinase, LKB1, are enhanced in ovarian cancer spheroids (Chapter 3). Further pharmacological activation of the AMPK pathway is detrimental to adherent cells but much less so when cells are in suspension. Interestingly, targeted knockdown experiments demonstrated that LKB1 is crucial for suspension-induced spheroid formation and survival and that this
effect is AMPK-independent. These studies have begun to uncover the diverse range of signalling aberrations that occur when cells form multicellular spheroids and how these pathways interact to promote aggregation and survival in suspension.

1.7 References


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Chapter 2

2 BMP signalling controls the malignant potential of ascites-derived human epithelial ovarian cancer spheroids via AKT kinase activation

2.1 Introduction

Metastasis of epithelial ovarian cancer (EOC) is unique among most carcinomas in that spread occurs by direct dissemination of malignant cells from the primary tumour into the peritoneal cavity. EOC cells exist in suspension as single cells or aggregates called spheroids, until they adhere to the serosal surfaces of abdominal organs to establish and grow as secondary tumours\(^1\).\(^2\). It is becoming increasingly evident that EOC spheroids harbour unique characteristics that render them more resistant to chemotherapeutics, and perhaps more aggressive in establishing metastatic implants\(^3\). From an experimental perspective, we know that the gene expression patterns of cancer cells within multicellular spheroids more closely resemble that of the tumour, when compared with adherent monolayer cell cultures\(^4\),\(^5\). Therefore, cell culture systems that better mimic this metastatic program of EOC are favoured because they will more accurately reflect the pathophysiology of native EOC spheroids and provide relevant data regarding the signalling pathways important for spheroid formation and survival.

The transforming growth factor beta (TGF-\(\beta\))/bone morphogenetic protein (BMP) signalling superfamily has been implicated in numerous aspects of the pathogenesis of many different cancers including EOC\(^4\),\(^5\). Both normal human ovarian surface epithelial (OSE) cells and EOC cells possess the signalling components necessary for activation of this pathway in response to ligands of this superfamily, including TGF-\(\beta\), BMPs, activin and Mullerian inhibiting substance (MIS)\(^6\)-\(^13\). For example, EOC cells respond to exogenous TGF-\(\beta\) by inducing growth arrest due to upregulation of p15 expression\(^14\). MIS treatment targets EOC-initiating cells of both cell lines and patient ascites cells by reducing their stem-like characteristics and thereby blocking their tumour-forming ability when injected in mice\(^15\). BMP signalling through BMP4 increases the adhesion, motility and invasiveness of ascites-derived primary human EOC cells and induces epithelial-
mesenchymal transition (EMT); treatment with the BMP2/4 antagonist Noggin blocks these activities as well as autocrine BMP4 signalling. In addition, BMP2 expression in EOC cells from ascites fluid is elevated compared to matched solid tumour samples. Regulated expression of a constitutively-active BMP2/4 receptor in the human OVCA429 ovarian cancer cell line recapitulates many of the changes modulated by BMP ligands, however, the ability of these cells to form ascites and secondary tumours in immuno-compromised mice is dramatically reduced. Thus, we propose that BMP signalling has different effects at specific stages of EOC progression including dissemination from the primary tumour, spread through the ascites as spheroids, and reattachment to form secondary tumours. Determining the molecular changes controlled by activated BMP signalling in an in vitro cell culture system that closely mimics EOC pathogenesis would provide additional mechanistic insight into the functional implications of this pathway during the disease process in patients.

Herein, we describe the characterization of activated BMP signalling using a three-dimensional cell culture system whereby ascites-derived primary human EOC cells are grown in suspension where they naturally and rapidly form viable multicellular aggregates that closely resemble those observed directly in the malignant ascites collected from patients. Endogenous BMP signalling is decreased during EOC spheroid formation yet re-established during the process of spheroid reattachment. Ectopic expression of the constitutively-active BMP type I receptor ALK3QD, however, reduces the formation of large multicellular spheroids, yet enhances the immediate reattachment of EOC spheroids via increased cell motility. In addition, we provide evidence that activated BMP signalling in EOC cells and spheroids induces AKT phosphorylation, which is a necessary intracellular mediator of activated BMP signalling regulating the malignant features of metastatic disease.

### 2.2 Materials and Methods

#### 2.2.1 Cell culture

Ascitic fluid collected from chemotherapy-naive patients at time of paracentesis or debulking surgery was used to generate primary ascites cell cultures from patients with
stage III or IV ovarian cancer as described previously. Briefly, ascitic fluid containing cells was mixed 1:1 with growth medium [MCDB105 (Sigma, St. Louis, MO)/M199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, Quebec, Canada) and 50 µg/ml penicillin-streptomycin]. Cells were grown in a 37°C humidified atmosphere of 95% air and 5% CO2. All experiments with primary EOC cells were performed between passages 3 and 5.

Adherent cells were maintained on tissue culture-treated polystyrene (Sarstedt, Newton, NC). Non-adherent cells were maintained on Ultra Low-Attachment (ULA) cultureware (Corning, Corning, NY) which is coated with a hydrophilic, neutrally charged hydrogel to prevent cell attachment. Single-cell suspensions of 5 x 10^4 cells/mL were seeded to ULA plates to form spheroids over time.

### 2.2.2 Adenovirus vectors and cell transduction

The virus Ad-ALK3QD, which encodes constitutively-active BMP type IA receptor was previously constructed using the AdEasy Vector System (Qbiogene, Irvine, CA, USA). Adenovirus expressing green fluorescent protein (Ad-GFP) was a kind gift from Dr. B. C. Vanderhyden (Ottawa Health Research Institute). Primary ovarian cancer cells were transduced at 80% confluence with a multiplicity of infection of 25 with either Ad-ALK3QD or Ad-GFP in a minimal volume of medium containing 10% FBS for 2 hours with occasional agitation. Following transduction, complete growth medium was replenished. ALK3QD is tagged with a hemagglutinin (HA) epitope at the carboxyl-terminus, therefore expression was detected by western analysis using anti-HA. All experiments were performed or initiated 24 hours following transduction.

### 2.2.3 RNA expression analysis

Total RNA was isolated from cells grown either as a monolayer on tissue-culture-treated polystyrene or as spheroids on ULA cultureware using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Quantity and quality of purified RNA was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Monolayer and spheroid
RNA samples from five different primary EOC patient samples that were transduced with either Ad-GFP (control) or Ad-ALK3QD (activated BMP signalling) were hybridized to Affymetrix® Human Genome U133A GeneChips (Affymetrix, Santa Clara, CA) at Precision Biomarker Resources Inc. (Evanston, IL).

2.2.4 Real-time quantitative RT-PCR

Reverse transcription was performed using total RNA isolated from five independent patient samples (adherent & spheroid, Ad-GFP & Ad-ALK3QD-transduced) and Superscript II reverse transcriptase (Invitrogen) as per manufacturer’s instructions. PCR reactions were carried out using Brilliant® SYBR® Green QPCR Master Mix (Agilent Technologies/Stratagene) and a Stratagene Mx3000P machine with data exported to Microsoft® Excel for data analysis. Human-specific primers sequences and annealing temperatures used for CDH1, SNAI1, SNAI2, TWIST1, TWIST2, ZEB2, SMAD6, NOG, MSX2, TBX3, HEY1 and DLX2 are available upon request. GAPDH served as an internal control for RNA input using previously published primer sequences.11

2.2.5 Cell number a viability assays

Primary EOC cells were transduced in complete growth medium with either Ad-GFP or Ad-ALK3QD. Twenty-four hours following transduction, cells were seeded to either tissue-culture treated or ULA cultureware. Adherent cells were exposed to 0.25% trypsin-EDTA for 3 minutes and, following detachment, trypsin was inactivated using complete growth medium. Spheroids were exposed to 0.25% trypsin-EDTA for 10 minutes with vortexing and trituration to disaggregate spheroids. Trypsin was then inactivated using a small volume of FBS. To evaluate total cell number, single-cell suspensions were counted in a hemacytometer. To assess cell viability, single-cell suspensions were first diluted 1:1 in Trypan Blue reagent (Invitrogen, Carlsbad, CA) and all dye-excluding, viable cells counted in a hemacytometer. All treatments were performed in triplicate and two hemacytometer counts were performed per replicate.
2.2.6  Spheroid formation and reattachment assays

Primary EOC cells were transduced at 80% confluence with either Ad-GFP or Ad-ALK3QD. Twenty four hours later, spheroids were formed on ULA cultureware for three days at which point phase contrast images were captured of each well containing spheroids using an Olympus IX70 inverted microscope and ImagePro image capture software. The size of each of the spheroids was quantified for each image using the area measurement tool in the ImageJ image processing program (NIH, Bethesda, MD). In some cases, instead of transduction with virus, cells were incubated in media with minimal serum (0.5%-1%) for 24 hours prior to seeding to ULA culture ware, at which point, cells were treated with either Fc-Noggin or LDN-193189.

Spheroids were collected and re-plated to: (i) 18 mm diameter round glass coverslips placed in 22 mm diameter culture dishes for subsequent BrdU immunocytochemical analysis (see below), or (ii) directly to tissue-culture-treated 24-well polystyrene plates to quantify spheroid reattachment and dispersion. Phase contrast images were captured using an Olympus IX70 inverted microscope and ImagePro software of individual reattaching spheroids at initial point of attachment prior to dispersion (3 hours) and 24 hours following re-attachment. At this point, the experiment was terminated and re-attached spheroids were fixed and stained with using Hema-3 Stain kit (Fisher, Kalamazoo, MI). Spheroid dispersion was quantified using the area measurement tool in ImageJ (NIH, Bethesda, MD). Dispersion area at 24 hours was calculated as a percentage of the original spheroid size at 3 hours of attachment.

2.2.7  Spheroid disaggregation assay

Primary EOC cells were transduced as described above with either virus (Ad-GFP or Ad-ALK3QD) and 24 hours later plated to ULA cultureware to form spheroids. Spheroids that had formed for 3 days were then exposed to 0.25% trypsin-EDTA for specified periods of time (i.e., 2-30mins) at which point the trypsin was inactivated with a small volume of FBS and single cells were counted in a hemacytometer. All treatments were performed in triplicate and two hemacytometer counts were performed per replicate.
2.2.8 Flow cytometry

Primary EOC cells were transduced with Ad-GFP or Ad-ALK3QD and 24 hours later plated to ULA culture ware to form spheroids or to standard tissue culture plastic for adherent culture. After three days in culture, adherent cells and spheroids were detached and disaggregated, respectively, using 0.25% trypsin-EDTA. Cells were rinsed with PBS and fixed for 5 minutes using 10% neutral-buffered formalin. Cells were then rinsed with PBS/1% BSA and incubated with primary anti-E-cadherin antibody (#3195; Cell Signalling) for 1 hour, rinsed in PBS/1% BSA, and incubated with AlexaFluor 488-conjugated anti-rabbit secondary antibody (#4412; Cell Signalling). The proportion of E-cadherin-positive cells was determined using a Beckman Coulter Epics XL-MCL flow cytometer with at least 10,000 events counted per test. Four independent patient samples were tested in triplicate and included cells-only and secondary antibody-only controls for each.

2.2.9 BrdU cytochemistry

Spheroids formed over a 3 day period were allowed to re-attach and disperse on glass coverslips for 24 hours at which point they were pulse labelled overnight with 10µM bromodeoxyuridine (BrdU; GE Healthcare, Buckinghamshire, UK). Spheroids on coverslips were then fixed in a buffered 10% formalin solution, washed with PBS, and permeabilized with 0.1% TritonX-100 in PBS. This was followed by sequential washes and incubations in 2N HCl/0.5% TritonX-100 for DNA denaturation, 0.1M NaB₄O₄ pH 8.5 for neutralization, mouse anti-BrdU primary antibody (1:100; Becton Dickinson), anti-Mouse FITC-conjugated secondary antibody (1:100; Vector Laboratories), and 4',6-diamidino-2-phenylindole (DAPI; 1:5000; Sigma). Stained coverslips were washed in PBS, inverted and mounted on glass slides with VectaShield mounting medium (Vector Laboratories). Fluorescence images were captured using an Olympus AX70 upright microscope and ImagePro image capture software.

2.2.10 Western blotting

Total cellular protein was isolated from adherent and non-adherent EOC cells. Cells were washed once briefly in ice-cold PBS, dissolved in lysis buffer [50 mM HEPES
pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1X protease inhibitor cocktail (Roche, Laval, Quebec, Canada), clarified by centrifugation (20 min at 15,000 x g), and quantified by Bradford analysis (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Thirty to fifty micrograms of protein extract per lane were separated by SDS-PAGE in the presence of 1% β-mercaptoethanol using 8% or 12% gels. Proteins were then transferred to a polyvinylidene difluoride membrane (PVDF; Roche, Laval, Quebec, Canada), blocked with 5% skim milk in Tris-buffered saline with Tween-20 [TBST; 10 mM Tris.HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20]. Membranes were washed in TBST and incubated (overnight, 4°C) with appropriate antibodies (1:1000 in 5% skim milk/TBST or 5% BSA/TBST). Immunoreactive bands were visualized by incubating (1h, room temperature) with a peroxidase-conjugated anti-rabbit (1:10,000 in 1% skim milk/TBST; GE Healthcare) followed by exposure to enhanced chemiluminescence reagent (ECL Plus; GE Healthcare).

### 2.2.11 Antibodies and other reagents

Antibodies against phospho-Smad1/5/8 (#9511), phospho-Smad2 (#3108), phospho-Smad3 (#9520), total Smad1 (#9743), total Smad2 (#3122), total Smad3 (#9528), Smurf1 (#2174), and E-cadherin (#3195) were purchased from Cell Signaling Technologies (Danvers, MA). HA-probe (Y-11; sc-805) and Smad4 (H-552; sc-7154) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against ID1 was purchased from Biocheck (Foster City, CA). Antibody to detect phosphorylated AKT (Ser473) was purchased from Cell Signaling Technology (#9271; Danvers, MA), and for total AKT1/2/3 from Santa Cruz Biotechnology (H-136 sc-8312; Santa Cruz, CA). Anti-actin antibody (A 2066) was purchased from Sigma (Mississauga, ON).

Recombinant human Noggin (Fc-NG; 6057-NG) was purchased from R&D systems (Minneapolis, MN) and used at 50 or 100 ng/mL, as indicated. The BMP type I receptor inhibitor LDN-193189 was purchased from Stemgent (San Diego, CA) and prepared in DMSO: chloroform (3:1) according to manufacturer’s instructions, and used at a concentration of 10 or 100 nM, as indicated. Akt inhibitor VIII (Akti-1/2) was purchased...
from EMD/Calbiochem (Merck, Darmstadt, Germany), prepared in DMSO according to manufacturer’s instructions, and used at a concentration of 5 mM. The PI3K inhibitor LY294002 was purchased from (Cell Signaling)) and used at a concentration of 50 mM. Mammalian target of rapamycin inhibitors were used at a concentration of 20 nM for rapamycin (Sigma) and 500 nM for temsirolimus (Torisel®; Pfizer).

2.2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism® software. Data were expressed as mean ± SEM. Statistical analysis was performed using two-tailed Student’s t-tests or one-way ANOVA and Tukey’s post-hoc test with significances set at *p < 0.05, ** p < 0.01 and *** p < 0.001 as indicated.

2.3 Results

2.3.1 Reduced BMP signalling activity in primary human EOC spheroids

We have previously reported that primary human EOC cells possess an intact BMP signalling pathway11,12,17. To determine whether the critical BMP signalling components are present in EOC spheroids we prepared protein extracts from several independent primary human EOC cells that were grown as suspension cultures for three days. We have selected this time point because primary EOC cells will autonomously form multicellular aggregates or spheroids with morphological characteristics mimicking those observed directly in patient ascites within this time frame. Western blotting using protein lysates isolated from EOC spheroids and matched adherent cultures demonstrated expression of phosphorylated Smad1/5/8 levels. Interestingly, total Smad1/5 protein was significantly increased in all primary EOC spheroids as compared with their adherent cell counterparts (Figure 2.1A). Thus, when BMP-activated R-Smad levels are normalized to total Smad1/5 protein, endogenous BMP signalling activity is in effect decreased by >50% upon spheroid formation.

To follow this observation, we performed quantitative RT-PCR on RNA isolated from primary EOC adherent cells and spheroids and directly measured the expression of
Figure 2.1: BMP signalling is decreased during EOC spheroid formation.

(A) Western blot analysis of phosphorylated and total Smad1/5/8 in adherent [A] and spheroid [S] samples in two independent EOC patient samples. Densitometric quantification of phosphorylated and total Smad1/5/8 (n=8) levels from Western blots.

(B) Quantitative RT-PCR analysis of BMP2, BMP4, BMP6 and ID3 mRNA in adherent and spheroid samples in four independent EOC patient samples. (C, D) Western blot and densitometric quantification of ID3 and SMURF1 levels respectively in spheroids compared to adherent EOC cells (n=6). *p<0.05; **p<0.01; ***p<0.001 as determined by Student’s t-test.
ligands BMP 2, 4 and 6, known to be present in EOC cells\textsuperscript{7,9,17}. The mRNA levels of all three of these BMP ligands were significantly reduced in EOC spheroids compared with matched adherent cells (Figure 2.1B). Quantitative RT-PCR analysis of BMP7, however, did not yield a consistently detectable product in all samples analyzed (data not shown). In addition, the expression of the BMP signalling target gene ID3\textsuperscript{12,17} was significantly reduced in EOC spheroids (Figure 2.1C). To address the potential mechanism by which EOC spheroids exhibit increased Smad1/5 protein, we assessed the expression level of the E3 ubiquitin-protein ligase SMURF1. SMURF1 is a Smad1/5-specific ubiquitin ligase and functions to target the degradation of R-Smad1/5/8 as a form of negative feedback regulation\textsuperscript{18,19}. Indeed, SMURF1 protein levels were significantly decreased in EOC spheroids as compared to adherent cells from multiple patient samples (Figure 2.1D), which could account for the observed increase in total Smad1/5 in EOC spheroids.

To determine whether this phenomenon of downregulated signalling could be applied broadly to the TGFβ superfamily, we also assessed the levels of the related R-Smad2/3. Phosphorylated Smad2 and Smad3 were detectable in both adherent EOC cells and spheroids, but there was no statistically significant difference in expression between culture conditions (Figure 2.2). Additionally, levels of the common-mediator Smad4 were not significantly altered in EOC spheroids (data not shown). Thus, it appears that differential R-Smad expression and activity in three-dimensional EOC spheroids is specific to the BMP pathway, with the net result being a downregulation of its endogenous signalling capacity in EOC spheroids.
Figure 2.2: TGF-β signalling is not altered during EOC spheroid formation. (A) Western blot and densitometric analysis of Smad2 and Smad3 levels in adherent [A] and spheroid [S] EOC cells (n=4).
2.3.2 Forced BMP activity in EOC spheroids alters cell adhesion

Given that endogenous BMP signalling activity was decreased in EOC spheroids, we postulated that this change was important for the optimal formation of spheroids. To examine this further, we tested the effect of ectopic re-activation of BMP signalling within these structures. To accomplish this, we transduced primary human EOC cells grown as adherent monolayer with adenovirus constructs expressing an HA-tagged constitutively-active mutant of the BMP type I receptor ALK3 (Ad-ALK3QD), or control virus expressing green fluorescent protein (Ad-GFP). We chose this method to sustain BMP signalling during spheroid formation and reattachment experiments to ensure cell autonomous BMP signalling without the limitation of BMP ligand access to all cells within the three-dimensional multicellular aggregate during the time course of the experiment. Transducing cells as adherent cultures ensured homogeneous and efficient transduction and resultant expression of ALK3QD (Figure 2.3A); direct transduction of established spheroids yielded uptake of virus into surface cells only, as visualized by Ad-GFP (data not shown). To confirm that ALK3QD expression resulted in activation of BMP signalling in transduced EOC cells, western immunoblotting was performed to detect downstream targets of the pathway. As predicted, forced ALK3QD expression resulted in increased phosphorylated Smad1/5/8 and ID1 protein levels as compared with EOC cells transduced with Ad-GFP (Figure 2.3B).

Since endogenous BMP signalling is naturally reduced in EOC spheroids, we hypothesized that sustained BMP signalling activity via ALK3QD would abrogate their formation and resultant morphological phenotype. Indeed, primary human EOC cells expressing ALK3QD generates EOC spheroids that are much smaller in size as compared with Ad-GFP transduced control spheroids (Figure 2.3C&D). This result is consistent with previous results using OVCA429 cells expressing ALK3QD11.

In addition, we noted that ALK3QD-expressing spheroids consist of cells that are much more loosely-aggregated than the compact spheroids observed from control cells (Figure 2.3C). To verify that this phenotype is not just due to decreased cell viability from overexpression of ALK3QD, we performed viable cell counting of Trypan blue-excluding cells over 72 hours of spheroid formation. ALK3QD signalling had no effect on
Figure 2.3: Activated BMP signalling results in smaller EOC spheroids that are more loosely aggregated.
(A) ALK3<sup>QD</sup> expression was achieved by adenoviral transduction of primary EOC cells using Ad-ALK3<sup>QD</sup> [A] and compared to Ad-GFP [G] control vector. (B) ALK3<sup>QD</sup> expression results in activation of BMP signalling pathway as confirmed by increased phosphorylated Smad1/5/8 and ID1 protein levels 24 hours following transduction. (C) ALK3<sup>QD</sup> dramatically reduces the ability of primary EOC cells to form large multicellular spheroids as compared with Ad-GFP transduced controls. Scale bar = 200 µm. (D) ALK3<sup>QD</sup> reduces the size of EOC spheroids as quantified using ImageJ software and averaged among seven experiments using independent patient samples. (E) ALK3<sup>QD</sup> expression in EOC cells has no effect on cell viability within the first 72 hours of seeding to non-adherent culture as determined by Trypan blue exclusion. (F) ALK3<sup>QD</sup>-expressing primary EOC spheroids (5 out of 8 individual patient samples) are more readily disaggregated compared to Ad-GFP controls, as determined by single-cell counting after a 2-minute trypsinization. *p<0.05; **p<0.01 as determined by Student’s t-test.
EOC cell viability in suspension culture, indicating that enhanced anoikis is not triggered by elevated BMP signalling during spheroid formation (Figure 2.2E). To assay cell cohesion directly, spheroid disaggregation experiments were performed on several patient samples (n=8) expressing ALK3^QD, or Ad-GFP controls. Timed exposure to trypsin followed by quantification of single cells demonstrated that activated BMP signalling caused decreased cell cohesion of spheroids in 5 of 8 independent primary EOC samples (Figure 2.3F).

Activated BMP signalling in adherent EOC cells induces epithelial-mesenchymal transition (EMT), a hallmark of which is the downregulation of E-cadherin\(^ {11,13}\). Since E-cadherin may be involved in mediating cell-cell interactions in 3D spheroids\(^ {2,20}\), we sought to determine if ALK3^QD was downregulating E-cadherin expression via inducing EMT in EOC spheroids, thereby resulting in decreased cell cohesion. Using real-time quantitative RT-PCR analysis of several EMT markers, we observed that EOC cells naturally undergo an EMT response during spheroid formation with an upregulation of Snail, Slug, ZEB2, Twist1 and Twist2 transcriptional repressors and concomitant downregulation of E-cadherin (CDH1) expression (Figure 2.4A). In contrast, we observed that spheroid cells expressing ALK3^QD possess increased E-cadherin mRNA expression compared with control cells, and this correlated with an increase in the proportion of cells that were E-cadherin positive as determined by flow cytometry (Figure 2.4B). Therefore, constitutively active BMP signalling appeared to counteract the natural dynamics of transitions between epithelial and mesenchymal cell phenotypes in EOC spheroids.
Figure 2.4: EMT is induced during EOC spheroid formation.

(A) EMT is induced in EOC spheroids, both in the absence or presence of activated ALK3 activation signalling, as determined by real-time quantitative RT-PCR of E-cadherin (CDH1), Snail (SNAI1), Slug (SNAI2), TWIST1, TWIST2 and ZEB2. Fold expression was quantified against adherent cultures (set to 1) using pooled data from 5 independent patient samples performed in duplicate; GAPDH served as an internal control. (B) Flow cytometry for E-cadherin protein expression across 5 independent patient samples in the presence or absence of Alk3 activation signalling. *p<0.05; ***p<0.001 as determined by Student’s t-test.
The capacity of EOC spheroids for reattachment, growth and motility defines their ability to form secondary metastases\(^2\). Since activated BMP signalling consistently reduces cell-cell cohesion within EOC spheroids, we next sought to determine whether activated BMP signalling affects the ability of EOC spheroid cells to reattach and migrate. ALK3\(^{QD}\)-expressing EOC spheroids and GFP controls were plated for reattachment using standard tissue culture-treated plastic by directly transferring spheroids into new dishes with fresh growth medium. We observed an increased cell dispersion area and number of motile cells emanating from ALK3\(^{QD}\)-expressing EOC spheroids within the first 24 hours of replating, as compared with controls (Figure 2.5A&B). This was not due to cell proliferation since there was no significant difference in BrdU-incorporated cytochemistry in dispersing cells of ALK3\(^{QD}\)-expressing spheroids compared to GFP controls (data not shown). This observed effect on EOC cell motility upon spheroid reattachment was consistent with our previous results of increased motility in adherent primary human EOC cells using recombinant human BMP4 and ALK3\(^{QD}\) expression in conventional scratch wound assays\(^{11,13}\).

### 2.3.3 Inhibition of endogenous BMP signalling affects EOC spheroid adhesion

Given the enhanced effect of activated BMP signalling during EOC spheroid reattachment, we wanted to determine if the reduction in endogenous BMP signalling, which was observed during EOC spheroid formation, would be restored during reattachment. Indeed, there is a significant increase in the levels of phosphorylated Smad1/5/8 when normalized to total protein levels in a number of different EOC patient samples (Figure 2.5C). These results indicate that the activity of the BMP signalling pathway is restored during EOC spheroid reattachment.
Figure 2.5: Alk3QD expression enhances the movement of EOC cells from spheroids after reattachment.

(A) ALK3QD enhances the ability of EOC spheroids to migrate from the spheroid. (B) ALK3QD increases the dispersion area generated by reattached EOC spheroids as quantified using ImageJ software and averaged among six experiments using independent patient samples. Dispersion area was calculated 24 hours after spheroids have been replated to standard tissue culture plastic and normalized to the size of original spheroid. ALK3QD increases the number of dispersed cells 24 hours following spheroid reattachment as determined by counting DAPI-stained nuclei. (C) Western blot and densitometric analysis of phosphorylated and total Smad1/5/8 in adherent, spheroid and reattached EOC spheroid cells (n=7). (*p<0.05; **p<0.001; ***p<0.001 as determined by Student’s t-test). Scale Bar: 200µm.
Given our results in EOC spheroids thus far, we postulated that blocking endogenous BMP activity may further facilitate spheroid formation yet decrease subsequent reattachment and dispersion. Primary human EOC cells express several BMP ligands, chiefly BMP2, BMP4 and BMP6 (Figure 2.1B) and their ability to promote signalling in EOC cells can be efficiently blocked using natural antagonists such as Noggin (NG) and Chordin\(^9,13\). Additionally, LDN-193189 is a small molecule that selectively inhibits BMP type I receptors and can be used to block this pathway\(^21,22\). Treatment of EOC cells with a single bolus of 50 ng/mL Fc-NG or with a range of concentrations of LDN-193189 resulted in a rapid and sustained reduction in phosphorylated BMP R-Smad1/5/8 over 72 hours (Figure 2.6A). Primary EOC cells were seeded to Ultra-Low Attachment dishes and treatment with Fc-NG (50 ng/mL) or LDN-193189 (10 nM and 100 nM) was initiated immediately. From this, we observed a statistically significant increase in the average size of EOC spheroids as compared with control cultures (Figure 2.6B&C). In addition to this, when EOC spheroids were treated with either Fc-NG or LDN-193189 upon reattachment, dispersion areas were significantly reduced. A reduction in the number of dispersing cells was observed at 100 nM of LDN-193189, but no difference in the number of cells arising from the attached spheroids by treatment with Fc-NG. Taken together, these results suggest a functional requirement for differential regulation of EOC spheroid formation and subsequent motility of EOC cells upon spheroid reattachment by BMP signalling.
Figure 2.6: Inhibition of BMP signalling enhances EOC spheroid formation and decreases reattachment.

(A) Treatment of primary EOC cells with extracellular antagonist of BMP signalling Fc-Noggin (Fc-NG, 50 ng/mL) and small molecule inhibitor LDN-193189 results in potent inhibition of BMP signalling as visualized by Western blot analysis of pSmad1/5/8. (B) Treatment with Fc-NG (50 ng/mL) or LDN-193189 (100 nM) increases the ability of EOC cells to form large multicellular spheroids and a subsequent decreased ability to disperse on tissue culture plastic. Scale bar=50 μm. (C) Quantification of spheroid size and dispersion area following treatment with Fc-NG or LDN-193189 using ImageJ software (n=3; n=7 respectively). (**p<0.01; ***p<0.001 using Student’s t-test).
2.3.4 BMP signalling activates the AKT pathway in EOC cells & spheroids

The phenotypic changes in EOC spheroid adhesion and motility due to ALK3\textsuperscript{QD} signalling likely result from a combination of both direct and indirect alterations in expression of many genes and their products. Activation of BMP signalling leads to the regulation of target gene expression primarily via Smad-dependent mechanisms. Several genes are direct targets of BMP signalling in EOC cells, including genes encoding the inhibitory Smad6/7 and the helix-loop-helix negative regulatory transcription factors ID1 and ID3\textsuperscript{12,17}. Thus, to uncover many potential targets on a genome-wide scale, we performed an expression microarray using Ad-ALK3\textsuperscript{QD} and Ad-GFP-transduced cells of five independent EOC patient samples, and compared mRNA expression between adherent and spheroid cultures as well. We selected primary human EOC cells from patients with high-grade serous adenocarcinoma since they represent the most common histologic subtype of EOC and also to minimize potential experimental variability among patient samples. Using Affymetrix Human U133A 2.0 plus arrays, we uncovered a plethora of altered gene expression characteristics which were consistent among all five patient samples ($p<0.05$): 444 annotated genes were elevated in expression due to ALK3\textsuperscript{QD} in EOC spheroids compared to GFP controls, and 314 annotated genes were reduced (Table S2.1). The subset of genes that were commonly-regulated by ALK3\textsuperscript{QD} in both adherent EOC cells and spheroids, comprising 96 up-regulated and 45 down-regulated genes (Tables 2.1 & 2.2 and Figure 2.7A) were used to focus our analysis. Some known BMP target genes were identified in this subset, namely the transcription factors \textit{SMAD6} and \textit{SMAD7}, \textit{MSX2}, \textit{DLX2}, and \textit{JUND}. In addition, the BMP signalling antagonists \textit{NOG} (Noggin) and \textit{GREM2} (Gremlin 2) were also increased due to ALK3\textsuperscript{QD} signalling in both adherent cells and spheroids. We have since validated the expression of several of these genes, with \textit{SMAD6}, \textit{NOG}, \textit{MSX2}, \textit{HEY1} and \textit{TBX3} demonstrating reproducible upregulation by ALK3\textsuperscript{QD} signalling as determined by qRT-PCR (Figure S2.1).
Table 2.1: Up-regulated genes in response to Alk3QD expression and common between adherent and spheroid EOC cells.

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**Intracellular signalling proteins**

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**Structural proteins**

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**Transcription factors**

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* Expression was validated by real-time quantitative RT-PCR as described in Materials & Methods.
Table 2.2: Down-regulated genes in response to Alk3QD expression common between adherent and spheroid EOC cells.

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Uncovering potential novel mechanisms of disease hidden within a gene expression signature is more readily attainable when compared to the large number of published datasets available. One such useful resource is Connectivity Map (CMAP) that was established and made available by the Broad Institute and MIT (http://www.broadinstitute.org/cmap)\(^{23-25}\). CMAP facilitates the discovery of connections among human diseases, chiefly cancer, with gene expression changes and drug action. By uploading the up-regulated and down-regulated probe set lists (i.e. 96 up- and 45 down-regulated genes) from our microarray study to CMAP (Figure 2.7A), we identified that three of the top ten drugs are inhibitors targeting the phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway (Figure 2.7B). The PI3K inhibitors LY294002 and wortmannin were ranked first and seventh respectively, and the mTOR inhibitor sirolimus, also known as rapamycin, was ranked second. Interestingly, the compiled data of these three drugs from CMAP had a strong negative correlation with our ALK3\(^{QD}\) gene expression signature. Given that the ALK3\(^{QD}\) gene expression dataset was negatively-correlated with those from all three of the PI3K-mTOR pathway inhibitors, we reasoned that activated BMP signalling induces the PI3K-mTOR pathway in primary EOC cells. To determine if this was the case, we performed western blotting to detect phosphorylated AKT levels as a direct readout of PI3K-mTOR pathway activity in EOC cells and spheroids expressing the constitutively-active BMP receptor. Indeed, ALK3\(^{QD}\) expression significantly increased phospho-AKT in EOC cells (Figure 2.8C).

We and others have demonstrated that active PI3K-mTOR signalling is vital to promoting the metastatic potential of EOC cells and spheroids\(^{26-29}\). Indeed, treatment of reattaching primary EOC spheroids with any of three different inhibitors of the PI3K-mTOR pathway, LY294002, rapamycin and temsirolimus (Torisel\(^{®}\)), resulted in a significant reduction in cell dispersion (Figure S2.2). To address whether this pathway is required for the phenotypic changes imparted by active BMP signalling, we targeted its central mediator AKT directly and specifically using the AKT inhibitor, Akti-1/2. Reattaching EOC spheroids transduced with either Ad-ALK3\(^{QD}\) or Ad-GFP were treated with 5 \(\mu M\) AKTi-1/2, or DMSO as a vehicle control. As early as 24 hours after treatment with Akti-1/2, the dispersion area of ALK3\(^{QD}\) spheroids was reduced, although not
Figure 2.7: The PI3K-AKT-mTOR pathway is activated by BMP signalling in EOC cells and spheroids.

(A) Venn diagrams (based on Genespring analysis of microarray data using Affymetrix Human U133 plus 2.0 arrays) representing a total of 96 genes which are increased and 45 genes which are decreased in expression in response to ALK3QD in both EOC cells and spheroids. The up-regulated and down-regulated probe set lists from the microarray study were uploaded to Connectivity Map (CMAP) which was established and made available by the Broad Institute and MIT ([http://www.broadinstitute.org/cmap](http://www.broadinstitute.org/cmap)).

(B) The top ten drugs with gene expression signatures correlating with the ALK3QD gene expression signatures. Inhibitors targeting the PI3K-AKT-mTOR pathway, specifically LY294002, sirolimus, and wortmannin, result in gene expression patterns exhibiting a negative correlation with the ALK3QD gene expression signature.
completely down to levels observed in Ad-GFP control spheroids (Figure 2.8D). This suggests that enhanced AKT activity is required, at least in part, for the enhanced reattachment and dispersion of EOC cells observed as a result of active BMP signalling.
Figure 2.8: BMP-enhanced spheroid reattachment is partially mediated by AKT signalling.
(A) ALK3QD results in increased levels of phosphorylated AKT (at residue Ser473) in EOC cells. (B, C) Enhanced EOC spheroid dispersion due to ALK3QD is decreased by treatment with the AKT inhibitor, Akti-1/2 (5 mM).
2.4 Discussion

The multicellular spheroids found in EOC malignant ascites possess distinct biological properties due to their 3D architecture and downstream signalling afforded by the tumour sphere structure\textsuperscript{2,30}. For example, the formation of EOC multi-cellular clusters provides protection against anoikis allowing these cancer cells to survive in ascites and seed metastatic tumours\textsuperscript{31}. Therefore, identifying signalling pathways that contribute to the formation or disruption of EOC spheroids will provide new potential therapeutic targets that may also facilitate killing of solid tumour cells given their common 3D structure. We have shown previously that EOC cells express an intact and functional BMP signalling pathway, which directly impacts several key characteristics of a transformed cell phenotype\textsuperscript{12,13}. Here we have exploited the \textit{in vitro} EOC spheroid model system, to uncover novel actions of the BMP signalling pathway in EOC pathobiology (Figure 2.9). Interestingly, we have found that endogenous BMP signalling in EOC cells is down-regulated during spheroid formation. To determine whether this reduction in BMP signalling has functional implications for the formation of EOC spheroids we generated cells with constitutive activation of the pathway. Indeed, expression of the constitutively-active BMP type I receptor ALK3QD caused a decrease in EOC cell cohesion during spheroid formation. Additionally, enhanced BMP signalling activity subsequently resulted in increased motility of EOC cells dispersing from spheroids that attached to the culture dish. At the protein level we determined that activated BMP signalling resulted in activation of the AKT signalling pathway in human EOC cells. Therefore, the phenotypic changes induced by BMP signalling in EOC spheroids may, in part, be mediated by its effects on AKT signalling.

The process of forming multi-cellular aggregates in suspension, akin to what occurs in malignant ascites, results in the down-regulation of endogenous BMP signalling. The genes encoding several BMP ligands showed significantly reduced expression during spheroid formation when compared to proliferating adherent monolayer cells, which was directly correlated with reduced levels of phosphorylated BMP R-Smad protein, thus implying that autocrine activation of the pathway is reduced in spheroid EOC cells. The downregulation of BMP signalling in EOC spheroid cells could occur through several
mechanisms. Several extracellular antagonists exist for BMP signalling, including Noggin, Chordin and Gremlin and other related proteins\textsuperscript{32}. Moreover, BMP signalling induces the transcriptional activation of target genes encoding inhibitory Smad proteins, Smad6 and Smad7. Smad6/7 function at several points in the pathway, namely to inhibit R-Smad phosphorylation, and Smad complex formation and its transcriptional activity\textsuperscript{33-35}. This mechanism is likely not operational in EOC spheroid cells because Smad6/7 mRNA expression does not change in EOC spheroids, and Smad6 protein levels are not consistent among EOC cells and spheroids generated from patient samples (data not shown). We also demonstrate that increased turnover of R-Smads by SMURF1-mediated ubiquitinylation is not a likely mechanism, since SMURF1 levels are in fact decreased in expression in EOC spheroids. This indicates that turnover of Smads is reduced in spheroids leading to the subsequent accumulation of Smad1/5 as we observed. This increase in BMP R-Smad protein levels may represent a compensatory response to the decrease in endogenous BMP signalling during EOC cell spheroid formation. Consequently, the reduction in expression of the major BMP ligands expressed in EOC cells, \textit{i.e.} BMP4, BMP2, and BMP6, likely represents the mechanism for downregulated BMP signalling in EOC spheroids. This is the first time that decreased endogenous BMP signalling has been observed during spheroid formation. This is in contrast to the data demonstrating that BMP signalling is elevated in primary EOC cells and in solid tumour samples compared to normal ovarian surface epithelial cells\textsuperscript{7,12,17}. Thus, our results provide new insight about the dynamics of BMP signalling within EOC spheroids, which represent a unique transitional step for malignant cells between the primary tumour and secondary metastases\textsuperscript{2}.

The process of multicellular spheroid formation is complex and involves the coordinated action of different cell adhesion molecules. During the initial stages of spheroid formation, cell-cell adhesion is primarily mediated by the actions of integrins and cadherins\textsuperscript{20,36,37}. By analysing several markers for EMT, we observed that EOC cells induce an EMT phenotype during spheroid formation, defined by a substantial decrease in the gene expression of the cell-cell adhesion molecule E-cadherin. This implies that EOC spheroid compaction likely depends on additional molecules or processes, such as fibronectin, vimentin or actomyosin-mediated contractility as seen in other spheroid
systems. Since endogenous BMP signalling is reduced during spheroid formation, we propose that induction of the EMT phenotype in EOC spheroid cells does not require activation of BMP signalling. We noted, however, that the general induction of EMT in EOC spheroids is sustained in the presence of ectopic activated BMP signalling, a result which is supported by previous studies showing that BMP stimulation can induce EMT. We also observed an unexpected slight but significantly greater increase in the induction of E-cadherin expression in ALK3 expressing patient derived EOC spheroid cells relative to GFP transduced cells. This up regulation may be related to the unique microenvironment of the spheroid counteracting the EMT inducing properties of activated BMP signalling to maintain cell-cell contacts. The decreased spheroid integrity that occurs as a result of activated BMP signalling may be due to the lack of typical late-step spheroid compaction. In fact, the reduction in EOC spheroid compaction in the presence of activated BMP signalling may provide cells with an increased propensity to attach and disperse during secondary metastasis formation (as modeled by spheroid re-attachment and dispersion in vitro).

Reattachment of EOC spheroids to a hospitable substratum and the subsequent dispersion of cells and expansion via cell proliferation and motility are necessary to achieve secondary metastasis. In response to activated BMP signalling, EOC spheroids have a significantly increased ability to reattach and disperse due to increased cell adhesion and motility and not cell proliferation. This result conforms to previous data indicating that BMP signalling has no effect on EOC cell proliferation, but induces a cell spreading phenotype, enhances motility and adhesion to several ECM components.

The phenotypic response of altered cell motility due to BMP signalling usually occurs in a Smad-independent manner utilizing other converging intracellular signalling pathways. In this report, we determined that levels of phosphorylated AKT were significantly elevated in EOC cells and spheroids in response to activated BMP signalling, thus providing a further mechanism for BMP-mediated changes in cell adhesion and motility. There has been growing evidence for crosstalk between the BMP and PI3K-AKT-mTOR signalling pathways. For example, AKT kinase is activated by BMP2 stimulation of mouse myoblast C2C12 cells, an effect that is inhibited by the BMP
type I receptor specific inhibitor LDN-193189\textsuperscript{21}. Stimulation of vascular smooth muscle cells with BMP2 induces cell motility in a phospho-AKT dependent manner via the action of Rac1 and RhoA GTPases\textsuperscript{45}. In addition, activated BMP signalling enhances cell motility, invasion and EMT via the PI3K-AKT pathway in other cancer cell types\textsuperscript{46-50}. Thus, it will be important to identify whether BMP activation of AKT employs common or different mechanisms in EOC cells and spheroids, as well as the functional implications of this signalling on the malignant characteristics of EOC.

Our laboratory has independent evidence that the PI3K-AKT pathway is down-regulated endogenously during EOC spheroid formation, yet its activation is required again during spheroid reattachment and cell dispersion. Taken together, AKT and BMP signalling are co-ordinately down-regulated during EOC spheroid formation. Perhaps sustained AKT activity due to enforced BMP signalling leads to less-cohesive spheroid formation yet enhances cell dispersion after re-attachment (Figure 2.9). This idea is supported by our studies in which treatment of ALK3\textsuperscript{QD}-expressing spheroids with an Akt1/2 inhibitor results in a partial restoration of spheroid dispersion area to that of controls. Thus, we believe that activation of the AKT pathway is functionally required and plays an important role in BMP-induced changes in spheroid behaviour and ultimately EOC metastasis. As mentioned previously, the presence of BMP signalling in EOC is correlated with significantly shorter survival periods for patients with advanced stage disease\textsuperscript{7}. One mechanism for the deleterious effects of active BMP signalling in EOC may be downstream activation of the AKT pathway, another established marker for poor patient prognosis\textsuperscript{51,52}. Thus, further exploration of the interaction between these two pathways is currently underway and may determine the therapeutic potential of targeting BMP signalling or AKT activity during EOC metastasis.

Our previous and current studies implicate disparate roles for BMP signalling during different steps of the metastatic cascade in EOC pathogenesis. Overall, the data supports the notion that BMP signalling has bi-phasic influences: reduced activity may be required for EOC spheroid formation during dissemination of cells from the primary tumour, yet reactivation is required for more efficient establishment of secondary metastases (Figure 2.9). As such, the conflicting roles for BMP signalling during EOC progression are
similar to the multifaceted effects of the related TGFβ pathway at early and late stages of multiple human cancers\textsuperscript{53}. Collectively our data indicates the critical necessity to assess the effects of signalling systems at each step of the EOC metastatic process to assess the overall therapeutic potential of targeting a particular pathway.
Figure 2.9: Proposed model of BMP signalling in EOC metastasis.
Metastatic EOC cells disseminating from the primary tumour naturally aggregate to form multicellular spheroids while in suspension in ascites in the peritoneal cavity. EOC spheroids endogenously down-regulate both BMP and AKT signalling, which may be required for tight cell-cell cohesion. Upon reattachment of EOC spheroids, however, cells reactivate the BMP and AKT pathways for efficient adhesion and dispersion to establish secondary metastases (e.g. on peritoneal wall). Ectopic activation of BMP signalling using ALK3QD, with the concomitant increase in AKT activity, alters this dynamic process by rendering EOC spheroids more loosely-aggregated while in suspension, which ultimately enhances their ability to disperse upon reattachment.
2.5 References


Figure S2.1: Validation of microarray results by quantitative RT-PCR analysis of specific up-regulated genes.

SMAD6, NOG, MSX2, TBX3, and HEY1 mRNA expression was detected using human-specific primers for each and cDNA samples generated from adherent primary human EOC cells (A) and spheroids (B) transduced with either Ad-ALK3QD or Ad-GFP control virus. Relative expression was normalized to Ad-GFP transduced cells (set to 1) and GAPDH mRNA served as an internal control.
Figure S2.2: Inhibition of PI3K-mTOR signalling reduces EOC cell dispersion upon spheroid reattachment.
EOC cells from patient ascites samples (EOC30 and EOC67) were seeded to ULA cluster plates to generate spheroids over three days. EOC spheroids were individually seeded to standard tissue culture plastic and treated with LY294002, rapamycin, or temsirolimus (Torisel®) or DMSO vehicle control. Images of spheroids were captured (>40 per treatment group) at 24 h post-reattachment and dispersion area was quantified using ImageJ software. (**p<0.01; ***p<0.001 using Student’s t-test).
Table S2.1: Genes with increased expression due to Alk3QD in adherent EOC cells.

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Table S2.3: Genes with decreased expression due to Alk3QD in adherent EOC cells.

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| RAB7B, member RAS oncogene family          | RAB7B   | 1533982_a_at | 2.551 |
| ubiquitin protein ligase E3 component n-recognition 4 | UBR4     | 231889_at | 2.551 |
| hypothetical LOC151658                     | LOC151658 | 238283_at | 2.545 |
| tensin 3                                   | TNS3    | 217853_at | 2.545 |
| calcium/calmodulin-dependent protein kinase 2, beta | CAMKK2   | 207390_at | 2.538 |
| dynein                                     | DYM     | 220774_at | 2.538 |
| unc-51-like kinase 2 (C. elegans)          | ULK2    | 215154_at | 2.538 |
| ATP/GTP binding protein-like 2             | AGBL2   | 220390_at | 2.538 |
| discs, large (Drosophila) homolog-associated protein 1 | DILGAP1  | 206489_s_at | 2.538 |
| hypothetical LOC100129550                  | LOC100129550 | 229699_at | 2.532 |
| low density lipoprotein-related protein 2  | LRP2    | 205710_at | 2.525 |
| TAP binding protein (tapasin)              | TAPBP   | 210294_at | 2.525 |
| transmembrane protein 163                  | TMEM163 | 1552626_a_at | 2.519 |
| chromosome 11 open reading frame 35       | C1orf25 | 236050_at | 2.519 |
| tumor necrosis factor receptor superfamily, member 21 | TNFRSF21 | 214531_at | 2.519 |
| potassium inwardly-rectifying channel, subfamily J, member 12 | KCNJ12 | 232289_at | 2.513 |
| chromosome 17 open reading frame 103      | C1orf103 | 226657_at | 2.513 |
| cathepsin F                                | CTSF    | 203657_s_at | 2.506 |
| aldehyde dehydrogenase 6 family, member A1 | ALDHHA1 | 204290_s_at | 2.506 |
| 4-hydroxyphenylpyruvate dioxygenase        | HPO     | 206024_at | 2.500 |
| Mov1010, Moloney leukemia virus 10-like 1, homolog (mouse) | MOV101L1 | 239257_at | 2.500 |
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| microtubule-associated protein 7          | MAP7    | 202930_at | 2.500 |
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| spermatid perinuclear RNA binding protein  | STGBP   | 223246_s_at | 2.494 |
| chromosome 8 open reading frame 83        | CT8f83  | 224156_s_at | 2.494 |
| growth arrest-specific 7                  | GAS7    | 202192_s_at | 2.488 |
| paired box 8                               | PAX8    | 221990_at | 2.488 |
| glutamate decarboxylase 1 (brain, 67kDa)   | GAD1    | 205278_at | 2.481 |
| ATP-binding cassette, sub-family A (ABC1), member 9 | ABCA9   | 235335_at | 2.481 |
| hypothetical gene supported by AK026416    | FLJ22763 | 233804_at | 2.475 |
| complement component 1, s subcomponent     | C15S    | 208747_s_at | 2.475 |
| ets variant 4                             | ETV4    | 1554576_a_at | 2.463 |
| collagen, type XVI, alpha 1               | COL16A1 | 204345_at | 2.457 |
| acyl-CoA thiosterase 4                    | ACOT4   | 229534_at | 2.457 |
| Kallmann syndrome 1 sequence              | KAL1    | 205206_at | 2.457 |
| carboxymethylenebutenolidase homolog (Pseudomonas) | CMBL   | 227522_at | 2.457 |
| chromosome 5 open reading frame 13        | C5orf13 | 201310_s_at | 2.457 |
| phospholipase A2, group X                 | PLA2G10 | 207222_at | 2.451 |
| C6D molecule                              | C6D     | 231955_at | 2.445 |
| proprotein convertase subtilisin/kexin type 6 | PCSK6  | 211262_at | 2.445 |
| GM2 ganglioside activator                 | GM2A    | 209727_at | 2.439 |
| caspase 10, apoptosis-related cysteine peptidase | CASP10  | 205467_at | 2.433 |
| receptor tyrosine kinase-like orphan receptor 1 | ROR1  | 232060_at | 2.433 |
| hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 /// hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 | LOC151658 | 238283_at | 2.545 |
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| ureidopropionase, beta                    | UPB1    | 220507_s_at | 2.433 |
| C-terminal binding protein 1              | CTP1    | 1557714_at | 2.427 |
| glutaminase                               | GLS     | 203159_at | 2.421 |
| chromosome 10 open reading frame 54       | C10orf54 | 223572_at | 2.416 |
| family with sequence similarity 3B, member B | FAM3B  | 222908_at | 2.410 |
| ring finger protein 144B                  | RNF144B | 228153_at | 2.398 |
| keratinocyte growth factor-like protein 2 | KFLF2   | 231031_at | 2.398 |
| cell adhesion molecule 4                  | CADM4   | 222293_at | 2.398 |
| K(lysine) acetyltransferase 2B            | KAT2B   | 203845_at | 2.392 |
| aldehyde oxidase 1                        | AOX1    | 205082_s_at | 2.392 |
| ubiquitin specific peptidase 2            | USP2    | 229337_at | 2.392 |
| chromosome 6 open reading frame 123       | C6orf123 | 207638_at | 2.387 |
| tumor necrosis factor receptor superfamily, member 21 | TNFRSF21 | 218856_at | 2.387 |
| ADAM metallopeptidase domain 12           | ADAM12 | 215613_at | 2.387 |
| phosphoinositide-3-kinase, regulatory subunit 3 (gamma) | PIK3R3 | 202743_at | 2.381 |
| solute carrier family 39 (metal ion transporter), member 11 | SLC39A11 | 227046_at | 2.381 |
| myelin-associated oligodendrocyte basic protein | MOBP | 242755_at | 2.375 |
| chromosome 18 open reading frame 2        | C18orf2 | 224045_s_at | 2.375 |
| superoxide dismutase 2, mitochondrial     | SOD2    | 216841_s_at | 2.375 |
| coiled-coil domain containing 80          | CCD80   | 225242_s_at | 2.375 |
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Table S2.3 cont’d.

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Table S2.4: Genes with decreased expression due to Alk3QD in EOC spheroids.

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| ephrin-A5                        | EFNA5   | 227955_s at                                                                      |
| phosphonositide-3-kinase interacting protein 1 | PIKIP1 | 221756_at                                                                      |
| CD302 molecule                   | CD302   | 203798_at                                                                      |
| coxsackie virus and adenovirus receptor pseudogene 1 | CXADR1 | 239155_at                                                                      |
| spermatogenesis associated 6     | SPATA6  | 238459_x_at                                                                     |
| nucleoredoxin                    | NXN     | 219489_s at                                                                      |
| mucin 1, cell surface associated | MUC1    | 213693_s at                                                                      |
| decorin                          | DCN     | 211813_x_at                                                                     |
| neuropeptide Y receptor Y5       | NPY5R   | 207400_at                                                                      |
| sepin B                          | 08-Sep  | 226627_at                                                                      |
| nicotinamide N-methyltransferase | NNMNT   | 202238_s at                                                                      |
| chromosome 1 open reading frame 53 | C1orf53 | 1558507_at                                                                     |
| mucin 1, cell surface associated | MUC1    | 207847_at                                                                      |
| carbohydrate (chondroitin 4) sulfotransferase 11 | CHST11 | 219634_at                                                                      |
| dipeptidyl-peptidase 4           | DPP4    | 203716_s at                                                                      |
| ELL associated factor 2          | EAF2    | 219551_at                                                                      |
| transducin (beta)-like 1X-linked | TBL1X   | 201868_s at                                                                     |
| OMA1 homolog, zinc metallopeptidase (S. cerevisiae) | OMA1 | 226019_at                                                                      |
| retinoic acid receptor responder (tazarotene induced) 2 | RARRES2 | 204946_at                                                                      |
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Chapter 3

3 LKB1 signalling protects dormant ovarian cancer spheroids from cell death in an AMPK-independent manner

3.1 Introduction

Ovarian cancer is the most lethal gynecologic malignancy in the western world, the overall survival of which has remained unchanged for more than 50 years\textsuperscript{1,2}. Models that can be used to uncover the molecular events important for disease dissemination are crucial since the majority of women with ovarian cancer (over 75%) are diagnosed at advanced stage\textsuperscript{3}. Intraperitoneal implants identified in these patients with advanced-stage disease are the result of single cells and multicellular aggregates, or spheroids, that adhere to the mesothelial lining of various abdominal organs to establish secondary lesions\textsuperscript{4-6}. In many cases, this is accompanied by accumulation of ascites fluid within the peritoneal cavity, where cells in suspension are exposed to a unique set of microenvironmental cues, allowing this population of cells to form secondary metastases\textsuperscript{3-5,7}. These non-adherent metastatic cells provide unique therapeutic challenges for treatment of ovarian cancer\textsuperscript{3}.

The biological significance and clinical relevance of multicellular spheroids has been documented in many different tumour types\textsuperscript{8-14}. It is well accepted that spheroids more closely mimic the cell-cell, cell-matrix interactions, metabolic gradients, cellular viability and differentiation of malignant cells within a solid tumour than do conventional monolayer cultures\textsuperscript{15}. We have shown that ascites-derived ovarian cancer cells in suspension form dormant multicellular aggregates characterized by quiescence and decreased Akt activity\textsuperscript{16}. These dormant cells are subsequently able to re-enter the cell cycle and grow when they reach an adherent substratum\textsuperscript{16}. Ovarian cancer cells that are able to resist anoikis and survive within ascitic fluid most likely have uniquely adapted key cell survival pathways to meet the nutrient and energy demands of this particular microenvironment.
A fundamental requirement of all cells is the ability to respond to various forms of metabolic stress and balance ATP consumption and generation in response. Under conditions where nutrients are low, AMPK acts as a metabolic checkpoint by activating catabolic processes and inhibiting anabolic metabolism\textsuperscript{17,18}. AMPK is a heterotrimeric complex containing a catalytic $\alpha$-subunit and two regulatory subunits, $\beta$ and $\gamma$. When intracellular ATP levels are low, AMP or ADP directly bind to the $\gamma$ regulatory subunits. This causes a conformation change in the complex that allows AMPK to be phosphorylated at threonine 172 on the $\alpha$ subunit\textsuperscript{17}. The primary kinase responsible for phosphorylation at this site is LKB1\textsuperscript{19-21}.

It has been suggested that AMPK may function as a context-dependent tumour suppressor or oncogene\textsuperscript{22}. Modest activation of AMPK may be cell protective, but prolonged or enhanced activation can be detrimental and result in growth arrest or cell death\textsuperscript{18}. The most thoroughly characterized mechanism through which the LKB1/AMPK pathway regulates cell growth is by suppression of mTORC1 signalling. LKB1, on the other hand, is commonly regarded as a tumour suppressor, and is mutated in the rare hereditary autosomal dominant Peutz Jeghers Syndrome. These patients experience benign intestinal hamartomatous polyps and have an increased risk of developing malignant tumours\textsuperscript{23}. Despite this, LKB1 mutations have been identified in relatively few sporadic cancers.

Previous studies have shown that metabolic stress is induced when normal epithelial cells lose ECM attachment, resulting in a decreased ATP:ADP ratio and subsequent activation of AMPK\textsuperscript{24-26}. However, this suspension-induced AMPK activation has yet to be examined in tumour spheroids. In our study, we use a disease-relevant spheroid model to interrogate the function of the LKB1/AMPK pathway in ovarian cancer cells. Our results indicate that LKB1 and AMPK serve distinct functions in ovarian cancer cells and spheroids to promote dormancy and anoikis-resistance.
3.2 Materials and Methods

3.2.1 Culture of cell lines, ascites-derived cells and isolation of native ascites spheroids

Ascites fluid from patients diagnosed with advanced stage (II-IV), high-grade serous epithelial ovarian cancer (Table S1) was used to establish primary cell cultures as previously described\textsuperscript{27}. The iOvCa147-E2 and iOvCa198 cell line were isolated from the EOC147 and EOC 198 ascites samples respectively. All work with patient materials has been approved by The University of Western Ontario Health Sciences Research Ethics Board (Protocol # 12668E and 16391E; Appendix B). Spheroids were isolated directly from ascites fluid by filtration through a 40 µm cell strainer (Becton Dickinson), washed with phosphate-buffered saline (PBS) into a collection tube with protein lysis buffer for immunoblot or embedded directly in OCT to obtain fresh frozen sections.

3.2.2 TCGA Analysis

Datasets from The Cancer Genome Atlas analysis of ovarian serous cystadenocarcinoma samples were downloaded from the University of California Santa Cruz Cancer Genomics Browser (https://genome-cancer.ucsc.edu)\textsuperscript{28} and from the Memorial Sloan-Kettering Cancer Center’s cBioPortal for Cancer Genomics (http://www.cbioportal.org/)\textsuperscript{29}. Array comparative genomic hybridization data was acquired at the Broad TCGA genome characterization center using the Affymetrix Genome-Wide Human SNP Array 6.0 platform. Raw data was analyzed using the GISTIC2 method to generate gene-level copy-number variation (CNV) estimates and downloaded as either thresholded copy-number calls or as log2-transformed CNV values. Protein expression data was generated and processed at the MD Anderson Cancer Center TCGA proteome characterization center using reverse-phase protein array (RPPA) technology as described\textsuperscript{30} and downloaded either natural log-transformed values or as z-scores.
### 3.2.3 Immunoblotting and Immunofluorescence

Whole cell protein lysates were generated from cell lines and ascites-derived cells in adherent and spheroid culture as previously described\(^3\). Antibodies used for immunoblot against p-AMPKα Thr172 (#2535), AMPKα (#5832), p-LKB1 Ser428 (#3482), LKB1 (#3050), p-p70S6K1 Thr 389 (#9234), p-ACC (#3661), ACC (#3676) and p70S6K1 (#2708) were obtained from Cell Signaling Technology (Danvers, MA). Anti-Tubulin antibody was obtained from Sigma. AICAR was purchased from Caymen Chemical Company (Ann Arbor, MI) and A-769662 from Tocris Bioscience (Bristol, UK). Immunofluorescent (IF) analysis was performed on fresh frozen sections that were fixed (4% formaldehyde), permeabilized (0.1% Triton X-100 in PBS), and blocked (5% BSA in 0.1% Triton X-100) before incubation with p-AMPKα antibody (#ab51110) from abcam® Inc. (Cambridge, MA). Following primary antibody incubation and PBS washes, sections were incubated for 1 hour with anti-rabbit FITC secondary antibody (1:250; Sigma-Aldrich). After further washing, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI; 1:1000) and slides were mounted with Vectashield (Vector Laboratories, Burlingame CA, USA). Fluorescence images were captured using an Olympus AX70 upright microscope and ImagePro image capture software.

### 3.2.4 Cell Viability and ATP assays

Cells were seeded to either 24-well tissue culture plastic or ultra-low attachment (ULA) plates at a density of 1.0x10\(^4\) to form adherent cultures or 5.0x10\(^3\) per well to form spheroids, respectively. Treatment was initiated at time of seeding for cells in suspension while cells under adherent conditions were given 12 hours to adhere prior to commencing treatment. CellTiter-Glo® reagent (Promega, Madison, WI) was prepared according to manufacturer’s instructions. At 72h post-treatment, spheroids were collected, pelleted and left in a minimal volume of media (100 µL), at which point CellTiter-Glo® reagent was added in a 1:1 volume ratio. Under adherent conditions, cells were harvested directly in CellTiter-Glo® reagent (1:1 reagent/media) after a 20 minute incubation period. All samples were subject to a freeze/thaw cycle prior to analysis. Approximately 200µL of the mixture was added to a white-walled 96-well micro-plate and luminescence signal was detected using a microplate spectrophotometer (Wallac 1420 Victor 2; Perkin-Elmer,
Waltham, MA). Treatments were conducted in at least duplicate wells and luminescence readings normalized to cells treated with vehicle control.

3.2.5 siRNA transfections

All siRNA transfections were performed in a 6-well format. The day prior to transfection, cells were plated at a density of approximately $1 \times 10^5$ cells per well in antibiotic-free media. The next day, DharmaFECT transfection reagent (DharmaFECT1 for OVCA429 and iOVCA147-E2 and DharmaFECT3 for SKOV3) was used to transfect cells, as per manufactures protocol. Briefly, 1 µl of DharmaFECT1 or 4 µl of DharmaFECT3 was combined with 10nM siRNA in a volume of 1mL of media (Wisent) and incubated for 20 min; the complexes of DharmaFECT and siRNAs were then added directly to each well. Media was removed 24 hours following transfection and replaced with fresh antibiotic-free growth media. At this point, the cells were incubated until nearly confluent, approximately 72 hours following transfection. PRKAA1 and STK11 siRNAs (M-005027-02 and M-005035-02 respectively) were obtained from Dharmacon (Thermo Fisher Scientific Inc., Waltham, MA). All siRNAs used were siGENOME SMARTpool predesigned pools of four oligos.

3.2.6 Graphing and Statistical Analysis

All graphs were generated using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Data were expressed as Mean ± SEM, as indicated. All statistical analysis (Student’s t-test and Analysis of Variance (ANOVA) with Tukey’s Multiple Comparison Test) was performed using GraphPad Prism 5. Tests of significance were set at $p < 0.05$. 
3.3 Results

3.3.1 AMPKα1 is expressed in metastatic ovarian tumour samples and is associated with a high frequency of copy-number gains and amplifications.

AMPK has been described in many instances to serve as a tumour suppressor despite the lack of genetic evidence to demonstrate a loss of AMPK function in cancer. In order to assess AMPK activity in a large number of serous ovarian tumours, the majority of which (91.1%) are from metastatic, stage III-IV cases, we made use of level 3 array comparative genomic hybridization (aCGH) and reverse phase protein array (RPPA) data from The Cancer Genome Atlas (TCGA). This analysis revealed copy-number gain of the PRKAA1 gene (encoding AMPKα1) in 36% (111/311) of samples (Figure 3.1A). To determine whether PRKAA1 copy-number correlated with protein expression, we plotted RPPA data against copy-number calls for both phosphorylated (T172) and total AMPKα1. This demonstrated a significant increase in both phosphorylated (Figure 3.1B) and total AMPKα1 (Figure 3.1C) in samples with copy-number gain. Using log2-transformed copy-number data, we also performed regression analysis to measure the correlation between PRKAA1 copy-number and protein expression. This revealed a positive correlation between copy-number and AMPKα1 protein expression (both phosphorylated and total; Figure 3.1D&E). In addition, we also noted a positive correlation between AMPKα1 protein expression and activity (Figure 3.1F). To verify AMPKα1 expression and activity in fresh tumour specimens, we performed western blots on lysates harvested from metastatic tumour samples obtained by our lab (Figure 3.1G). Indeed, our direct results demonstrate that AMPKα1 is expressed and active in metastatic ovarian tumours.
Figure 3.1: The AMPK pathway is active in metastatic ovarian tumour samples.
(A) Gene copy-number calls at the PRKAA1 locus are depicted for 311 ovarian serous cystadenocarcinoma tumors (red & pink = high-level & low-level amplification, respectively; teal & blue = heterozygous & homozygous deletion, respectively). OncoPrint obtained from cBioPortal.org. (B,C) Phosphorylated and total AMPKα1 protein (quantitative RPPA; n=398) expression data were transformed to z-scores and depicted as functions of copy-number. One-way ANOVA with Tukey’s Test was performed (*p<0.05; ***p<0.001). (D,E) In-transformed protein expression (n=397; re-transformed to log2 values) data depicted as a function of log2-transformed copy number values. (F) Phosphorylated AMPKα1 protein expression depicted as a function of total protein. Correlation and linear regression analysis performed: line of best fit, Pearson’s r, Goodness-of-fit R², and p values all reported. (G) Lysates were generated from metastatic tumour samples from seven ovarian cancer patients and immunoblot was performed to examine AMPK activity in these samples.
3.3.2 Multicellular aggregates filtered from patient ascites fluid exhibit enhanced AMPK activity.

Our lab has previously demonstrated that ovarian cancer cells which form multicellular aggregates in vitro enter a dormant state, a process which is aided by decreased AKT activity\(^{16}\). Herein, we postulate that the AMPK pathway is another pathway, that mediates spheroid formation-induced dormancy, due to its unique ability to respond to stresses, such as nutrient deprivation and hypoxia. In order to evaluate this, we analyzed AMPK activity in native ascites spheroids filtered directly from patient ascites fluid by western blot and immunofluorescence. Lysates generated from ascites spheroids filtered directly from a number of different patient ascites samples revealed a significant increase in AMPK activity in spheroids compared to matched adherent samples from the same patient (Figure 3.2A). Additionally, immunofluorescence on ascites-derived spheroids revealed intense expression of phosphorylated AMPK\(\alpha\)1 in the cytoplasm and along the cell membrane (Figure 3.2B). These data indicate that AMPK activity is enhanced in actively metastatic cells in spheroids within malignant ascites fluid.

3.3.3 Ovarian cancer cell lines and ascites-derived cells in suspension exhibit decreased levels of ATP and enhanced AMPK activity.

Following our observation that AMPK activity is enhanced in native ascites spheroids, we sought to investigate regulation of this phenomenon further using spheroids formed in vitro. We hypothesized that as a result of spheroid formation induced-dormancy, the metabolic state of cells within these multicellular aggregates is decreased. To assess this, we used Cell Titer Glo® luminescence-based ATP assay to determine levels of intracellular ATP in ovarian cancer cells in adherent and spheroid form. Indeed, ATP levels were significantly lower in spheroids compared to their adherent counterparts (Figure 3.3A). Correspondingly, western blot analysis of adherent and spheroid ovarian cancer cells revealed a significant increase in AMPK activity associated with spheroid formation (Figure 3.3B,C). Taken together, these data demonstrate decreased ATP levels that are associated with increased AMPK activity when ovarian cancer cells aggregate to form multicellular clusters or spheroids.
**Figure 3.2: Native ascites spheroids have enhanced AMPK activity compared to adherent cells.**

(A) Lysates were prepared from filtered spheroids [S] and passage 0 adherent cells [A] from 5 independent patient ascites samples. Immunoblot (shown representative image from two samples) and subsequent densitometry were performed to determine levels of phosphorylated AMPKα1 compared to total protein. Bars: Mean ± SEM. Levels of phosphorylated AMPKα1 were compared using Student’s t-test (*p<0.05). (B) Spheroids filtered from patient ascites fluid for immunofluorescence analysis compared to early passage adherent cells from the same patient [EOC 169]. Nuclei (blue) and pAMPKα1 (green) are visible. Scale bar: 100µm.
Figure 3.3: Spheroids formed from EOC cell lines and ascites-derived cells have decreased levels of ATP and corresponding increases in AMPK activity.

(A) Quantification of ATP levels in EOC cell lines cultured in both adherent and spheroid conditions using luminescence-based ATP assay CellTiter Glo®. Difference in viable cells between each culture condition was accounted for by normalizing results to total protein levels for each sample. Bars: Mean ± SEM. Student’s t-test was used to compare levels of ATP between culture conditions (**p<0.001). (B) Immunoblot performed on EOC cell lines and ascites-derived EOC cells to determine levels of phosphorylated and total AMPKα1 protein. (C) Densitometry was performed on cell lines and primary EOC cells (n=14) to compare levels of phosphorylated AMPKα1 between adherent and spheroid cultured cells. Bars: Mean ± SEM. Student’s t-test was used to determine statistical significance (*p<0.05).
3.3.4 LKB1 protein is expressed in metastatic ovarian tumour samples.

Activity of the key upstream AMPK kinase LKB1 is commonly thought to be tumour suppressive\textsuperscript{32}. Multiple studies have suggested that single allelic inactivation of the STK11 gene encoding LKB1 is sufficient to promote tumorigenesis, while other data suggests that biallelic loss may be required\textsuperscript{33-36}. In order to examine status of LKB1 in serous ovarian tumours we again made use of data from the TCGA. Whereas copy-number gain was common for PRKAA1, heterozygous deletion of STK11 was detected in 84\% of samples (262/311; Figure 3.4A). This single allelic loss correlated with decreased protein expression compared to samples with normal copy-number (Figure 3.4B), and a positive correlation between STK11 copy-number and LKB1 protein expression when we performed regression analysis on log2-transformed copy-number data (Figure 3.4C). When we sought to determine LKB1 expression in metastatic tumour samples obtained by our lab, however, we consistently observed expression of phosphorylated and total LKB1 (Figure 3.4D). Therefore, despite single allele loss of STK11, LKB1 protein expression is maintained in metastatic ovarian cancer cells and may in fact serve an important function in late-stage disease.

3.3.5 Suspension-induced activation of AMPK signalling is accompanied by enhanced LKB1 signalling and inhibition of mTORC1.

We next wanted to determine the specific components of the AMPK pathway that may also be altered when ovarian cancer cells are put in suspension. We first focused on LKB1 as a critical upstream activator of AMPK signalling. Immunoblot revealed increased phosphorylation of LKB1 at Serine 428 in spheroids formed from a number of ovarian cancer cell lines and ascites-derived cells compared to cells grown under adherent conditions (Figure 3.5A,B). Although its phosphorylation state does not affect its catalytic activity, phosphorylation at Ser428 has been shown to be important for the tumour suppressive functions of LKB1\textsuperscript{37}. LKB1 can be localized to the nucleus or cytoplasm, and the cytoplasmic pool of LKB1 has been shown to contribute to the tumour suppressive function of this kinase\textsuperscript{32}. We determined by cellular fractionation and
immunoblotting that LKB1 protein in adherent and spheroid-cultured cells is located in the cytoplasm (Figure S3.1).

Since the LKB1/AMPK signalling pathway has been identified as a key negative regulator of mTORC1 signalling, we next focused on this pathway as a downstream readout for AMPK’s ability to rewire cellular metabolism in these clusters of cells. Immunoblot performed on spheroids from cell lines and ascites-derived cells revealed a significant decrease in mTORC1 activity as determined by p70S6K1 phosphorylation (Figure 3.5C). Taken together, these results provide additional evidence for decreased anabolic metabolism that occurs when cells detach into suspension and form spheroids, indicating that the LKB1/AMPK/mTORC1 signalling axis may be a crucial mediator of cell survival in this context.

3.3.6 Spheroids are much less sensitive to further activation of the AMPK pathway than adherent ovarian cancer cells.

It has been previously demonstrated that treatment of ovarian cancer cells with AMP mimetic AICAR results in increased AMPK activity and decreased viability of adherent cells\textsuperscript{38,39}. Similarly, in our study, we demonstrate robust activation of AMPK after treatment of ovarian cancer cells with 1 mM AICAR, which corresponds with decreased mTORC1 activity (Figure 3.6A). In addition, AICAR treatment of various ovarian cancer cell lines and ascites-derived cells results in decreased viability in cells cultured under both adherent and suspension conditions. Importantly, the detrimental effects of AICAR treatment on spheroid cell viability are not observed until later time points compared to their adherent counterparts (Figure 3.6B). When spheroids are treated with AICAR during reattachment, however, a significant reduction in dispersion area was observed highlighting the detrimental effect that AMPK activation has on cell proliferation (Figure 3.6C).

To further explore mechanisms of AMPK activation, we also tested a more specific allosteric AMPK activator, A-769662, which stimulates AMPK directly without affecting the kinase domain\textsuperscript{40}. Treatment of ovarian cancer cells with A-769662 (100µM) results in activation of AMPK as indicated by enhanced phosphorylation of ACC (Figure
A

Ovarian Serous Cystadenocarcinoma (TCGA provisional dataset)

STK11

<table>
<thead>
<tr>
<th>Amplification</th>
<th>Homozygous Deletion</th>
<th>Gain</th>
<th>Heterozygous Deletion</th>
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B

Figure 3.4: Despite heterozygous deletion, LKB1 protein is expressed in metastatic ovarian tumour samples.

(A) Gene copy-number calls at the STK11 locus are depicted for 311 ovarian serous cystadenocarcinoma tumors (red & pink = high-level & low-level amplification, respectively; teal & blue = heterozygous & homozygous deletion, respectively). OncoPrint obtained from cBioPortal.org. (B) STK11 protein (quantitative RPPA; n=398) expression data was transformed to z-scores and depicted as functions of copy-number. One-way ANOVA with Tukey’s Test was performed (**p<0.001). (C) In-transformed protein expression (n=397; re-transformed to log2 values) data depicted as a function of log2-transformed copy number values. Correlation and linear regression analysis performed: line of best fit, Pearson’s r, Goodness-of-fit R2, and p values all reported. (D) Lysates were generated from metastatic tumour samples from seven ovarian cancer patients and immunoblot was performed to examine LKB1 expression in these samples.
Figure 3.5: Spheroids formed from EOC cell lines and ascites-derived cells express LKB1 protein and have decreased levels of mTORC1 signalling.

(A) Immunoblot performed on EOC cell lines and ascites-derived EOC cells to determine levels of phosphorylated and total LKB1 protein. (B) Densitometry was performed on cell lines and primary EOC cells (n=16) to compare levels of phosphorylated and total LKB1 between adherent and spheroid cultured cells. Bars: Mean ± SEM. Student’s t-test was used to determine statistical significance (*p<0.05). (C) Immunoblot performed on EOC cell lines and ascites-derived EOC cells to determine levels of phosphorylated and total p70S6K1 protein as a read-out of mTORC1 activity.
Figure 3.6: AICAR treatment of EOC cell lines and ascites-derived cells decreases cell viability in adherent and spheroid cells.

(A) Immunoblot performed on EOC cells treated for 24 hours with various doses of AICAR as indicated. (B) Viability of ovarian cancer cell lines and ascites-derived cells following 3 and 6 days of AICAR (1mM) treatment in adherent and spheroid culture conditions. Bars: Mean ± SEM. Effect of treatment at each timepoint was determined using Student’s t-test. Results are significant (p<0.05) unless otherwise indicated. (C) Reattachment of spheroids (72h) formed from ascites-derived cells (n=9) treated with AICAR (1mM) at time of seeding to suspension culture. Quantifications performed using ImageJ and Student’s t-test used to compare areas between groups (***p<0.001). Representative image of one patient sample (EOC154) depicted. Scale bar: 100µm.
Similarly to AICAR treatment, this compound also reduces viability of ovarian cancer cells in adherent culture. However, there is no effect of A-769662 treatment on viability of cells in spheroids (Figure 3.7B). These results suggest that actively proliferating, adherent ovarian cancer cells are more sensitive to AMPK activation, but quiescent cells in multicellular spheroids are less impacted by a further increase in the activation state of this pathway.

Following the observation that both AICAR and A-769662 have detrimental effects on ovarian cancer cells in adherent culture, we further characterized this reduced viability by determining whether cells were undergoing apoptosis or arresting in a particular phase of the cell cycle. Both compounds result in a decreased proportion of cells in the S-phase of the cell cycle as early as 24 hours after treatment. The effect observed with AICAR treatment is more robust across different cell lines than that observed with A-769662 (Figure S3.2). We also used Caspase-Glo® luminescence-based assay which uses caspase 3/7 activity as a read-out for apoptosis. We found that 72 hours following treatment with AICAR (1mM), there was a significant induction of apoptosis, an effect which was not observed in A-769662-treated cells (Figure S3.2). These results suggest that these two compounds have different mechanisms of action in ovarian cancer cells: AICAR decreases ovarian cancer cell viability largely by apoptosis, whereas A-769662 elicits a cytostatic response thereby blocking ovarian cancer cell growth.

3.3.7 LKB1, but not AMPK, is required for ovarian cancer cell survival in suspension.

Given the relative insensitivity of ovarian cancer cells in suspension to further activation of AMPK, we assessed the functional impact of attenuation of the LKB1/AMPK pathway in spheroids. In order to do this we transfected ovarian cancer cell lines with siRNAs targeting either PRKAA1 (AMPKα1) or STK11 (LKB1). A number of established ovarian cancer cell lines were screened for LKB1 and AMPK expression and activity in adherent culture (Figure S3.3) and those with the highest activity were used in knockdown experiments. Effective knockdown of STK11 and PRKAA1 was achieved in cells in both adherent (Figure 3.8A) and suspension (Figure 3.8B) cultures. Cells in adherent culture were not sensitive to knockdown of either AMPKα1 or LKB1 with
respect to cell viability, most likely since these cells are proliferating and not under metabolic stress, therefore not requiring this pathway. Surprisingly, knockdown of AMPKα1 had no impact on the survival of cells in suspension, while loss of LKB1 significantly reduced viability of cells in spheroids (Figure 3.8C). These results point to AMPK-independent LKB1 signalling as an important stress adaptation used by ovarian cancer cells in suspension in order to avoid anoikis.
Figure 3.7: Allosteric AMPK activator A-769662 decreases viability of EOC cells in a context-dependent manner.

(A) Immunoblot performed on EOC cells treated for 24 hours with various doses of A-769662 as indicated. AMPK activation determined by levels of phosphorylated ACC. (B) Viability of ovarian cancer cell lines following 3 and 6 days of A-769662 (100µM) treatment in adherent and spheroid culture conditions. Bars: Mean ± SEM. Effect of treatment at each timepoint was determined using Student’s t-test. Results are significant (p<0.05) unless otherwise indicated.
Figure 3.8: siRNA-mediated knockdown of STK11 but not PRKAA1 results in a decrease in viability of ovarian cancer spheroids. (A,B) Immunoblot performed for proteins as indicated on adherent and spheroid ovarian cancer cells 72 hours after transfection or 3 days after spheroid formation respectively. (C,D) Cell viability as determined by Cell Titer Glo® assay on ovarian cancer cell lines in adherent or suspension culture respectively after three days (seeded to these conditions 72 hours after transfection). (E) Images of day 3 OVCA429 spheroids following siRNA knockdown as indicated. Scale Bar: 100µm.
3.4 Discussion

The distinct mode of metastatic spread whereby EOC cells transit the peritoneal cavity in suspension, presents unique therapeutic challenges for treatment of advanced-stage ovarian cancer. Characterization of this unique population of non-adherent cells will provide insights into novel targets for treatment of this deadly disease. Our laboratory has previously shown that ovarian cancer cells in suspension have a propensity to aggregate and form dormant multicellular clusters or spheroids. This dormancy is reversible upon reattachment to an adherent substratum, a process that is dependent upon AKT$^{16}$. Following up on this observation, we show in this report that cells in dormant EOC spheroids have reduced metabolic activity and as such induce the LKB1/AMPK metabolic stress response pathway. Indeed, we demonstrate that AMPK activity is enhanced in quiescent ovarian cancer spheroids. Also, direct pharmacologic activation of AMPK in proliferating adherent ovarian cancer cells leads to cytostasis and ultimately a decrease in cell viability. Our surprising new result, however, is that where maintenance of LKB1 expression is required for ovarian cancer cell survival in suspension, AMPK is not necessary. This implies an AMPK-independent role for LKB1 in mediating anoikis-resistance in ovarian cancer cells. This is the first study to demonstrate a pro-survival function for LKB1, a kinase that has been traditionally considered to be a tumour suppressor.

Expansive tumour growth is typically dependent on over-proliferative malignant cells that lack the normal response to induce protective growth arrest. Under nutrient-rich conditions, proliferative cancer cells should have low to absent levels of active AMPK signalling. Indeed, we show that expression of phosphorylated AMPKα is marginal in the majority of cultured EOC cells assessed; however, AMPK activity is significantly elevated upon spheroid formation. Ectopic activation of this pathway in proliferating cells using the AMP mimetic AICAR or an allosteric AMPKα activator A-769662 result in decreased viability. These agents most likely produce this effect through different mechanisms. Although both compounds induce a potent cytostatic response, AICAR treatment eventually results in significant cell death due to induction of apoptosis whereas A-769662 does not. A recent report highlighted the difference between these two
compounds, demonstrating that the growth-suppressive effects of AICAR are actually independent of AMPK in a glioma model\textsuperscript{41}. Thus, the decreased viability observed in ovarian cancer spheroids treated with AICAR may in fact occur via AMPK-independent mechanisms. Taken together, this indicates that suppressed AMPK signalling is required to sustain active tumour growth, and supports a general idea that this pathway possesses classical tumour suppressor function under these conditions. However, when cells are in suspension AMPK is activated to facilitate cellular quiescence, and further activation is of lesser consequence on viability. These results indicate that AMPK acts like a typical tumour suppressor, yet its function may be utilized to promote protection from apoptosis during later stages of ovarian cancer progression.

Along this same reasoning, it is not unreasonable to postulate that LKB1 function may be context-specific during ovarian cancer progression given its array of downstream targets. A recent report of a conditional mouse model for serous ovarian carcinoma determined that loss of one Stk11 allele in the context of Pten loss within the OSE leads to the development of high-grade papillary serous ovarian carcinomas\textsuperscript{42}. This is not the first study to demonstrate synergism between LKB1 and other tumour suppressors or oncogenes. In fact, LKB1 has been shown to accelerate tumorigenesis in conjunction with p53\textsuperscript{43}, Kras\textsuperscript{44}, and c-myc\textsuperscript{45}. This provides additional support for the complex function of this important kinase. Indeed, the molecular signature of a particular tumour is likely another factor that impacts the operational role of LKB1 in a particular type or stage of cancer\textsuperscript{18}. Although the study by Tanwar and colleagues\textsuperscript{42} implicates loss of LKB1 function in the initiation of ovarian cancer, knockout is performed in the ovarian surface epithelium and not the secretory epithelium of oviduct; the secretory epithelial cells of the distal fallopian tube is now considered the site of origin for high-grade serous ovarian cancer\textsuperscript{46-48}. Since loss of Stk11 in premalignant serous tubal intraepithelial carcinoma lesions in humans has not yet been documented, studies in this regard would provide additional insight into the role of LKB1 in ovarian cancer initiation.

In this report, we show that analysis of the serous ovarian cancer provisional dataset from cBioPortal indicated that 84\% of tumours exhibit heterozygous loss of Stk11 the gene encoding LKB1\textsuperscript{29}. Despite this, we show that numerous EOC solid tumour
specimens, as well as established ovarian cancer cell lines, express LKB1 protein. Interestingly, we also show copy number gains or amplifications in PRKAA1, which encodes AMPKα1, in 36% of samples. This suggests that there may be compensatory mechanisms to upregulate AMPK activity in late-stage ovarian tumours which harbour reduced LKB1 in order to maintain a functional pathway for tumour cell survival during metastasis. It is also possible that this discordance in LKB1 and AMPK copy number variations indicates that these kinases may not necessarily be acting in concert in advanced-stage ovarian cancer. Our data imply that although STK11 haploinsufficiency may occur and predispose to ovarian cancer initiation, maintenance of functional LKB1 signalling pathway is likely essential during metastatic progression particularly to fuel recurrence of chemo-resistant ovarian cancer.

The LKB1/AMPK signalling pathway represents an immediate response to metabolic stress and reduced energy supply to downregulate anabolic metabolism and shunt pathways to utilize alternative energy substrates. Previous data from our laboratory has shown a decrease in AKT activity upon spheroid formation and an accompanying induction of dormancy and autophagy (Correa, Shepherd, DiMattia, unpublished). We now show that the LKB1/AMPK pathway is induced in EOC spheroids, which has opposing regulatory effects on mTORC1 compared with what would be observed by AKT. Indeed, we show that mTORC1 activity is reduced in spheroids. This result indicates that the LKB1/AMPK signalling cascade acts to reduce protein translation and induce autophagy, likely in concert with downregulated AKT activity.

It has been shown in other cell systems that LKB1/AMPK is an important mediator of protecting detached epithelial cells from anoikis. Interestingly, targeted knockdown of STK11 (LKB1) in our study demonstrated a significant reduction in spheroid cell viability yet there was no effect when AMPKα1 activity was reduced. We confirmed that there was no compensatory effect of AMPKα2 expression in PRKAA1-knockdown spheroid cells that could explain this lack of effect (Appendix A). This implies that LKB1 plays an important role in mediating anoikis-resistance and dormancy in EOC spheroids independent of AMPK. AMPK is the most studied downstream target of LKB1. However, LKB1 has been called a “master kinase” given its ability to phosphorylate at
least 12 other downstream proteins, referred to as AMPK-related kinases (ARKs: MARK1, MARK2, MARK3, MARK4, SIK1, SIK2, SIK3, BRSK1, BRSK2, SNRK, NUAK1, NUAK2)\textsuperscript{49}. The ARKs have been shown to play roles in many important aspects of cell function including cell polarity (MARK, BRSK)\textsuperscript{50,51}, cell proliferation (NUAKs)\textsuperscript{52,53} and CREB-regulated gene transcription (SIKs)\textsuperscript{54-56}. It seems likely that one or more of these ARKs are important downstream mediators of the LKB1-dependent decrease in cell viability we observe in ovarian cancer spheroids. One likely candidate, microtubule affinity-regulating kinase 4 (MARK4), is able to phosphorylate Raptor on the same residue as AMPK, resulting in inhibition of mTORC1 signalling\textsuperscript{57}. Another of these kinases, NUAK1 may also be an interesting target to investigate in our system, as it is able to promote cell survival during periods of nutrient deprivation\textsuperscript{58}. Further studies are needed to determine which of the numerous substrates downstream from LKB1 are mediating this requirement to maintain cell viability in ovarian cancer spheroids.

Although literature supports the idea that STK11 acts as a tumour suppressor during early steps of tumorigenesis, our STK11 knockdown results provide yet another example of a protein, in this case LKB1, exhibiting an important reciprocal metastasis-promoting function during late-stage disease. EOC spheroids have the capacity to harbour a niche of chemotherapy-resistant cells. Our data supports this idea, and importantly we provide the first evidence that AMPK-independent LKB1 signalling may play a significant role in adaptive resistance mechanisms in these metastasis-promoting structures.
3.5 References


Figure S3.1: LKB1 is located in the cytoplasm in adherent and spheroid EOC cells. Immunoblot performed on whole-cell (T), cytoplasmic (C), and nuclear (N) protein extracts isolated from ovarian cancer cell lines to determine subcellular localization of LKB1 protein. Lamin A/C and tubulin used as nuclear and cytoplasmic loading controls, respectively.
Figure S3.2: AICAR and A-769662 have different effects on cell cycle progression and apoptosis in adherent ovarian cancer cells.

(A) Cell cycle analysis (BrdU and PI) by flow cytometry on ovarian cancer cells following 24 and 72 hours of treatment with either AICAR or A-769662 (n=2 for each cell line). (B) Caspase 3/7 activity 72 hours following treatment with either AICAR or A-769662 as determined by Caspase-Glo®. Bars: Mean ± SEM. Effect of treatment determined by One-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).
Figure S3.3: LKB1 and AMPK are expressed in EOC cell lines under adherent conditions but activity is low.
(A, B) Immunoblot performed on a number of ovarian cancer cell lines cultured under adherent conditions in order to determine levels of phosphorylated and total LKB1 and AMPK as indicated.
Chapter 4

4 Discussion

4.1 Summary of findings

The high level of a mortality associated with high-grade serous ovarian cancer has been directly attributed to intra-abdominal metastases, which recur despite aggressive surgical and chemotherapeutic interventions. The presence of microscopic disease and dormant, chemotherapy-resistant cancer cells is likely the cause of these recurrences. Therefore, experimental models that allow us to better understand the biology and pathogenesis of dormant high-grade ovarian cancer cells are crucial to uncover more effective treatment options for patients with advanced-stage disease. Our studies use a highly-relevant, tractable, non-adherent culture system to examine the molecular underpinnings of multicellular spheroid formation and subsequent reattachment to an adherent substratum. These structures induce dormancy and mimic the spheroid state of ovarian cancer cells in ascites, which facilitates the spread of this cancer.

I focused on two distinct cellular signalling systems and discovered in the first instance that BMP signalling is decreased upon spheroid formation, a process that is reversed when these clusters reattach to an adherent substratum (Chapter 2). To examine the functional implications of this dynamic regulation, I constitutively activated BMP signalling and discovered a detrimental effect on spheroid formation, resulting in smaller, more loosely aggregated clusters. Activation of this pathway during spheroid reattachment on the other hand, resulted in increased cellular dispersion. These phenotypic alterations in spheroid formation and reattachment observed in response to BMP signalling, were shown to be mediated, in part, by interaction with the PI3K/AKT signalling pathway. Specifically, constitutive activation of BMP signalling resulted in enhanced AKT activity, which was shown to contribute to increased spheroid reattachment observed as a result of overactive BMP signalling.

Given the ability of the PI3K/AKT and BMP signalling pathways to act together to alter spheroid formation and reattachment, I chose to examine other key cell survival pathways, which might act in concert with AKT in EOC cells. The objective was to
identify signalling pathways that may interact to maintain the viability of ovarian cancer cells and allow seeding of recurrent disease. In doing so, we expect to identify new potential therapeutic targets for which drugs may already exist or lay the groundwork for use of specific combinatorial treatments. Additional rationale for this route of investigation was provided by other work in our laboratory demonstrating an AKT-dependent induction of dormancy and autophagy in EOC spheroids. Investigating this area of research revealed number of studies that reported AMPK-dependent induction of autophagy under conditions of stress such as glucose deprivation and hypoxia. Therefore, I hypothesized that, in addition to the AKT pathway, the LKB1/AMPK signalling cascade would be an important contributor to spheroid-formation induced dormancy.

AMPK has a unique ability to integrate extracellular nutrient and energy signals in order to control the metabolic function of cells. I discovered that AMPK activity is greatly enhanced in ovarian cancer spheroids and this is associated with enhanced phosphorylation of its upstream kinase, LKB1 (Chapter 3). Activation of AMPK is detrimental in adherent, proliferating cells but has little effect on dormant, multicellular spheroids. On the other hand, targeted knockdown experiments highlight an AMPK-independent role for LKB1 in survival of cells in suspension. This is the first demonstration of a pro-survival function for LKB1, a kinase that has been primarily considered to be a tumour suppressor.

The data presented in this thesis discusses two signalling pathways that play distinct roles in EOC spheroid formation and survival. Both pathways however, present potential therapeutic targets for the unique population of non-adherent ovarian cancer cells within ascites fluid.

4.2 BMP signalling plays context-specific roles during ovarian cancer spheroid formation and reattachment

Although a dichotomous role for TGF-β in carcinogenesis is well-established, this is not the case for BMP signalling. More in vitro and in vivo evidence is required to determine whether this pathway is indeed oncogenic or tumour suppressive and in which
types and stages of cancers this is true. BMP signalling is generally considered a critical pathway during development controlling cell differentiation. Therefore, it is reasonable to suppose that induction of BMP signalling in cancer cells might be anti-oncogenic causing a differentiation-related program to force cancer cells out of the cell cycle. It is also possible that BMP signalling has been co-opted by cancer cells in a pro-oncogenic manner to facilitate maintenance of the cancer cell phenotype.

Our study focused on dissecting the contribution of the BMP signalling cascade during the various phases of ovarian cancer progression using a biologically-relevant, tractable in vitro model system. This model takes into account the unique mode of ovarian cancer metastasis, whereby multicellular spheroids represent an important conduit through which cells are able to survive until they reach a mesothelial surface where re-implantation and invasion are possible. It has been suggested that ovarian cancer cells within multicellular aggregates or spheroids undergo EMT, acquiring more mesenchymal characteristics, preparing them to reattach and invade, forming secondary metastases when conditions are favourable\textsuperscript{4,5}.

The data presented in Chapter 2 confirms that spheroids formed from primary ovarian cancer cells undergo EMT, characterized by decreased expression of the E-cadherin gene. This is supported by previous evidence that E-cadherin expression is reduced in cells isolated from ascites compared to their solid tumour counterparts\textsuperscript{6} as well as other studies demonstrating that ovarian cancer cell lines with more mesenchymal characteristics have an increased propensity for compact spheroid formation\textsuperscript{5}. The autonomous down-regulation of BMP signalling during spheroid formation and the decreased propensity for compact spheroid formation when this pathway is activated suggests that BMP signalling may in fact be opposing the natural EMT response of EOC spheroids. This is concordance with a study published in 2011 by the Weinburg lab in which they documented that the BMP pathway had the ability to antagonize TGF-β-induced EMT in mammary epithelial cells\textsuperscript{7}. Indeed, activation of BMP signalling within EOC spheroids results in increased E-cadherin expression when compared to controls, suggesting that inhibition of EMT could be a potential mechanism through which BMP signalling decreases spheroid compaction.
Although activated BMP signalling in spheroids results in smaller, more loosely aggregated clusters this does not result in an overall decrease in cell viability. Therefore, it should not be assumed that activation of this pathway is detrimental. Rather, it may ‘prime’ cells within these aggregates to more readily attach and disperse. Indeed, when spheroids with constitutively active BMP signalling are exposed to an adherent substratum, they have an increased propensity for reattachment and dispersion. This is supported by previous work demonstrating that BMP4 has the ability to enhance motility and invasion of adherent primary ovarian cancer cells\textsuperscript{8,9}.

Taken together, these findings suggest that inhibition of BMP signalling may in fact be a viable therapeutic target through which to prevent EOC spheroid reattachment and formation of secondary metastatic lesions. We show that inhibition of the BMP pathway using a small molecule inhibitor as well as BMP antagonist, noggin, does in fact decrease the ability of cells to disperse from a spheroid following re-introduction to an adherent substratum. Since this does not completely prevent spheroids from reattaching, it is likely that the BMP signalling pathway acts in conjunction with other pathways during this process.

Our studies uncovered an interaction between the BMP and AKT signalling cascades and demonstrated that these two pathways act in concert to promote EOC spheroid reattachment. This finding is supported by other reports demonstrating that the PI3K/AKT pathway is required for BMP-induced migration and invasion in gastric, colon and pancreatic cancer cells\textsuperscript{10,11}. This highlights the potential for targeting these pathways in combination for treatment of metastatic ovarian cancer. Xenograft models will be important to determine whether this interaction is translatable in an \textit{in vivo} setting. Weroha and colleagues recently published elegant work validating the use of a novel ‘tumourgraft’ model whereby ovarian cancer patient tumour material is minced and intraperitoneally injected into immune-compromised mice\textsuperscript{12}. The tumours formed in mice recapitulate the clinical and molecular characteristics of the patient tumour but also mimic the patient’s clinical response to chemotherapeutic treatment. This is an extremely exciting, highly translatable \textit{in vivo} model with which to test the potential response of an patient’s tumour to a targeted therapeutic. Using this model, we would be able to test the
therapeutic response of a number of ovarian cancer tumour specimens to treatment with the small molecule inhibitors of BMP and AKT signalling that I have in the studies described in this thesis. Molecular analysis of these ‘tumourgrafts’ would allow us to determine patient-specific responses to modulation of these two signalling pathways prior to and following treatment.

This work highlights the fact that the BMP signalling pathway exerts the majority of its effects during spheroid reattachment and dispersion. Therefore, it would be interest to examine the contribution of tumour-stroma interactions in this process and the role that the BMP signalling pathway plays in mediating these interactions. Carcinoma-associated fibroblasts (CAFs) have become increasingly recognized as important components of the tumour microenvironment that can aid in the initiation and progression of a number of different cancers including breast, prostate and ovarian. Interestingly, normal fibroblasts co-cultured with carcinoma cells results in an irreversible conversion to a CAF phenotype, suggesting that fibroblasts exposed to cancer cells exhibit permanent, heritable changes. Recent studies in ovarian cancer have demonstrated that the presence of CAFs can contribute to tumour progression, omental and lymph node metastases, in addition to being associated with poor patient prognosis. Fu and colleagues were able to obtain primary cultures of CAFs from ovarian cancer patients and co-culture these with EOC cells. Analysis of conditioned medium from this co-culture system revealed the presence of number of soluble factors including TGF-β and a number of BMPs. Using our in vitro system, it would be of interest to determine the effects of this co-culture media on spheroid reattachment both under ambient conditions as well in spheroids with constitutively activated BMP signalling and those treated with noggin. These studies would provide an additional layer of complexity to our model system, allowing us to not only determine the contribution of CAFs to spheroid reattachment and dispersion but also allow us to determine the interaction between the BMP signalling pathway and this population of cells (Figure 4.1).

The results presented in this thesis have uncovered an important role for BMP signalling in cellular cohesion and EMT during spheroid formation and cellular motility.
during reattachment. This has laid the groundwork for preclinical models to investigate the utility of targeting this pathway for treatment of advanced-stage ovarian cancer.

4.3 LKB1 has AMPK-independent effects on cellular viability in ovarian cancer spheroids

Given the important roles that the AKT signalling pathway plays in EOC spheroids both in mediating responses to BMP signalling but also in contributing to spheroid formation-induced dormancy, I wanted to identify other candidate pathways known to interact with the PI3K/AKT pathway. Taking into account the dormant state of the cells within spheroids and their decreased metabolism an obvious candidate for investigation was the AMPK signalling cascade given its unique ability to respond to changes in extracellular energy and nutrient supply. The AMPK and PI3K/AKT signalling pathways converge on mTORC1 and have opposing regulatory effects on this complex. We have demonstrated that in ovarian cancer spheroids decreased AKT activity and induction of cellular dormancy is associated with enhanced and sustained AMPK activity (Figure 4.1).

Other studies in our laboratory have linked cellular dormancy and quiescence within multicellular spheroids with induction of autophagy (Correa, DiMattia, and Shepherd, unpublished data). AMPK has the ability to directly induce autophagy by phosphorylating and positively regulating ULK1, a critical protein for autophagy initiation. AMPK can also indirectly induce autophagy through its ability to inhibit mTORC1 by phosphorylation of TSC2 and Raptor. A study by Avivar-Valderas and colleagues in 2012 highlighted the potential for interaction between mTORC1, AMPK and a member of the unfolded protein response (UPR) pathway, protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) in autophagy induction and anoikis-resistance in mammary epithelial cells. It was noted that in response to loss of ECM attachment PERK was able to activate AMPK through its upstream kinase, LKB1 although the precise mechanism through which this occurs remains unclear. This suspension-induced AMPK activity was required for inhibition of mTORC1 and induction of autophagy. This was the first study to implicate the LKB1/AMPK/mTORC1 signalling cascade as a key regulator of anoikis in epithelial cells.
A number of pathways and processes are uniquely altered in ovarian cancer spheroids, aiding in cellular aggregation and induction of dormancy. Specifically, the BMP signalling pathway is down-regulated during ovarian cancer spheroid formation and aids in cellular aggregation and cohesion. Alternatively, the LKB1/AMPK signalling cascade is up-regulated during spheroid formation and contributes to spheroid formation-induced dormancy. Many of these signalling aberrations are reversed during spheroid reattachment. We propose that upon spheroid reattachment to serosal surfaces of various peritoneal organs (omentum, appendix, intestine), carcinoma-associated fibroblasts (CAFs) secrete a number of cytokines, such as BMPs and TGF-βs, to enhance proliferation, motility and invasion.

Figure 4.1: Contribution of BMP and LKB1/AMPK signalling pathways to ovarian cancer metastasis.
Applying these findings to our system, I chose to focus on LKB1 and mTORC1 as critical upstream and downstream mediators of AMPK signalling in EOC spheroids. As discussed in this thesis, suspension-induced AMPK activation in ovarian cancer cells is in fact associated with enhanced phosphorylation of its upstream kinase LKB1 and decreased activity of mTORC1. Surprisingly, targeted knockdown of AMPK did not have any effect on viability of cells within ovarian cancer spheroids. This was unexpected given the important role AMPK is known to play in cell survival under nutrient replete conditions. However, targeted knockdown of LKB1 results in significant reduction in viability of cells within multicellular aggregates, indicating an important role for this kinase in anoikis-resistance of ovarian cancer cells independent of AMPK.

As described in the introduction to this thesis, LKB1 phosphorylates 12 other kinases in addition to AMPK, termed the AMPK-related kinases (ARKs). Interestingly, one of these kinases, microtubule affinity-regulating kinase 4 (MARK4) has very recently been shown to phosphorylate Raptor on the same residue as AMPK, resulting in inhibition of mTORC1 signalling\(^{25}\) (Figure 4.2). Another of these kinases, NUAK1 may also be an interesting target to investigate in our system as it has been shown to be a target of AKT in addition to LKB1\(^{26}\). Similar to AMPK, NUAK1 is able to promote cell survival during times of nutrient deprivation\(^{27}\). Although their functions are not as well characterized as AMPK, it is likely that one or more of the ARKs may be important mediators of the pro-survival functions of LKB1 in our system. Targeted knockdown of each of these ARKs (MARK1-4, NUAK1, NUAK2, SIK 1-3, SNRK, BRSK1, BRSK2) and assessment of cell viability in suspension would be the most effective method through which to determine our target(s) of interest.

These studies are the first to identify a pro-survival function for the tumour suppressor LKB1 in a metastatic cancer setting. Further studies will focus on examining the effect that LKB1 loss has on cellular quiescence and autophagy induction in ovarian cancer spheroids, as well as determining downstream mediators of LKB1 in our system. Perhaps we have uncovered a unique LKB1 signalling axis crucial in mediating anoikis-resistance in ovarian cancer cells.
Figure 4.2: Proposed mechanisms of dormancy induction in ovarian cancer spheroids.
AMPK and AKT have opposing regulatory effects on mTORC1 via phosphorylation of TSC2. mTORC1 activity is decreased in multicellular spheroids and this corresponds with decreased AKT activity and enhanced AMPK activation. LKB1 not only functions as an upstream kinase for AMPK but also for a number of AMPK-related kinases (ARKs). One of these ARKs, MARK4, phosphorylates Raptor leading to inhibition of mTORC1. This is another potential kinase with elevated activity in dormant ovarian cancer spheroids. We propose that LKB1 is an important mediator of spheroid-formation induced dormancy independent of AMPK.
4.4 BMP and LKB1 signalling: Is there a connection?

The ability to avoid anoikis is an adaptation that is afforded to all metastatic ovarian cancer cells. In fact this biologic phenomenon is probably important in a variety of cancers that induce ascites formation or pleural effusions including mesothelioma\textsuperscript{28}, gastric cancer\textsuperscript{29} and pancreatic cancer\textsuperscript{30}. We have shown that under non-adherent conditions these cells have a propensity to aggregate and form multicellular clusters or spheroids, conferring them with a survival advantage. Therapeutic targeting of this population of cells while they are suspended within the peritoneal cavity or at the point of reattachment to form secondary metastatic lesions would be beneficial since the majority of ovarian cancer patients succumb to recurrent, metastatic disease. In this thesis I have specifically focused on two signalling pathways with diverse functions in ovarian cancer spheroid biology.

One of the few studies linking LKB1 to the TGF-β/BMP signalling pathway demonstrated that LKB1 is able to phosphorylate Smad4, preventing it from binding DNA, resulting in inhibition of both TGF-β and BMP signalling cascades\textsuperscript{31}. Future studies could focus on examining potential interaction between these two signalling cascades through Smad4 or other transcriptional targets. It would also be important to determine whether there may be a functional link between these pathways. Perhaps decreased cellular cohesion that occurs as a result of activated BMP signalling will render cells in suspension even more susceptible to knockdown of LKB1. It would also be interesting to determine the activity of the TGF-β/BMP pathways in cells that have LKB1 knocked down. This may provide additional mechanistic evidence for the AMPK-independent effects of LKB1 in our system.

Both studies discussed in Chapter 2 and 3 of this thesis could be expanded with the use of the recent Pax8-Cre p53\textsuperscript{num}/PTEN\textsuperscript{−/−}/BRCA1/2\textsuperscript{−/−} mouse model of high grade serous cancer (HGSC)\textsuperscript{32}. In this model, disease begins in the fallopian tube secretory epithelium with the development of STIC lesions which progress to HGSC, which metastasizes throughout the peritoneum, similar to the human disease. This is an extremely relevant model, as it highly resembles the progression of human ovarian cancer and provides opportunities to study the earliest events in initiation of HGSC.
The majority of studies to date have focused on BMPs in the ovary and OSE. However, now that it is clear the OSE is not the origin of high-grade serous ovarian cancer, it would be of interest to determine the role that the BMP signalling pathway plays in the fallopian tube. To set the stage it will be relevant to determine the expression of various BMPs and their receptors by IHC in STIC lesions from human and mouse fallopian tube. Based on the expression levels, knockout of these specific components of the pathway using the aforementioned mouse model would provide insight into the role of this pathway in initiation and early pathogenesis of high-grade serous ovarian cancer. This would nicely complement the studies presented in this thesis where we demonstrate a role for this pathway in advanced-stage HGSC.

Very little is known about the role of LKB1 in ovarian cancer pathogenesis. However, recent studies from the Teixeria lab demonstrate that loss of Pten and STK11 in the OSE results in formation of high-grade papillary serous carcinomas. This suggests that loss of LKB1 may be important in tumour establishment, but based on the findings presented in this thesis, LKB1 also aids in cell survival during later metastatic stages. However, the transgenic mouse model of high-grade serous ovarian cancer discussed above is a more relevant model with which to test this hypothesis since it provides a more accurate representation of disease initiation. Knockout of STK11 in addition to Pten Tp53 and Brca1/2 specifically in the secretory epithelium of the fallopian tube may in fact result in more rapid disease progression in this model. Ideally, all combinations of this knockouts focusing on this gene set would provide the most relevant information regarding the importance of LKB1 in the initiation and progression of HGSC. These mouse models would indicate which genes, in combination with homozygous or heterozygous loss of LKB1 activity might predispose secretory epithelial cells to transformation and metastasis to the ovary and peritoneal cavity. These studies would help to determine at which stage of disease progression LKB1 activity is important for either disease progression or inhibition, providing additional support for the findings discussed in Chapter 3.
4.5 Synthesis

The goal of this thesis was to contribute to our knowledge of ovarian cancer metastasis, particularly focusing on multicellular spheroids as major contributors to formation of secondary metastatic lesions. The data presented in Chapters 2 and 3 characterizes two signalling pathways that are dysregulated in EOC spheroids and discusses the potential for targeting these pathways therapeutically. Overall, this body of work has contributed to the field of ovarian cancer metastasis by uncovering unique and complex interactions between a number of signalling cascades and provided rationale for investigating these pathways further in preclinical models. Understanding the unique characteristics afforded to non-adherent ovarian cancer cells is critical for the identification of more effective treatment regimes particularly for late-stage recurrent disease.

4.6 References


Appendix A: Additional Figures

Figure 1: siRNA-mediated knockdown of PRKAA2 does not affect levels of AMPKα. Immunoblot performed on proteins as indicated 72 hours after transfection of adherent EOC cells with siRNA. AMPKα expression and activity is decreased by PRKAA1 knockdown but not PRKAA2.
Appendix B: Ethics Approval

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Gabriel DiMattia
Review Number: 12696E
Review Level: Delegated
Approved Local Adult Participants: 90
Approved Local Minor Participants: 0
Protocol Title: Development of Biological Models for the translation ovarian cancer research initiative.
Department & Institution: Biochemistry, University of Western Ontario
Sponsor:
Ethics Approval Date: December 09, 2011
Date

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<td>Revised Study End Date</td>
<td>The study end date has been extended to December 31, 2015 to allow for project completion.</td>
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This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines, and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 000006940.
Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. T.G. Shepherd
Review Number: 16391E
Review Date: August 12, 2009
Protocol Title: Investigating key signalling pathways in secondary tumour implants formed during ovarian cancer metastasis
Department and Institution: Oncology, London Health Sciences Centre
Sponsor: CIHR-CANADIAN INSTITUTE OF HEALTH RESEARCH
Ethics Approval Date: August 28, 2009
Expiry Date: September 30, 2014
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.
Appendix C: Summary of Clinical Data for EOCs

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<th>Grade</th>
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<td>3</td>
<td>IIIC</td>
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*, stage was defined as at least IC for this patient.
n.a., not available
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Curriculum Vitae

Teresa Marie Peart

EDUCATION

University of Western Ontario, London, Ontario
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University of Western Ontario, London, Ontario
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McKinnon Park Secondary School, Caledonia, Ontario
OSSD and Ontario Scholar Award  2003

RESEARCH-RELATED EXPERIENCE

University of Western Ontario, London, Ontario
• My research examines the role of key signalling pathways and their relation to ovarian cancer pathogenesis
• Using novel cell culture methods to examine hypotheses
• Presented findings at a number of local and National conferences
• Author on several peer-reviewed publications

• Studied the muscle physiology of exercising grass carp
• Responsible for developing and carrying out methods for exercising grass carp as well as accurately sampling blood and muscle
• Performed various in-vitro tests
• Responsible for keeping accurate laboratory records
• Presented findings in two conferences

TEACHING EXPERIENCE

Undergraduate Teaching assistant, Medical Science 4461, University of Western Ontario  Jan 2012-April 2012

Undergraduate Teaching assistant, Medical Science 4900, University of Western Ontario  Sept 2011-present
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Arlan Walsh, Department of Biochemistry, University of Western Ontario
Sept 2010-April 2011
• Responsible for training student in proper laboratory techniques, overseeing daily experiments as well as providing expertise crucial for project completion

Anton Shimanovsky, Department of Biology, McMaster University
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Dominik Dobransky, Department of Biochemistry, University of Western Ontario
Sept 2009-April 2010
• Responsible for training student in proper laboratory techniques, overseeing daily experiments as well as providing expertise crucial for project completion

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Sept 2008-April 2009

SCHOLARSHIPS

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CIHR student training program in Cancer Research Sept. 2009-Sept. 2013

PUBLICATIONS

Peer-Reviewed Publications


Abstracts


Peart T, Bertrand M, Sugimoto AK, Prefontaine M, DiMattia GE and Shepherd TG (2011) Activated BMP signalling modulates multicellular spheroid formation and reattachment of ascites-derived human epithelial ovarian cancer cells in an in vitro model of metastasis. Published in AACR 102nd Annual Meeting Program; Denver, CO.


Peart T, Bertrand M, Sugimoto AK, Prefontaine M, DiMattia GE and Shepherd TG (2012) The 5’-AMP-activated protein kinase (AMPK) pathway is upregulated in ovarian cancer spheroids to promote the dormant phenotype. Canadian Conference on Ovarian Cancer Research; Quebec City, Quebec; awarded prize for oral presentation.

Peart T, Bertrand M, Sugimoto AK, Prefontaine M, DiMattia GE and Shepherd TG (2012) The 5’-AMP-activated protein kinase (AMPK) pathway is upregulated in ovarian cancer spheroids to promote the dormant phenotype. UWO Oncology Research and Education Day.