Features of Dormancy in Metastatic Ovarian Cancer Cells

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Graduate Program in Biochemistry  
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy  
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CHARACTERIZING QUIESCENCE AND AUTOPHAGY AS FEATURES OF DORMANCY IN METASTATIC OVARIAN CANCER CELLS

(Thesis format: Integrated Article)

by

Rohann Jonathan Mark Correa

Graduate Program in Biochemistry

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

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The University of Western Ontario
London, Ontario, Canada

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Abstract

The most prevalent subtype of ovarian cancer – high-grade serous (HGS) carcinoma – is also the most lethal, since the majority of cases are characterized by advanced-stage (metastatic) presentation. Metastasis of this cancer proceeds by an intra-peritoneal route, involving detachment of cells from the primary tumour and dissemination throughout the peritoneal cavity as multicellular aggregates, or spheroids. Herein, we demonstrate that HGS patient-derived tumour cells cultured to form in vitro spheroids exhibit features of cancer dormancy, a cellular state known to promote therapeutic resistance and disease recurrence. We discovered that upon spheroid formation, cells became non-proliferative, exhibiting a cell cycle profile and protein expression pattern (elevated p27Kip1 and RBL2/p130) that was consistent with quiescence. This was accompanied by decreased AKT kinase activity, which may be important in mediating cell cycle exit via the SCF ubiquitin-ligase complex member p45/SKP2. Moreover, when spheroids were re-attached to an adherent substratum, quiescence was rapidly reversed in an AKT-dependent manner. Aside from quiescence, we also discovered that the cellular self-digestion mechanism autophagy was upregulated during spheroid formation. Induction of this process was also observed in adherent cells (and augmented in spheroids) by pharmacologic AKT inhibition. To determine autophagy’s effect on cell viability, we attempted to block it using siRNAs targeting critical autophagy-related (ATG) genes. Interestingly, depletion of Beclin1/ATG6 had no effect, despite its role as a canonical inducer of the process. Conversely, depletion of ATG5 and ATG7 led to efficient autophagy blockade, as did treatment with the classical autophagy inhibitor Chloroquine and the novel agent Spautin-1. These approaches caused a loss of viability in both adherent and spheroid cultures. Moreover, combining autophagy blockade with AKT inhibition synergistically reduced viability, thus implying that autophagy upregulation functions as a survival mechanism. Taken together, these data reveal that two cellular processes, quiescence and autophagy, are readily induced by metastatic ovarian cancer cells as features of a dormant phenotype. We therefore propose that therapeutically targeting these dormant cells will prove highly effective in combating metastasis, resistance, and recurrence in patients with metastatic HGS ovarian cancer.
Keywords

Ovarian cancer, high-grade serous ovarian carcinoma, metastasis, primary cultures, spheroid, quiescence, autophagy, dormancy, AKT, Beclin1
Co-Authorship Statement

All Chapters were written by Rohann Correa and edited by Dr. Gabriel DiMattia and Dr. Trevor Shepherd.

The data presented in Chapter 2 appear in the published manuscript “Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids”. Rohann Correa, Teresa Peart, Yudith Ramos-Valdes, Gabriel DiMattia, and Trevor Shepherd. *Carcinogenesis*. 2012, 33(1): 48-58. TP and TRV performed experiments appearing in Fig. 2.2 and Fig. 2.4, respectively. RC performed all other experiments and data analysis. The manuscript was written by RC and edited by GD and TS. References to figures were modified from the original publication to correspond with the formatting of this thesis.

In Chapter 3, experiments and data analysis were performed by Rohann Correa, with the exception of all immunofluorescence staining which was performed by Dr. Elena Fazio. Also, YRV performed some of the immunoblots appearing in Fig. 3.2, 3.3, and 3.6 as well as selected and tested OVCAR8 clones for expression of eGFP-LC3B.

In Chapter 4, experiments and data analysis were performed by Rohann Correa, with the exception of several EC50 and Combination Index Analysis experiments (~4 EOCs) which were performed by YRV.
Acknowledgments

I would like to begin by thanking my supervisors Dr. Gabriel DiMattia and Dr. Trevor Shepherd. Over the course of my training, their experience, insight, and guidance have proven invaluable to my project, while their determination and passion for science has been an inspiration to me as a researcher-in-training. I would also like to thank my advisory committee consisting of Dr. Fred Dick and Dr. Jim Koropatnick for sharing their wealth of experience and providing excellent guidance. Additionally, I wish to thank Dr. Chris Pin, Dr. Tom Drysdale, and Dr. Bonnie Deroo (as well as their post-doctoral fellows, research associates, and trainees) for contributing valuable insights during weekly multi-lab meetings.

Next I would like to thank the members of my own lab: Davis Dong, Dr. Elena Fazio, Teresa Peart, Samah Rafehi, Jessica Tong, as well as past lab members – all of whom have tolerated working alongside me for all of these years! I want to specifically thank our technician Yudith Ramos-Valdes, as she was responsible for much of my initial training and continues to be of great assistance. In addition, members of Dr. Fred Dick’s laboratory (specifically, Dr. Matthew Cecchini, Dr. Courtney Coschi, and Srikanth Talluri) have provided valuable technical assistance and advice throughout my training.

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Finally, I wish to acknowledge the women who have suffered from ovarian cancer in the past and those who continue their struggle with this disease, particularly those women who have agreed to participate in this study.
Dedication

To my parents and to Rachel
For your constant support, understanding, and love
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>4E BP1</td>
<td>Eukaryotic Translation Initiation Factor 4E Binding Protein 1</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AKT</td>
<td>Cellular homologue of the murine thymoma retroviral oncogene vAkt8</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate-Activated Protein Kinase</td>
</tr>
<tr>
<td>ARID1A</td>
<td>Gene encoding AT-Rich Interactive Domain-Containing Protein 1A</td>
</tr>
<tr>
<td>ATG</td>
<td>autophagy-associated (ATG) gene</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-Associated Death Promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-Associated X Protein</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 Homology Domain 3</td>
</tr>
<tr>
<td>BIM</td>
<td>Bcl-2-Like 11</td>
</tr>
<tr>
<td>BRAF</td>
<td>Gene encoding the B-RAF Kinase</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast and Ovarian Cancer Susceptibility Gene 1</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>C3 PI3K</td>
<td>Class III PI3K</td>
</tr>
<tr>
<td>CD44</td>
<td>Cell Surface Receptor for Hyaluronic Acid</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CIC</td>
<td>Cortical Inclusion Cyst</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin-Dependent Kinase Inhibitor</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CTNBB1</td>
<td>Gene encoding β-Catenin</td>
</tr>
<tr>
<td>CUL1</td>
<td>Cullin1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DP1</td>
<td>E2F Dimerization Partner 1</td>
</tr>
<tr>
<td>DREAM</td>
<td>DP1-RBL2/p130-E2F4-and-MuvB Complex</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor (ErbB1)</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic Translation Initiation Factor 4E</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Human Epidermal Growth Factor Receptor 2 (HER2/Neu)</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead Box O</td>
</tr>
<tr>
<td>G1</td>
<td>Gap phase 1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap phase 2</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal Stromal Tumor</td>
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</table>
GSK3  Glycogen Synthase Kinase 3
HAT   Histone Acetyltransferase
HCQ   Hydroxychloroquine
HDAC  Histone Deacetylase
HGS-OvCa  High-Grade Serous Ovarian Carcinoma
HIF1α  Hypoxia-Inducible Factor 1 Alpha
HIF2α  Hypoxia-Inducible Factor 2 Alpha
IP     Intra-Peritoneal
IRS1   Insulin Receptor Substrate 1
KRAS  Gene encoding the oncoprotein Kirsten-Ras
LKB1   Liver Kinase B1
M      Mitosis phase
mTOR  Mechanistic Target of Rapamycin
mTORC1 Mechanistic Target of Rapamycin Complex 1
mTORC2 Mechanistic Target of Rapamycin Complex 2
OSE   Ovarian Surface Epithelium
p110  Phosphatidylinositol-3-kinase catalytic subunit, encoded by PIK3CA
p70S6K Ribosomal Protein S6 Kinase 1
p85   Phosphatidylinositol-3-kinase regulatory subunit, encoded by PIK3R1
PARP  Poly(ADP)ribose Polymerase
PDGF  Platelet-Derived Growth Factor
PDGFR  Platelet-Derived Growth Factor Receptor
PDK1  Phosphoinositide-Dependent Kinase 1
PE    Phosphatidyl-ethanolamine
PERK  Pancreatic eIF2-Alpha Kinase / Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3 (EIF2AK3)
PHLPP1 PH-Domain and Leucine-Rich Repeat Protein Phosphatase 1
PHLPP2 PH-Domain and Leucine-Rich Repeat Protein Phosphatase 2
PI    Phosphatidylinositol
PI(3,4,5)P3 Phosphatidylinositol-3,4,5-trisphosphate
PI(4,5)P2 Phosphatidylinositol-4,5-bisphosphate
PI3K  Phosphatidylinositol-3-Kinase
PIK3CA Gene encoding the catalytic subunit (p110) of Phosphatidylinositol-3-kinase
PRAS40 Proline-Rich AKT Substrate of 40kDa
pRB   Retinoblastoma protein
PTEN  Phosphatase and Tensin Homologue, encoded by the PTEN gene
ROS   Reactive Oxygen Species
RTK   Receptor Tyrosine Kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>SCF</td>
<td>SKP2-CUL1-F-Box Protein Complex</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SKP1</td>
<td>S-phase Kinase-Associated Protein 1</td>
</tr>
<tr>
<td>SKP2</td>
<td>S-phase Kinase-Associated Protein 2</td>
</tr>
<tr>
<td>Spautin-1</td>
<td>Specific and Potent Autophagy Inhibitor 1</td>
</tr>
<tr>
<td>T-loop</td>
<td>Catalytic activation loop of a kinase</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid Cycle</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TIC</td>
<td>Tubal Intra-Epithelial Carcinoma</td>
</tr>
<tr>
<td>TP53</td>
<td>Gene encoding p53</td>
</tr>
<tr>
<td>TSC1</td>
<td>Tuberous Sclerosis Complex 1</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous Sclerosis Complex 2</td>
</tr>
<tr>
<td>ULK1</td>
<td>Uncoordinated-51-like kinase 1</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>USP10</td>
<td>Ubiquitin Specific Peptidase 10</td>
</tr>
<tr>
<td>USP13</td>
<td>Ubiquitin Specific Peptidase 13</td>
</tr>
<tr>
<td>UVRAG</td>
<td>Ultraviolet Irradiation Resistance-Associated Gene</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 is focused on understanding the biology of metastatic ovarian cancer cells, particularly those in three-dimensional clusters or spheroids. The introductory chapter begins (Section 1.2) by illustrating the complexity of ovarian cancer, focusing specifically on the origins, pathobiology, and lethality of its most prevalent and metastatic sub-type – high-grade serous carcinoma. Not only do tumour cells of this sub-type comprise our biological model, but our in vitro culture system also seeks to model high-grade serous metastasis. In Section 1.3, the oncogenic PI3K/AKT/mTOR signaling pathway is introduced, since our work in all data chapters characterizes the effects of its endogenous or pharmacologic modulation. One such effect is cellular quiescence, which is introduced in Section 1.4 since our findings demonstrate its reversible induction in spheroids (Chapter 2). Finally, Section 1.5 discusses the paradoxical cellular mechanism of autophagy, since work presented in Chapters 3 and 4 explore its regulation and pro-survival properties in ovarian cancer cells. The final Section summarizes the rationale for our studies and outlines the findings presented in this thesis.

1.2 Ovarian Cancer

1.2.1 Classification of ovarian neoplasms

The ovary is primarily responsible for gametogenesis (i.e., production of oocytes) and steroidogenesis (production of estrogen and progesterone) in mammals. Human ovaries are each 3-5cm at their longest dimension and located on either side of the
uterus. The ovaries are not physically attached to the uterus or the Fallopian tubes, but rather are affixed to the lateral pelvic walls by the broad ligament. Each ovary lies in close proximity to Fallopian tube fimbriae (fringes or finger-like terminations), as their brief physical interaction during ovulation allows entry of the oocyte for possible fertilization.

Tumours of the ovary can be broadly classified as non-epithelial and epithelial, based on their cell-type of origin. Although non-epithelial tumours (i.e., germ cell tumours or sex cord-stromal tumours) comprise ~40% of all ovarian neoplasms, the majority of these are benign. In contrast, epithelial ovarian tumours are most often malignant and represent >90% of all ovarian cancers. Epithelial ovarian cancers (EOCs) can be sub-classified into eight histologic types, the four most prevalent of which are serous (50-70%), endometrioid (7-25%), mucinous (~10%), and clear cell (<3-5%) (Fig. 1.1). These distinct histologies each resemble differentiated tissues of the female reproductive tract, namely secretory Fallopian tube epithelium (serous), endometrial glands (endometrioid), mucin-secreting endocervical glands (mucinous), and glycogen-filled vaginal “rests” (clear cell). The extent of differentiation toward these histologies is defined as tumour grade, where higher grade implies less differentiation or resemblance to the aforementioned tissues (among other criteria). Tumours are further classified as benign, borderline, or malignant based on their invasiveness and proliferative capacity. Notably, the most prevalent sub-type of EOC is malignant high-grade serous carcinoma, accounting for nearly 50% of all ovarian malignancies. Thus, epithelial ovarian tumours exhibit great heterogeneity in histologic appearance, degree of differentiation, and extent of proliferation/invasion. Importantly, they also exhibit differences in the genetic aberrations underlying their pathobiology.

1.2.2 Genetics of Epithelial Ovarian Cancer

In 2004, Shih and Kurman sought to reconcile some of the heterogeneity of EOCs by proposing a “two-pathway” model of tumour progression. They developed this model
Figure 1.1: Classification of Ovarian Neoplasms.
Non-epithelial tumours include those arising from either the granulosa, thecal, ovarian stromal, or germ cells (oocytes). Epithelial tumours develop via two divergent sequences: a Type I, Low-Grade (L.G.) pathway thought to arise primarily from ovarian surface epithelial cells, and a Type II, High-Grade (H.G.) pathway arising from secretory epithelial cells in the distal Fallopian tube. Type I tumours are malignancies of all histologic sub-types. Type II tumours are predominantly serous, but also include endometrioid, undifferentiated carcinomas, and carcino-sarcomas (mixed epithelial-stromal tumours). Note: tumours of mixed epithelial histology are also observed.
by integrating emerging molecular-genetic data with what has long been known about epithelial ovarian cancers: different histologic sub-types can exhibit widely divergent natural histories and clinical behaviors. According to this model, EOCs are divided into Type I and Type II tumours based on their pathobiology and associated genetic aberrations\(^8\). Type I tumours include low-grade serous, endometrioid, mucinous, and clear cell histotypes that develop as a result of a stepwise progression from precursor lesions (e.g., endometriosis\(^9,10\)) to benign, borderline, and malignant tumours\(^9-13\) (Fig 1.1). Such tumours are slow-growing, often diagnosed prior to extensive metastasis, characterized by mutations in genes such as \(KRAS\)\(^{14-23}\), \(BRAF\)\(^{14-16,18,24}\), \(PIK3CA\)\(^{10,14,16,25,26}\), \(PTEN\)\(^{16,17,27-30}\), \(CTNBB1\) (\(β\)-Catenin)\(^31\), and \(ARID1A\)\(^{32-34}\), and exhibit relatively little genomic instability\(^20\). In contrast, mutations in these genes are seldom observed in Type II tumours (Table 1.1). Instead, Type II malignancies exhibit germline and somatic \(BRCA1/2\) mutation\(^{35-41}\), near-universal \(TP53\) mutation\(^{13,15,42-44}\), as well as numerous copy-number abnormalities in a background of profound genomic instability\(^{20,42}\). Type II malignancies are primarily comprised of high-grade serous cancers (Fig. 1.1). These are highly proliferative tumours that metastasize early in their course, resulting in disseminated disease at the time of diagnosis. Therefore, according to Shih and Kurman’s model, epithelial ovarian tumorigenesis proceeds along two distinct paths, each of which is associated with its own set of enabling genetic aberrations.

1.2.3 Origins of Epithelial Ovarian Cancer

Traditionally, the ovarian surface epithelium (OSE), a single layer of relatively undifferentiated mesothelium covering the ovary\(^49\), was viewed as the source of all epithelial ovarian tumours. The “incessant ovulation” hypothesis originally proposed by Fathalla in 1971 maintained that ovulation-associated damage and repair of the ovarian surface exposed the OSE to sources of DNA damage, thereby predisposing it to malignant transformation\(^50\). This hypothesis was supported by epidemiologic evidence of decreased ovarian cancer risk with multiple pregnancies and oral contraceptive use in humans\(^51\), as well as experimental evidence of increased ovarian tumorigenesis in super-
<table>
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<tr>
<th>Gene</th>
<th>Clear Cell</th>
<th>Mucinous (or Mucinous Borderline)</th>
<th>Endometrioid</th>
<th>Low-Grade Serous (or Serous Borderline)</th>
<th>High-Grade Serous</th>
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<td>0.5&lt;sup&gt;28,29,42&lt;/sup&gt;</td>
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<sup>a</sup>Percent of mutated cases, given as a median value from the cited studies

Table 1.1: Mutations Frequencies in Type I and Type II Epithelial Ovarian Cancer<sup>a</sup>
ovulated hens\textsuperscript{52}. Tumours were thought to arise when OSE – which would have already accrued DNA damage over a lifetime of ovulations – became trapped in cortical inclusion cysts (CICs) and underwent malignant transformation and differentiation to one of the epithelial tumour histologies\textsuperscript{53}. By such a mechanism, OSE-CICs were believed to give rise to all ovarian tumours.

Despite this prevailing view, investigators began to question whether a single tissue-of-origin could yield the divergent genetic and clinical characteristics seen across epithelial ovarian tumours. Furthermore, while OSE/CIC-associated precursor lesions had been described for Type I tumours\textsuperscript{54}, no such precursor lesion had ever been identified for highly-prevalent, highly-metastatic Type II tumours.

Evidence for an alternate tissue-of-origin emerged when the precursor lesion of Type II tumours was discovered upon careful examination of prophylactically-removed Fallopian tubes from women at risk for developing ovarian cancer (\textit{BRCA1} or \textit{BRCA2} mutation carriers). Peik \textit{et al.} were the first to report in 2001 that 6/12 Fallopian tubes examined exhibited dysplastic changes in their epithelia, specifically in secretory (serous) cells\textsuperscript{55}. Additional studies went on to demonstrate that these tubal intra-epithelial carcinomas (TICs) were preferentially located in the fimbria\textsuperscript{56} and, importantly, were present in patients with serous carcinomas\textsuperscript{57}. Further analysis identified pre-malignant precursor lesions in normal Fallopian tube epithelium that preceded TICs. Specifically, Lee \textit{et al.} discovered pre-malignant foci of p53 immunoreactivity (due to accumulation of a non-functional mutant protein): they termed these “p53 signatures”\textsuperscript{58}. Similar to TICs, these ‘signatures’ were comprised exclusively of secretory (serous) cells and localized in the fimbria. Furthermore, signatures co-occurred with malignant TICs and serous carcinomas – sometimes even harboring identical p53 mutations\textsuperscript{58}. Therefore, the formation of pre-malignant p53 signatures and their progression to early-malignant TICs were heralded as the missing early phases of serous tumorigenesis.

These findings prompted Lee \textit{et al.} to improve upon the two-pathway model of Shih and Kurman. Type II high-grade serous tumours are now believed to originate in the
fimbria as p53 signatures. These can progress to TICs if they acquire additional genetic ‘hits’. By exfoliating from the fimbria and implanting on nearby ovarian, uterine, or peritoneal surfaces, TICs rapidly giving rise to high-grade serous carcinomas (Fig. 1.2A). Interestingly, this exfoliation-implantation model of tumorigenesis shares striking similarities with the process of intra-peritoneal dissemination (Fig. 1.2B), the predominant means of metastasis in ovarian cancer.

1.2.4 Pathobiology of Epithelial Ovarian Cancer Metastasis

Metastasis has traditionally been defined as the spread of cancer cells from the primary site to form new tumours in distant organs, requiring transit through the circulatory or lymphatic systems. However, in EOC, traditional blood-borne metastasis is uncommon. Instead, dissemination occurs primarily by an intra-peritoneal (IP) route whereby tumour tissue spreads throughout the abdominal/peritoneal cavity (recently reviewed by Sodek and colleagues). Associated with IP metastasis is the production of ascites, a fluid accumulation in the peritoneal cavity seen in ~75% of advanced-stage ovarian cancers. It is etiologically linked to increased filtration from peritoneal capillaries (mediated in large part by tumour-secreted vascular endothelial growth factor, or VEGF) and impaired drainage through peritoneal lymphatics (caused by physical blockage of lymphatic drainage ducts by tumour tissue). Large quantities (litres) of ascites fluid serve not only as a source of significant discomfort for patients, but also create a means of dispersal for metastasizing cells.

Exfoliated tumour cells suspended in ascites fluid are readily detectable and are sometimes collected for diagnostic cytology. They are found as solitary cells and as multicellular aggregates or “spheroids” (Fig. 1.2B). Isolation and analysis of spheroids was reported in 1987 by Allen and colleagues, who described them as compact to loosely-adherent clusters of variable size and morphology. Since this initial report, ovarian cancer spheroids have been isolated and analyzed by several groups. Moreover, in vitro model systems have also been developed to facilitate more detailed investigation
Figure 1.2: High-grade serous ovarian cancers exhibit similarities in their mechanisms of tumorigenesis and intra-peritoneal metastasis.

(A) High-grade serous ovarian tumours originate in the fimbrial epithelium as “p53 signatures” comprised of normal secretory cells. These progress to tubal intraepithelial carcinomas (TICs) through acquisition of additional genetic “hits”. TIC cells exfoliate and implant on nearby ovarian, uterine, or peritoneal surfaces, giving rise to high-grade serous carcinomas. (B) Cells again undergo exfoliation from established tumors. Suspended in ascites, single-cells or multicellular aggregates (spheroids) disperse throughout the peritoneal cavity. Re-attachment to peritoneal surfaces leads to secondary tumour formation, and ultimately, widespread intra-peritoneal disease (carcinomatosis).
of their biology\textsuperscript{69-72}. Thus far, the study of spheroids has focused on their mechanism of formation and their ability to re-attach to a substratum (reviewed by Shield \textit{et al.}\textsuperscript{73}). Aggregation of cells to form spheroids is mediated through extracellular matrix (ECM) proteins and the cell-surface receptors that bind them (integrin heterodimers and CD44): function-blocking antibodies against these receptors impair aggregate formation\textsuperscript{74,75}, whereas incubation with soluble matrix proteins promotes aggregation\textsuperscript{75}. Once formed, ascites spheroids maintain a propensity for re-attachment to ECM components as well as mesothelial cell monolayers\textsuperscript{71,72,76} that was shown to be integrin-dependent\textsuperscript{77}. Sodek \textit{et al.} demonstrated that compact spheroids possess invasive properties and rely on matrix metalloproteases to breach collagen I matrices\textsuperscript{78,79}. Recent work from the Brugge lab has also demonstrated that once attached, EOC spheroids use myosin-generated force for mechanical displacement of mesothelial cells, allowing them to gain access to the sub-mesothelial ECM for subsequent invasion\textsuperscript{69}.

These reports have taken the first steps in understanding spheroid formation and re-attachment, but important questions about spheroid pathobiology remain. For instance, various stresses are associated with cell exfoliation, like the loss of cell-cell and extracellular matrix (ECM) attachment [e.g., accumulation of reactive oxygen species (ROS) and a sharp drop in ATP levels\textsuperscript{80}], which leads to the eventual induction of detachment-mediated apoptosis (anoikis)\textsuperscript{81,82}. It will be important to determine whether spheroids represent a means by which exfoliated tumour cells mitigate some of these cellular stresses, allowing their survival and subsequent seeding of metastatic lesions.

Since an exfoliation-implantation process has been described as a necessary early step in high-grade serous tumorigenesis, cellular mechanisms that promote tumour cell survival upon detachment may be ‘hard-wired’ from early on, equipping cells to not only establish primary tumours, but to also survive the challenges of intra-peritoneal metastasis. Therefore, investigating how these cells subsist under conditions of detachment – in other words, understanding the pathobiology of spheroids – should uncover key metastasis-promoting mechanisms.
1.2.5 Lethality of High-Grade Serous (HGS) Ovarian Cancer Metastasis

It is estimated that 225,500 women worldwide\textsuperscript{83}, including 22,240 in the United States\textsuperscript{84} and 2,600 in Canada\textsuperscript{85}, were diagnosed with ovarian cancer in 2012. Although not among the most prevalent, this disease has the third-highest death-to-incidence ratio of all cancers, exceeded only by lung and pancreatic cancer\textsuperscript{83-85}. Of these deaths, nearly 70\% occurred in patients with HGS cancer\textsuperscript{3,5,42}. Moreover, since HGS cancers are Type II malignancies, the majority of patients (~61\%) present with early and extensive intraperitoneal metastasis (Stage III-IV) – these patients experience a 5-year relative survival rate of only 27.3\%\textsuperscript{86}. Synthesizing this information, it is clear that the vast majority of ovarian cancer-associated deaths occur among patients with HGS, particularly those with metastatic disease. Therefore, a critical need exists to better understand the metastatic process of HGS tumours since this is a direct contributor to the high rate of mortality associated with this disease.

1.2.6 Treatment of Ovarian Cancer

Although several improvements have been made in the treatment of ovarian cancer, its 5-year relative survival rate has climbed from 36\% in 1977 to 44\% in 2009\textsuperscript{86}. Cytoreductive surgery in patients with metastatic ovarian cancer is critical to dramatically reducing tumour burden\textsuperscript{87} and optimal surgical de-bulking (<1.5-2cm of remaining macroscopic disease) is associated with increased overall survival\textsuperscript{88}. Chemotherapy has been utilized since the 1960s, but the introduction of platinum agents, their combination with taxanes\textsuperscript{89}, and the switch from cisplatin to carboplatin\textsuperscript{90} have all made incremental improvements in survival. In addition to chemotherapeutics, agents targeting VEGF (vascular endothelial growth factor; for example, Bevacizumab) have also shown promise for increasing progression-free survival\textsuperscript{91,92}. Furthermore, the poly(ADP)ribose polymerase (PARP) inhibitor Olaparib demonstrated increased progression-free survival when used simultaneously with carboplatin-taxol and when continued as maintenance therapy\textsuperscript{93}. Currently, clinical trials for numerous targeted agents are underway in ovarian
cancer, including those targeting members of the PI3K/AKT/mTOR (Phosphatidylinositol-3-Kinase/AKT/mechanistic Target of Rapamycin) signaling pathway. Despite these initially effective treatments, the aggressiveness of metastatic disease and its propensity for recurrence remain major obstacles to improving long-term survival\textsuperscript{6,87}. Therefore, substantial need still exists for novel therapeutic strategies – preferably those targeting mechanisms uniquely-vital for metastatic and recurrent disease.

1.2.7 Summary

Ovarian tumours can be broadly grouped into non-epithelial and epithelial neoplasms. Almost all malignant tumours are epithelial in histology, yet the development of these tumours follows two divergent paths, each involving unique genetic aberrations and different originating cell types. The Type II pathway yields high-grade cancers that metastasize early in their course by virtue of exfoliated cells and spheroids suspended in ascites fluid. These metastatic, high-grade serous cancers are unfortunately the most prevalent and most responsible for the high mortality associated with advanced-stage disease. Therefore, an improved understanding of the metastatic process is essential to more effectively treat women suffering from this disease.

1.3 PI3K/AKT/mTOR Signaling in Ovarian Cancer

1.3.1 Overview

Intra-cellular signaling can exert control over nearly all aspects of a cell’s behaviour. The PI3K/AKT/mTOR pathway is an essential controller of protein translation, cell growth, proliferation, and survival. Members of this pathway are proto-oncogenes, as their activities are commonly upregulated in cancer and contribute to most of Hanahan and Weinberg’s original “Hallmarks of Cancer”\textsuperscript{94}. This section will describe
how the PI3K/AKT/mTOR pathway becomes activated, how it contributes to oncogenesis, and the upregulation of its members in high-grade serous ovarian cancer.

1.3.2 Pathway Activation

1.3.2.1 Phosphatidylinositol-3-Kinases (PI3K)

The first PI3 kinase was identified in 1985 by Cantley and colleagues for its ability to phosphorylate the inositol rings of membrane phospholipids (phosphatidylinositols) at their 3’-OH position\(^9^5\). Further studies revealed the existence of numerous PI3 kinases grouped into three classes: Class I (A & B), II, and III. Class IA PI3K is most associated with downstream AKT/mTOR signaling\(^9^6\), whereas Class IB is more often associated with small G-protein (e.g., Ras) and G-protein coupled receptor signaling\(^9^7\). Furthermore, out of all the PI3Ks, mutations in the Class IA genes \textit{PIK3CA} (encoding the Class IA p110\(^\alpha\) catalytic subunit)\(^2^6\) and \textit{PIK3R1} (encoding the Class IA p85 regulatory subunit)\(^9^8\) are associated with cancer.

In its inactive state, Class IA PI3K exists in the cytosol as a heterodimer of regulatory (p85) and catalytic (p110) subunits. This heterodimer must interact with trans-phosphorylated tyrosine residues on the cytoplasmic tails of activated Receptor Tyrosine Kinases (RTKs) to become catalytically active\(^9^9\). It does so using the Src homology 2 (SH2) domain of p85 either directly or through adaptor proteins like Insulin Receptor Substrate 1 (IRS1)\(^1^0^0\). RTK interaction recruits PI3K to the inner leaflet of the plasma membrane, relieves p85-mediated inhibition of the p110 catalytic subunit\(^1^0^1\), and brings the kinase into close proximity with its lipid substrate phosphatidylinositol-4,5-bisphosphate [PI(4,5)P\(_2\)]. It should be noted that p110 can also associate with the Ras oncprotein (via its Ras-binding domain) to promote catalytic activity, thereby using oncogenic Ras signaling to drive PI3K/AKT/mTOR activity\(^1^0^2\). Once activated, PI3K then catalyzes the conversion of PI(4,5)P\(_2\) to phosphatidylinositol-3,4,5-trisphosphate.
[PI(3,4,5)P₃], which acts as a membrane-docking site for kinases containing plexstrin-homology (PH) domains¹⁰³ (Fig. 1.3).

1.3.2.2   AKT Kinases

The AKT kinases are the central PH-domain containing proteins that are activated downstream of PI(3,4,5)P₃ production. These kinases are cellular homologues of the viral oncogene v-akt¹⁰⁴ discovered by Staal and colleagues in 1987¹⁰⁵-¹⁰⁷. AKT kinases 1, 2, and 3 are encoded by separate genes, each with its own tissue-specific pattern of expression: AKT1 is ubiquitously expressed¹⁰⁸, AKT2 is expressed in insulin-sensitive tissues (liver, muscle, adipose) as it is important in regulating glucose uptake¹⁰⁹, and AKT3 is expressed primarily in brain and testes¹¹⁰. All the kinases share the same domain structure consisting of an N-terminal PH domain, a linker region, a kinase domain, and a hydrophobic motif¹⁰³.

To become activated, the AKT kinases are first recruited to the plasma membrane through interaction of their PH domains with PI(3,4,5)P₃¹⁰³ which has been generated by active PI3K. PH-mediated membrane recruitment relaxes the conformation of AKT, allowing two subsequent phosphorylation events necessary for its activation¹¹¹. The first phosphorylation of AKT1/2/3 occurs on Ser473/474/472 of the C-terminal hydrophobic motif and is deposited by mTOR complex 2 (mTORC2)¹¹². Subsequently, phosphorylation on Thr308/309/305 in the kinase domain activation loop (T-loop) is achieved by 3'-phosphoinositide-dependent kinase 1 (PDK1), which has already been recruited to the membrane due to the higher affinity of its PH domain for PI(3,4,5)P₃¹¹³. T-loop phosphorylation is thought to depend upon phosphorylation of the hydrophobic motif since this motif interacts with PDK1, stabilizing and bringing the kinase into close proximity with AKT for efficient phosphorylation¹¹⁴,¹¹⁵ (Fig. 1.3). Importantly, both of these phosphorylation events are necessary for maximal AKT activation⁹₆,¹¹₂,¹¹⁴. Once active, AKT goes on to phosphorylate multiple substrates, some
Figure 1.3: Activation of the PI3K/AKT/mTOR Pathway. 

Activation of Receptor Tyrosine Kinases (RTKs) recruits Phosphatidylinositol-3-Kinase (PI3K) to the membrane to activate its catalytic activity. PI3K generates phosphatidylinositol-3,4,5-trisphosphate \([\text{PI}(3,4,5)\text{P}_3]\) which acts as a membrane-docking site for the AKT kinases. AKT is recruited to the membrane and activated by phosphorylation on Ser473 by the mechanistic Target of Rapamycin Complex 2 (mTORC2), and on Thr308 by 3' phosphoinositide-dependent kinase 1 (PDK1). AKT phosphorylates and de-stabilizes TSC1/2, promoting activation of Rheb (Ras homolog enriched in brain). GTP-Rheb activates mTORC1, provided that it is localized to the lysosomal membrane in an amino-acid-dependent manner. mTORC1 phosphorylates ribosomal protein S6 kinase 1 (p70S6K) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). This pathway can be attenuated by the Phosphatase and Tensin Homologue (PTEN), which reverses \([\text{PI}(3,4,5)\text{P}_3]\) production, and the LKB1/AMPK (liver kinase B1/AMP-activated protein kinase) pathway, which inhibits mTORC1.
of which are critical in regulating the activity of mTOR.

1.3.2.3 *Mechanistic Target of Rapamycin (mTOR) Kinase Complexes*

mTOR kinase was named for its role as the mechanistic (formerly mammalian) target of Rapamycin, an anti-fungal agent produced by the *Streptomyces hygroscopicus* bacteria discovered on Easter Island, or Rapa Nui, in 1975\textsuperscript{116}. In mammalian cells, mTOR exists in two large kinases complexes: mTORC1 and mTORC2. mTOR complex 2 (mTORC2) has already been mentioned, as it functions upstream of AKT to directly phosphorylate and promote its activation\textsuperscript{112}. The upstream regulation of mTORC2 remains under investigation, but appears to be controlled by PI3K signaling in a ribosome-dependent manner\textsuperscript{117}.

In contrast to mTORC2, the activation of mTORC1 is more thoroughly characterized (Fig. 1.3). Much of its regulation converges upon the Tuberous Sclerosis Complex, a heterodimer of TSC1 (Hamartin) and TSC2 (Tuberin). TSC1/2 negatively regulates mTORC1 by converting the GTPase Rheb (Ras homolog enriched in brain) to its inactive GDP-bound state \textsuperscript{118}. AKT directly phosphorylates TSC\textsubscript{2} \textsuperscript{119}, destabilizing the TSC1/2 complex leading to Rheb-mediated mTORC1 activation\textsuperscript{120}. AKT also promotes mTORC1 activation independently of TSC1/2 through the phosphorylation of proline-rich AKT substrate of 40kDa (PRAS40), preventing it from binding and sequestering the mTORC1 subunit Raptor\textsuperscript{121}. Recent work has also revealed that for mTORC1 to be activated by these signals, it must translocate to the lysosomal membrane, a process that is dependent upon amino acid abundance and facilitated by Rag GTPase/Ragulator interactions \textsuperscript{122,123}. Thus, mTORC1 is activated by upstream AKT activity, but its regulation is also closely tied to nutrient availability.

Once activated, mTORC1 phosphorylates several proteins, but its two canonical targets are the ribosomal protein S6 kinase 1 (p70S6K) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) \textsuperscript{124}. Phosphorylation of p70S6K promotes
its activation, and in turn, the assembly of factors involved in protein translation\textsuperscript{125}. Phosphorylation of 4E-BP1 prevents it from binding and sequestering the eukaryotic translation initiation factor 4E (eIF4E), freeing this protein to effect further complex assembly at the 5’-cap of mRNA\textsuperscript{126}. Through phosphorylation of these and several other targets, mTORC1 executes its role as a key mediator of protein translation.

### 1.3.2.4 Pathway Attenuation

The pathway members discussed above can also be de-activated by inhibitory feedback mechanisms. One key mechanism is the reversal of second-messenger production that is achieved by the phosphatase and tensin homologue (PTEN). This lipid phosphatase catalyzes the conversion of PI(3,4,5)P\textsubscript{3} back to PI(4,5)P\textsubscript{2}\textsuperscript{127}, reducing the available docking sites for PH-domain-containing kinases like AKT, and in doing so, attenuating all downstream pathway activity\textsuperscript{128} (Fig. 1.3). Aside from lipid phosphatases, the PHLPP1 and PHLPP2 (PH-domain and Leucine rich repeat protein phosphatases) can also attenuate pathway activity by directly de-phosphorylating AKT\textsuperscript{129} and p70S6K\textsuperscript{130}. Other mechanisms of pathway attenuation downstream of AKT do not involve phosphatases: one important example is signaling through LKB1/AMPK (liver kinase B1/AMP-activated protein kinase). AMPK is activated by LKB1 in response to an elevated cellular AMP:ATP ratio (i.e., energy depletion conditions), depositing an activating phosphorylation on TSC2 to promote TSC1/2 activity and inhibit mTORC1\textsuperscript{118}. Therefore, multiple mechanisms exist for negatively regulating this pathway, both upstream and downstream of AKT.

### 1.3.3 Consequences of PI3K/AKT/mTORC1 Pathway Activation

The activation of this pathway in cancer contributes to most of Hanahan and Weinberg’s original *Hallmarks of Cancer*\textsuperscript{94} (Fig 1.4). Through amplification, mutation, and overexpression of RTKs and their ligands\textsuperscript{131-135}, autocrine signaling loops are established to constitutively activate this pathway, thus providing *self-sufficiency in*
Figure 1.4: Cellular Activities Regulated by PI3K/AKT/mTOR Signaling. AKT or mTORC1 phosphorylate the indicated targets to facilitate their activation (arrow) or inhibition (blocking arrow). Targets include the cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1, Hypoxia-Inducible Factor 1 & 2 alpha (HIF1α & HIF2α), Bcl-2 Homology Domain 3 (BH3)-only pro-apoptotic proteins, Forkhead Box O (FOXO) transcription factors, Matrix Metalloproteinases (MMP), Sterol Regulatory Element-Binding Protein 1 or 2 (SREBP1/2), the ribosomal protein S6 kinase 1 (p70S6K), and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). The modulation of these targets leads to many of the *Hallmarks of Cancer* (Hanahan and Weinberg, 2000), as indicated by color-coded oval rings.
growth signals. The phosphorylation of cyclin-dependent kinase (CDK) inhibitors p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} by AKT kinases leads to their cytoplasmic sequestration, allowing nuclear cyclin/CDK complexes to signal cell cycle progression in a dysregulated fashion\textsuperscript{136,137}. To fuel this rapid cycling, high levels of mTORC1 activity promote protein translation\textsuperscript{125,126,138}, lipid biosynthesis\textsuperscript{139}, and energy production\textsuperscript{139}. Therefore, upregulated activity of both AKT and mTORC1 drives unrestrained proliferation, endowing cancer cells with insensitivity to anti-growth signals. AKT/mTORC1 signaling also promotes angiogenesis by activating pro-angiogenic genes (e.g., VEGF) in a hypoxia-inducible factor alpha (HIF1\textalpha{} & HIF2\textalpha{})-dependent manner\textsuperscript{140}. AKT also negatively regulates the function and expression of Bcl-2 homology domain 3 (BH3)-only pro-apoptotic proteins (e.g., BAD, BIM) through direct phosphorylation\textsuperscript{141,142}, or by phosphorylation of Forkhead Box O (FOXO) transcription factors to prevent their expression\textsuperscript{143}. In so doing, AKT prevents these pro-apoptotic genes from being transcribed and their protein products from inducing cell death, ultimately facilitating evasion of apoptosis. Finally, AKT kinase overexpression also results in increased adhesion, invasion, and migration\textsuperscript{144}, properties that promote tissue invasion and metastasis. Therefore, given it’s ability to endow tumour cells with many of the Hallmarks of Cancer, the PI3K/AKT/mTOR pathway is clearly a driver of oncogenesis.

1.3.4 Aberrant Pathway Activation in High-Grade Serous (HGS) Ovarian Cancer

The PI3K/AKT/mTOR pathway exhibits oncogenic activation in numerous cancers including ovarian. In HGS ovarian cancer, although mutations in pathway members are rare (Table 1.1), gene copy-number alterations are more common. Upstream of PI3K activation, alterations in RTKs that directly activate PI3K have been reported. The ErbB family of RTKs is an important example since its members, namely EGFR (epidermal growth factor receptor) and ERBB2 (Human Epidermal Growth Factor Receptor 2; HER2/Neu) are amplified in a subset of HGS ovarian cancers\textsuperscript{42,134,145,146}. ERBB3 also exhibits increased activity in ascites-derived tumour cells from HGS
patients\textsuperscript{131}. In addition, the platelet-derived growth factor receptors (PDGFRs) are overexpressed\textsuperscript{133} and exhibit autocrine activation\textsuperscript{132} in serous cancers. In fact, their ligand (PDGF) was initially discovered due to its abundance in malignant ascites from ovarian cancer patients\textsuperscript{135}.

Downstream of RTKs, PI3K is also altered in HGS cancers: amplification/copy-number increases are relatively common\textsuperscript{25,26,147}, as is increased PI3K activity as determined by \textit{in vitro} kinase assays\textsuperscript{148}. The AKT kinases also exhibit alteration in HGS cancers. One of the first ever reports of AKT2 gene alteration described its amplification and overexpression in ovarian cancer cell lines and tumour specimens\textsuperscript{149}. Additionally, copy number increases and/or mRNA over-expression of all three AKT genes\textsuperscript{25,150-152}, elevated phospho-AKT (representing activation of all three AKT kinases)\textsuperscript{145,146,153}, and elevated AKT2 activity (based on \textit{in vitro} kinase assays)\textsuperscript{148} are observed in HGS tumours.

Downstream of AKT, mTORC1 signaling also exhibits increased activity. Although not mutated or amplified, the canonical mTORC1-targets p70S6K and 4E-BP1 exhibit hyper-phosphorylation\textsuperscript{154,155}. Finally, the deletion of phosphatases that attenuate pathway activation are also found in HGS tumours. Specifically, the lipid phosphatase PTEN (which was initially discovered due to its abrogation in cancer\textsuperscript{156}) is deleted in a subset of HGS tumours\textsuperscript{28,29,152}, as are the AKT- and p70S6K-inactivating protein phosphatases (PHLPPs).

In addition to published studies, an assessment of publicly-available genomics data also reveals activating alterations in several PI3K/AKT/mTOR pathway members. The Cancer Genome Atlas (TCGA) Project continually collects hundreds of tumour specimens from multiple disease sites to perform cross-platform genomic analysis. These data are constantly being generated as samples are acquired and processed, thus representing the most current and comprehensive resource for cancer genomics that is also publicly available. Using TCGA datasets from 538 patients with high-grade serous ovarian tumours, a visual “OncoPrint” diagram\textsuperscript{157} was generated to depict amplification, deletion, mRNA expression and mutation of all PI3K/AKT/mTOR pathway members.
previously discussed (Fig. 1.5). These data suggest that activation of this pathway – suggested by alteration of any one of these genes – is observed in 404/538 (75%) of HGS ovarian cancers.

1.3.5 Summary

Activation of the PI3K/AKT/mTOR pathway is initiated by RTKs that stimulate the lipid kinase PI3K to generate second messenger phospholipids. These recruit AKT to the membrane, activating it and the downstream kinase complex mTORC1. The activity of these effector kinases promotes cell motility/invasion, angiogenesis, proliferation, as well as increased protein translation and lipid biosynthesis – all of which are cellular activities fueling oncogenesis. In HGS ovarian cancer, amplification of RTK genes, PIK3CA, and AKT genes, as well as deletion of PTEN, is prevalent. Therefore, the PI3K/AKT/mTOR pathway is a key driver of oncogenesis that is hyper-activated in a large proportion of HGS ovarian tumours.

1.4 Quiescence

1.4.1 Overview

Cellular quiescence is defined as a reversible exit from the cell division cycle. This section will provide a brief overview of the cell division cycle and its regulation followed by a discussion of the molecular mediators of quiescence, regulatory mechanisms governing its maintenance, and finally, the relevance of quiescence to cancer biology and therapy.
Figure 1.5: Alteration of the PI3K/Akt/mTOR Pathway in High-Grade Serous Ovarian Cancer.
Using datasets from The Cancer Genome Atlas (TCGA) Project, 538 high-grade serous ovarian cystadenocarcinoma tumour samples were profiled for mRNA expression (Agilent G4502A_07 microarray; data transformed to z-scores) and Gene Copy Number (Affymetrix Genome-Wide Human SNP Array 6.0; data processed by GISTIC2 to yield gene-level copy-number calls). Memorial Sloan-Kettering Cancer Center’s cBio Portal for Cancer Genomics (http://www.cbioportal.org/) was used to generate a visual “OncoPrint”. Each patient tumour sample (single vertical line), can exhibit any of the following: no alteration (grey), mutation (green), amplification (red), deletion (blue), mRNA over-/under-expression (>2 z-score; red or blue outline).
Figure 1.5: Alteration of the PI3K/Akt/mTOR Pathway in High-Grade Serous Ovarian Cancer.

1.4.2 The cell division cycle

The process of cellular reproduction is collectively known as the cell division cycle. In the most basic sense, this consists of a synthesis (S) phase in which DNA is replicated and a mitotic (M) phase in which nuclear contents and cytoplasm are divided between two daughter cells\textsuperscript{158}. Indeed in early embryos, the cell division cycle is comprised exclusively of these two phases, with cells rapidly copying their DNA and dividing\textsuperscript{159}. However, once a multicellular embryo develops, two additional “gap” phases arise: gap phase 2 (G2) allows time for the repair of DNA damage and replication errors following S-phase, while gap phase 1 (G1) is a critical pause where the cell decides whether another round of replication will ensue or whether it will undergo terminal differentiation or apoptosis\textsuperscript{158}. Alternatively, cells may exit the cell cycle temporarily (quiescence)\textsuperscript{159} or permanently (senescence)\textsuperscript{160}. The inputs influencing this decision are complex and numerous, involving various environmental cues and signaling pathways\textsuperscript{158}. However, most of these inputs converge upon a set of key cell cycle regulators: the pocket protein family members p107, p130, and pRB (Retinoblastoma protein)\textsuperscript{161} (Fig. 1.6).

1.4.3 Regulation of the cell division cycle

The pocket proteins interact with the E2F-family of transcription factors to exercise control over cell cycle progression. p107 and pRB are predominantly expressed during the cell division cycle\textsuperscript{161}. In contrast, p130 expression is negligible in cycling cells, but is highly expressed during quiescence\textsuperscript{162}. In their hypo-phosphorylated state, both pRB and p107 bind to E2Fs, a family of transcription factors that transactivate the expression of genes required for cell cycle progression\textsuperscript{161,163}. Binding obscures the transactivation domains of E2Fs\textsuperscript{164,165}, thus preventing the transcriptional activation of S-phase genes such as cyclin E and c-myc\textsuperscript{166,167}. When bound to E2Fs at their target gene
promoters, pRB and p107 also recruit chromatin-remodeling factors such as histone deacetylases (HDACs) that induce a compact chromatin structure and make E2F target

Figure 1.6: The cell division cycle and regulation of quiescence.
The cell division cycle consists of a synthesis (S) phase and mitotic (M) phase separated by gap (G) phases G1 and G2. (A) In early to late G1 phase, cyclin-dependent kinase (CDK) complexes are low in abundance or bound by repressive CDK inhibitors (CKIs). The Retinoblastoma protein (pRB) and p107 remain hypo-phosphorylated, repressing the transcription of E2F-target genes via E2F binding and recruitment of histone deacetylases (HDACs). (B) To enter S-phase, CKIs are destabilized and degraded, allowing CDK complex-mediated phosphorylation of pRB and p107. They dissociate, freeing E2Fs to recruit histone acetyltransferases (HAT) and induce the
expression of target genes. (C) In quiescent (G0) cells, p130 is the most abundant RB family member, forming repressive DREAM (DP, RBL2/p130, E2F4, and MuvB) complexes at hundreds of E2F target gene promoters. The CKI p27<sup>Kip1</sup> is also predominantly expressed in quiescent cells, repressing the activity of any CDK complexes. Genes less accessible for transcription<sup>168,169</sup>. Therefore, through the actions of hypo-phosphorylated p107 and pRB, cells remain arrested in G1 and are prevented from transitioning into S-phase (Fig. 1.6A).

In contrast, when hyper-phosphorylated, pRB and p107 are unable to bind and sequester their respective E2Fs, freeing them to bind target gene promoters, recruit histone acetyltransferases (HATs) to loosen chromatin structure, and subsequently transactivate the expression of genes for cell cycle progression<sup>161,170</sup>. Complexes of Cyclin Dependent Kinases (CDKs) and their activating Cyclins are responsible for pRB and p107 phosphorylation<sup>158,171</sup>. During G1 phase, Cyclin D-CDK4/6 complexes phosphorylate and partially inactivate p107 and pRB<sup>171-173</sup>. As a consequence, E-type Cyclin genes (which themselves are E2F-targets) are expressed and their protein products go on to bind and activate CDK2. Newly-formed Cyclin E-CDK2 complexes then initiate a feed-forward loop that results in hyper-phosphorylation and complete inactivation of pRB and p107, thereby freeing the E2Fs to execute a G1-to-S transition<sup>171-173</sup> (Fig. 1.6B).

In addition to regulation by Cyclins, CDK activity can also be controlled by two families of cyclin-dependent kinase inhibitors (CKIs): the INK4 and CIP/KIP proteins<sup>174</sup>. INK4 proteins (p15<sup>INK4a</sup>, p16<sup>INK4b</sup>, p18<sup>INK4c</sup>, p19<sup>INK4d</sup>) all selectively bind to CDK4 and CDK6, preventing their association with D-type cyclins and thereby inactivating Cyclin D–CDK4/6 complexes<sup>175</sup>. In contrast, CIP/KIP inhibitors (p27<sup>Kip1</sup>, p57<sup>Kip2</sup>, and p21<sup>Cip1</sup>) bind both the substrate-interacting domain of cyclins and the catalytic domain of CDKs to prevent ATP entry, thereby inhibiting all Cyclin-CDK complexes<sup>174,176</sup>. By inhibiting Cyclin-CDK activity, CKIs prevent phosphorylation and inactivation of pRB and p107, halting cell cycle progression (Fig. 1.6A). Upon mitogenic stimulation, however, these CKIs can be phosphorylated by a variety of kinases, resulting in their ubiquitination and degradation and the subsequent liberation of Cyclin-CDK complexes<sup>171,174</sup> (Fig. 1.6B).
1.4.4 Quiescence: exit from the cell division cycle

Cells that have exited the cell division cycle and are not actively proliferating are said to be quiescent, or in a G0 state. Quiescence can be induced by growth factor and/or nutrient depletion as well as contact inhibition. At the molecular level, quiescence harbors unique features that distinguish it from G1-phase arrest. For instance, quiescent cells exhibit reduced RNA content, as they significantly decrease the transcription of genes encoding ribosomal and translational RNA\(^{177,178}\). Quiescent cells are also differentiated by their predominant expression of p130 in favor of pRB and p107 – in quiescent cells, p130 is the most abundant of the three pocket proteins\(^{161,162}\).

The accumulation of p130 is not simply a marker of quiescence, but an essential mediator of the G0 state. During quiescence, abundant p130 protein complexes with E2F4 to repress E2F target genes and execute cell cycle exit\(^{162,179-182}\). Recently, Litovchick \textit{et al.} demonstrated that p130, but not pRB or p107, forms repressive DREAM complexes (\(\text{DP1/2, RB-like/p130, E2F4/5, and MuvB}\)) at the promoters of over 800 E2F target genes involved in cell cycle progression, mitochondrial biogenesis, and metabolism\(^ {183,184}\) (Fig. 1.6C). DREAM occupancy was necessary to repress transactivation of these genes, as depletion of several complex members led to their increased expression. Moreover, overexpression of DREAM complex members (or overexpression of p130 alone) was sufficient to retain cells in G0 despite mitogenic stimuli\(^ {183}\). Therefore, p130 is an abundant and essential mediator of the G0 state.

In addition to p130, establishment and maintenance of quiescence also requires high levels of the CKI p27\(^{Kip1}\)\(^ {174}\) (Fig. 1.6C). Like p130, p27\(^{Kip1}\) is highly expressed in quiescent cells\(^ {185-187}\). It has been implicated as an essential mediator of quiescence as its deletion impairs cell cycle exit\(^ {188,189}\), whereas its over-expression can induce growth-factor insensitivity and a G0 state in cancer cells\(^ {190}\). Therefore, in parallel with p130, p27\(^{Kip1}\) is also a major contributor to cellular quiescence.
1.4.5 Regulation of quiescence

1.4.5.1 Glycogen Synthase Kinase 3 (GSK3)

In quiescent cells, p130 accumulates as a result of post-translational modifications, since its mRNA level remains relatively constant. Multiple phosphorylation events are known to govern the activities of pocket proteins, including p130. Like pRB and p107, Cyclin-CDK complexes act on p130 to generate a hyper-phosphorylated species that is incapable of binding E2Fs and is associated with cell cycle progression. Unlike pRB and p107, however, p130 undergoes specific phosphorylation events unique to G0 that are partly responsible for assembly of repressive p130-E2F4 complexes. Interestingly, this quiescence-specific phosphorylation of p130 occurred independently of Cyclin-CDK complexes. Recently, glycogen synthase kinase 3 (GSK3) was identified as a G0-specific p130 kinase. Its activity resulted in the phosphorylation of three residues in the Loop domain of p130 (a non-conserved region that does not share sequence homology with pRB or p107), protecting the protein from proteasome-mediated degradation and thus enhancing its stability. Therefore, by phosphorylating p130, GSK3 contributes to its elevated expression in G0 (Fig. 1.7).

1.4.5.2 AKT Kinases

In addition to GSK3, upstream AKT kinases also control the expression of quiescence-associated proteins. As discussed previously, AKT promotes cell proliferation through activation of the cell cycle machinery and inactivation of its inhibitors. On the other hand, downregulation of AKT activity by nutrient and/or growth-factor depletion has the opposite effect – not only does it stall proliferation, but it also stabilizes proteins critical for establishment of a G0 state. In fact, it is AKT attenuation that permits downstream GSK3 activation and p130 phosphorylation in quiescent cells (discussed above), since AKT kinases are canonical repressors of GSK3. In addition to activating
Figure 1.7: AKT and DYRK1A/B regulate quiescence.
AKT down-regulation de-stabilizes the SCF ubiquitin ligase complex due to under-phosphorylation of SKP2, an essential complex member. As a result, p130 and p27Kip1 (SCF-targets), become stabilized, and accumulate to promote quiescence. AKT also negatively regulates GSK3, p27Kip1, and the FOXO3a/4 transcription factors, thus its down-regulation allows p130 and p27Kip1 to accumulate and promote quiescence. Finally, DYRK1B directly phosphorylates the LIN52 DREAM complex subunit (promoting repressive DREAM complex formation at E2F-target gene promoters) and p27Kip1 (stabilizing and allowing its accumulation), thereby promoting quiescence.
GSK3, AKT attenuation also promotes p130 and p27Kip1 accumulation via the SCF (Skp1/Cul-1/F-box) ubiquitin ligase complex: when inactive, AKT fails to phosphorylate the critical SCF subunit p45/SKP2, causing its destabilization and preventing proper SCF complex formation\textsuperscript{197,198}. This results in stabilization and accumulation of SCF-targets, namely p130\textsuperscript{199,200} and p27Kip1\textsuperscript{188}. AKT attenuation contributes to p130 and p27 expression by a third mechanism involving Forkhead transcription factors FOXO4 and FOXO3a: while AKT activity normally results in their nuclear export, under conditions of AKT attenuation, FOXOs are retained in the nucleus where they transactivate the expression of both p130 and p27Kip1\textsuperscript{202}. Therefore, in cells with reduced AKT signaling, multiple mechanisms drive cell cycle exit by promoting the accumulation of p27Kip1 and p130 (Fig. 1.7).

1.4.5.3 \textit{DYRK Kinases}

In addition to AKT and GSK3, an additional mechanism regulates quiescence via the DYRK kinases. Friedman’s group has pioneered the characterization of DYRK1B in quiescence, first demonstrating its transcriptional downregulation in mitogen-activated cells\textsuperscript{203}. They have also uncovered G0-specific phosphorylation events catalyzed by DYRK1B, specifically phosphorylation of Cyclin D1 leading to its de-stabilization\textsuperscript{204} and phosphorylation of p27Kip1 leading to its accumulation, nuclear localization, and inhibition of CDK2\textsuperscript{205}. Recent work from DeCaprio’s group has also demonstrated that both DYRK1A and DYRK1B directly phosphorylate a key member of the DREAM complex to facilitate its assembly; blocking DYRK activity not only prevented complex formation, but also impaired entry into the G0 state\textsuperscript{206}. Taken together, these findings imply that the DYRK kinases function independently of AKT/GSK3 to actively promote cell cycle exit to a quiescent state (Fig. 1.7).
1.4.6  Quiescence in cancer and therapeutic resistance

Quiescence is highly relevant to cancer biology, since quiescent tumour cells are associated with therapeutic resistance. Most chemotherapeutic agents depend upon rapid proliferation for their activity: for instance, agents such as paclitaxel function by inhibiting mitotic spindle assembly, chromosome segregation, and cytokinesis\textsuperscript{207}. Therefore, quiescent tumour cells are not subject to the primary modes of action of such drugs and accordingly exhibit chemotherapeutic resistance\textsuperscript{208,209}. Additionally, quiescence endows cancer cells with increased survival under nutrient-poor and hypoxic conditions\textsuperscript{186,187}; indeed, an increased fraction of non-cycling (Ki67-negative) cells are observed in under-vascularized regions of ovarian tumours\textsuperscript{210}. Furthermore, quiescence in ovarian cancer cells promoted survival under sub-optimal growth conditions and upon treatment with chemotherapeutics\textsuperscript{185,211}. Consequently, quiescence serves as a survival mechanism contributing to therapeutic resistance in cancer cells.

1.4.7  Summary

Cellular quiescence is more than just cell cycle arrest. Instead, it is a molecularly definable state distinct from the cell division cycle that is actively entered into and maintained. By repressing E2F-target genes and inhibiting Cyclin-CDK complexes, respectively, p130 and p27Kip1 represent the best-known mediators of this state. Through their regulation by AKT, GSK3, and DYRK kinases, quiescence can be induced, maintained, but also reversed upon mitogenic stimulation. Importantly, G0 cells are commonly observed in tumours, representing a barrier to chemotherapeutic efficacy and possibly a mediator of resistance and disease recurrence.
1.5 Autophagy

1.5.1 Overview

Autophagy is a cellular process that holds the potential to sustain cell viability when cancer cells are quiescent or during times of cellular stress and deprivation. The term autophagy is derived from the Greek words ‘auto’, meaning self, and ‘phagy’ meaning to eat. As this name implies, autophagy is a cellular self-digestion mechanism that facilitates the sequestration of cytoplasmic constituents in double-membrane vesicles (autophagosomes) and their delivery to lysosomes for degradation. This section will discuss autophagy’s regulation and execution by autophagy-related (ATG) proteins, its cellular functions, and its importance in cancer.

1.5.2 Discovery of Autophagy and Autophagy-Related (ATG) Genes

Autophagy was discovered in 1957 when Clark & Novikoff, using transmission electron microscopy, first observed mitochondria sequestered within cytoplasmic vesicles. Further studies revealed that these vesicles also contained lysosomal hydrolases, and that organelles within them (mitochondria, endoplasmic reticulum) were partly-digested. The term ‘autophagosome’ subsequently emerged to define a double-membrane vesicle containing cytoplasmic constituents undergoing degradation. This early work was all based on morphological analysis using electron microscopy – the study of autophagy was transformed, however, with the use of yeast genetic screens to identify essential autophagy-associated (ATG) genes.

The Ohsumi group conducted the first of such screens, identifying numerous Atg genes comprising the core molecular machinery responsible for regulating and executing autophagic degradation. Homologs of these genes have since been discovered in higher eukaryotes, including humans, demonstrating the evolutionary conservation of this
process. The core machinery can be divided into three functional groups: (i) the uncoordinated-51-like kinase 1 (ULK1) complex (regulated by mTORC1), (ii) the Class III PI3K (PI3K C3) complex, and (iii) the Atg5-Atg12 and Atg8/LC3 ubiquitin-like conjugation systems. To perform autophagic degradation, the cell must deploy this core molecular machinery in a regulated and coordinated series of steps\textsuperscript{218}, outlined in Figure 1.8.

1.5.3 Molecular Mechanisms of Autophagy

1.5.3.1 \textit{mTORC1 connection to the ULK1/2 Kinase Complex}

In addition to discovering Atg proteins, in 1998 Ohsumi’s group also revealed that yeast TOR kinase is a key autophagy regulator\textsuperscript{219}. Its mammalian homologue, mTORC1, was similarly recognized as an important repressor of autophagy\textsuperscript{220}.

Only recently has mTORC1’s mechanism of autophagy repression been elucidated (Fig. 1.9A). This involves ULK1 or ULK2 (the mammalian homologues of yeast Atg1), either of which can exist in complex with ATG13, FIP200 (focal adhesion kinase family interacting protein of 200kDa), and ATG101 in mammalian cells. This complex localizes to the isolation membrane to promote autophagosome formation\textsuperscript{221-223}, but is under the direct control of mTORC1: when active, mTORC1 associates with the ULK complex\textsuperscript{222}, phosphorylating both ULK1/2 and ATG13 to suppress its catalytic activity at the membrane\textsuperscript{221-223}. However, upon mTORC1 inhibition (e.g., by starvation, Rapamycin treatment, or energy depletion), mTORC1 dissociates, preventing the ULK complex from being repressively phosphorylated. ULK1/2 then phosphorylates residues on both ATG13 and FIP200, promoting catalytic activity of the whole complex and subsequently promoting autophagy\textsuperscript{221-223}. The substrates of ULK1/2 that link its activity to the autophagic machinery have yet to be fully characterized, but a recent report has identified Ambra1 as a direct target whose phosphorylation by ULK1/2 promotes autophagy\textsuperscript{224}. Autophagy is therefore negatively regulated by the interaction of mTORC1
Figure 1.8: Autophagic Degradation.
Autophagy digests cytoplasmic material through a process of membrane sequestration and lysosome-mediated destruction. (A) Vesicle nucleation: isolation membrane thought to arise from the endoplasmic reticulum (ER). (B) Vesicle Elongation: nascent autophagosome lengthens around cytoplasmic material. (C) Vesicle Completion: autophagic membranes completely envelop and seal cargo within mature autophagosomes. (D) Docking & Fusion: mature autophagosome fuses with lysosomes, introducing cargo to lysosomal hydrolases. The autophagosome-lysosome fusion is known as an autophagolysosome (or autolysosome). (E) Degradation & Recovery: Lysosomal enzymes mediate digestion of autophagic cargo. Liberated constituent biomolecules are returned to the cytoplasm via permeases.
Figure 1.9: Regulation of Autophagy Initiation.

(A) mTORC1 can be activated to suppress autophagy or inactivated to induce autophagy by the stimuli listed (center). When active (left), it associates with and phosphorylates ATG13 and ULK1/2 to block catalytic activity of this complex. Upon inactivation (right), mTORC1 dissociates and is unable to conduct these repressive phosphorylation. Thus, ULK1/2 can phosphorylate ATG13 and FIP200, activating the entire complex and promoting autophagy. (B) Formation of the Class III PI3K complex results in its allosteric activation and proper localization to the Isolation Membrane, where the catalytic activity of hVps34 (Class III PI3K) promotes membrane elongation. It also receives regulatory inputs from the ULK complex, through its activating phosphorylation of Ambra1.
with the ULK complex; when mTORC1 dissociates, the resulting ULK complex activation promotes autophagy induction.

### 1.5.3.2 The Class III PI3K Complexes

The Class III PI3K (PI3K C3 or hVps34) is the most conserved of the PI3Ks. Its integral role in vesicle nucleation and expansion of the isolation membrane thus highlights the ancestral and conserved nature of this process. In mammalian cells, PI3K C3 exists in a complex with its regulatory subunit p150, as well as Beclin1 and Atg14L (Fig. 1.9B). Complex formation mediates the allosteric activation of PI3K C3, which then catalyzes the conversion of PI to PI(3)P. Proper complex localization is facilitated by Atg14L, ensuring that formation of PI(3)P occurs at early autophagosomes (isolation membranes) and their endoplasmic reticulum-derived precursors (“omegasomes”). PI(3)P enrichment recruits additional effector proteins important for membrane elongation.

It should also be noted that an alternate PI3K C3 complex exists. This complex retains p150 and Beclin1 as subunits, but replaces Atg14L with UVRAG (UV-radiation resistance associated gene). Although the UVRAG-PI3K C3 complex can promote autophagy, recent research argues for other autophagy-independent functions: specifically, in membrane-trafficking during phagocytosis, endocytosis, and cytokinesis. Nonetheless, the canonical function of PI3K C3 is to direct the early phases of autophagosome formation, which it does in a core complex with p150, Beclin1, and Atg14L.

### 1.5.3.3 The Ubiquitin-Like Conjugation Systems

Two highly-conserved ubiquitin-like conjugation systems are absolutely essential for the continued formation and completion of autophagosomes. These systems...
utilize E1-like, E2-like and E3 ubiquitin ligase-like enzymes for the conjugation of the ubiquitin-like proteins ATG12 and LC3 to their targets\textsuperscript{237} (Fig. 1.10).

The first system, which facilitates the conjugation of ATG12 to ATG5, involves activation and covalent binding of ATG12 to the E1-like enzyme ATG7\textsuperscript{238}, subsequent transfer to the E2-like enzyme ATG10\textsuperscript{239}, and final covalent attachment to its target ATG5\textsuperscript{240}. The ATG5-ATG12 conjugate then associates non-covalently with ATG16L, and through self-oligomerization, forms a multimeric complex of ATG5-ATG12/ATG16L units \textsuperscript{235}. This complex associates with their membranes during the elongation phase but dissociates as the autophagosomes approach completion \textsuperscript{235,241}.

Maturation and completion of autophagosomes also requires the conjugation of MAP1LC3A/B/C (Microtubule-Associated Protein 1 Light Chain 3 A/B/C; a mammalian homologue of yeast Atg8) to the lipid phosphatidylethanolamine (PE). Full-length MAP1LC3A/B/C (LC3) is first cleaved by the cysteine protease ATG4 to expose a C-terminal glycine residue, resulting in the species known as LC3-I\textsuperscript{242}. This residue is the site of covalent attachment to the E1-like enzyme ATG7\textsuperscript{238} and subsequent transfer to the E2-like enzyme ATG3\textsuperscript{243}. The final conjugation of LC3 to PE is performed by the previously-generated ATG5-ATG12 conjugate, which possesses E3 ligase-like activity\textsuperscript{244}. Once LC3 has been conjugated to PE (forming the species ‘LC3-II’), ATG5-ATG12-ATG16L also regulates its proper localization\textsuperscript{245}, ensuring its insertion into both the inner and outer membranes of autophagosomes where it serves as a scaffold supporting membrane expansion and curvature\textsuperscript{237}. Therefore, both ubiquitin-like conjugation systems converge on the membrane-insertion of LC3-II, thereby facilitating the expansion of autophagosomes around their cargo.
Figure 1.10: Regulation of Autophagosome Formation and Maturation.
Two ubiquitin-like conjugation systems are essential for the generation of autophagosomes. The first (left), requires activation and covalent binding of ATG12 to the E1-like enzyme ATG7, subsequent transfer to the E2-like enzyme ATG10, and final covalent attachment to ATG5. The ATG5-ATG12 conjugate then associates with ATG16L, eventually forming a multimeric complex that localizes to nascent autophagosomes. The second conjugation system (right) begins with proteolytic cleavage of ATG8/LC3-I by ATG4, its transfer to ATG7 (E1-like), then to ATG3 (E2-like), and finally – via the previously-generated ATG5-ATG12 conjugate – its ligation to phosphatidylethanolamine (PE) and insertion into the membranes of maturing autophagosomes. This processed form (LC3-II) is essential for autophagic vesicle expansion and completion.
1.5.4 Functions of Autophagy

1.5.4.1 Homeostatic Autophagy

Autophagy is active at a basal level in all eukaryotes and maintains cellular homeostasis by clearing damaged organelles, large protein aggregates, and intracellular pathogens\textsuperscript{227,246-249}. The importance of this cellular quality control function is highlighted by the fact that mice harboring targeted deletions of \textit{Atg5} or \textit{Atg7} accumulated damaged mitochondria and poly-ubiquitinated protein aggregates\textsuperscript{250-253}. These accumulations led to dysfunction and death of mouse neurons and cardiomyocytes, causing progressive neurodegeneration and heart failure, respectively\textsuperscript{250,251,253}. In humans, the deregulation of basal autophagy contributes to similar disease pathologies\textsuperscript{254}. For example, in neurodegenerative disorders such as Parkinson’s disease, the characteristic accumulation of misfolded protein aggregates and dysfunctional mitochondria in neurons is exacerbated by inherited mutations in the \textit{PARK2} gene that impair autophagy\textsuperscript{255}. Similarly, Danon disease (characterized by the accumulation of dysfunctional mitochondria in cardiomyocytes and myocytes) is associated with mutation in \textit{LAMP2}, an autophagy-associated protein important for the docking/fusion of autophagosomes with lysosomes\textsuperscript{256}. Therefore, basal autophagy plays an important role in maintaining cellular homeostasis to prevent disease.

1.5.4.2 Autophagy Induction

In addition to functioning at a basal level to maintain cellular homeostasis, autophagy can also be upregulated as an adaptive response to cellular stresses. Numerous cellular stresses are capable of inducing autophagy, including starvation\textsuperscript{257}, energy depletion\textsuperscript{258}, ECM-detachment\textsuperscript{259}, ER-stress and the Unfolded Protein Response (UPR)\textsuperscript{227}, metabolic stress\textsuperscript{260}, hypoxia\textsuperscript{261}, ammonia\textsuperscript{262}, and reactive oxygen species (ROS)\textsuperscript{263}.
These stimuli utilize a variety of mechanisms to induce autophagy, yet many converge upon mTORC1 and/or AMPK. As discussed above, attenuation of mTORC1 activity liberates the ULK1 complex, thereby triggering autophagy induction. The LKB1/AMPK pathway induces autophagy in an mTORC1-dependent manner by phosphorylating TSC2$^{118}$ and Raptor$^{264}$ to attenuate mTORC1 activity. Recent work has also revealed that AMPK directly phosphorylates ULK1 to promote its activity$^{265}$ and that AMPK activation causes phosphorylation and stabilization of p27$^{Kip1}$ $^{266}$, both of which result in autophagy induction in an mTORC1-independent manner. Although other pathways have been identified, mTORC1 and AMPK represent the best-characterized mechanisms of autophagy induction that integrate various cellular stress signals.

Autophagy induction is a pro-survival mechanism since its targeted disruption in yeast, slime molds, plants, and nematodes consistently reduced their viability under conditions of metabolic stress$^{249}$. Likewise, $\text{Atg}5^{-/-}$, $\text{Atg}7^{-/-}$, $\text{Atg}3^{-/-}$ mice die within 24 hours of birth due to their inability to survive the neonatal starvation period$^{252,267,268}$. To promote survival, autophagy essentially recycles existing cytoplasmic structures (organelles, proteins, membranes), re-directing their constituent biomolecules (nucleosides, sugars, amino-acids, fatty acids) toward biosynthetic or energy-generating pathways$^{227,247,249,269,270}$. This mechanism is supported by studies in which administration of methyl pyruvate (a substrate of the TCA cycle) was sufficient to rescue survival of autophagy-deficient cells exposed to prolonged metabolic stress$^{269,270}$. Thus, autophagy induction by starvation promotes sustained viability by re-purposing intracellular resources and fueling ATP production.

### 1.5.5 Autophagy in Cancer

One of the first diseases to be associated with autophagy was cancer$^{271}$. Following years of study, however, the precise role of autophagy in cancer remains paradoxical, as both tumour-suppressive and tumour-promoting functions have been demonstrated.
1.5.5.1 Autophagy is Tumour-Suppressive

Autophagy is considered a tumour-suppressive mechanism based on evidence from human cancer and mouse models (Table 1.2). The human chromosomal locus 17q21, which contains the Beclin1 gene (BECN1), exhibits single-copy loss in prostate272, breast273,274, and ovarian cancers275-279. Likewise, another PI3K C3 complex member, UVRAG, is mutated in gastric280 and colon281 cancers. Moreover, mice with heterozygous disruption of the Beclin1 gene (Beclin1+/−) develop spontaneous tumours (lymphoma, lung carcinoma, hepatocellular carcinoma) and mammary hyperplasia at higher rates than wild-type littermate282,283. Additionally, the E3 ligase Parkin (PARK2) – which plays an essential role in autophagic degradation of defective mitochondria284 – is also deleted in a subset of breast and ovarian tumours285 and Park2+/− mice develop hepatocellular carcinomas286. Finally, mice harboring liver-specific Atg7 deletion or systemic mosaic deletion of Atg5 develop benign liver adenomas287. Taken together, these key examples provide strong evidence supporting a tumour-suppressive role for autophagy.

The mechanism underlying autophagy’s tumour-suppression, although not yet fully elucidated, appears to involve the maintenance of genomic stability. It has been demonstrated that both Beclin1+/− tumours and Uvrag+/− mouse cells exhibit increased DNA damage, gene amplifications, aneuploidy, and genomic instability in response to metabolic stress260,295,296. Likewise, the benign liver adenomas found in Atg7+/− (liver specific) and Atg5−/− (mosaic) mice were associated with markers of genomic damage287. Genomic instability in autophagy-deficient cells is thought to occur as a result of abnormal accumulation of aggregated proteins and dysfunctional mitochondria – indeed, such structures have been observed in Atg5−/− liver adenomas287 as well as Parkin-deficient cells297,298. These abnormal accumulations lead to ROS production and increased oxidative stress, which in turn can cause DNA damage and ultimately genomic instability260,295,299. Therefore, oxidative stress resulting from autophagy-deficiency can contribute to genomic instability and ultimately tumorigenesis.
### Table 1.2: Selected Mouse Models of Autophagy Disruption

<table>
<thead>
<tr>
<th>Genotype &amp; Phenotype</th>
<th>Conditional Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype &amp; Phenotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Class III PI3K Complex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Becn1</em></td>
<td>Viable; Malignant lymphoma &amp; carcinoma</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td><em>Pik3c3</em></td>
<td>Embryonic lethal</td>
<td>288</td>
</tr>
<tr>
<td><em>Bif1</em></td>
<td>Viable; Malignant lymphoma &amp; carcinoma</td>
<td>289</td>
</tr>
<tr>
<td><em>Ambra1</em></td>
<td>Embryonic lethal</td>
<td>290</td>
</tr>
<tr>
<td><em>Atg4c</em></td>
<td>Viable; Carcinogen-induced fibrosarcomas</td>
<td>291</td>
</tr>
<tr>
<td><strong>Conjugation Systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Atg3</em></td>
<td>Perinatal lethal</td>
<td>267</td>
</tr>
<tr>
<td><em>Atg5</em></td>
<td>Perinatal lethal</td>
<td>Benign liver adenoma</td>
</tr>
<tr>
<td><em>Atg7</em></td>
<td>Perinatal lethal</td>
<td>Benign liver adenoma</td>
</tr>
<tr>
<td><em>Atg161</em></td>
<td>Viable</td>
<td>292</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulk1</em></td>
<td>Viable</td>
<td>293</td>
</tr>
<tr>
<td><em>Park2</em></td>
<td>Viable; Hepatocellular Carcinoma</td>
<td>286</td>
</tr>
<tr>
<td><em>Lamp2</em></td>
<td>Viable</td>
<td>294</td>
</tr>
</tbody>
</table>

*Gene disruption via hypomorphic alleles; b chimeric deletion; cliver-specific deletion*
1.5.5.2  **Autophagy is Tumour-Promoting**

Despite its well-documented suppressive role, autophagy is often upregulated in established tumours, serving to promote cancer cell growth and survival (Fig. 1.11). Autophagy induction in tumours has been demonstrated by elements of their microenvironment (e.g., hypoxia\textsuperscript{261,300} and byproducts of their own dysregulated metabolism (ammonia\textsuperscript{262}, ROS\textsuperscript{301}). A recent immunohistochemical analysis found that elevated expression and punctate staining of LC3B was common across a number of tumour types and was associated with increased proliferation, metastasis, and poor patient outcome\textsuperscript{302}, thus suggesting a tumour-promoting role for autophagy. Indeed, in cultured tumour cells, autophagy upregulation has been shown to promote proliferation\textsuperscript{303,304} and preserve cell survival \textsuperscript{305-308}. Moreover, autophagy disruption impeded growth of Ras-driven xenograft tumours\textsuperscript{309,310} and delayed tumour progression in an established mouse model of breast cancer\textsuperscript{303}.

The tumour-promoting function of autophagy is tied to aberrant tumour metabolism. Tumours often exhibit deregulated metabolism characterized by insufficient oxygen and substrates for energy production, as well as an abundance of metabolic waste products \textsuperscript{311,312}. Akin to its role in starving cells (described above), autophagy feeds these metabolically-hungry tumours by degrading cytosolic components and re-generating substrates needed to fuel the TCA cycle\textsuperscript{303,309,310,313}. Autophagy simultaneously rids tumour cells of metabolic waste products and damaged organelles that would otherwise compound oxidative stress, cause excessive cellular damage and genomic instability, and eventually lead to apoptotic or non-apoptotic cell death\textsuperscript{304,306,314-317}. Therefore autophagy in tumours not only fuels metabolic demands, but also mitigates the cellular collateral damage that results from metabolic dysregulation.

Importantly, autophagy can also be induced by anti-cancer therapies (radiation\textsuperscript{318}, chemotherapy\textsuperscript{319}, and targeted agents\textsuperscript{320}). This therapy-induced autophagy was initially thought to cause type II, or autophagic, cell death – however, careful experimentation has revealed that in many such cases, autophagy only accompanied, rather than caused, cell
Figure 1.11: Autophagy in Cancer.
Autophagy functions to suppress tumorigenesis by limiting accumulation of reactive oxygen species (ROS), DNA damage, and genomic instability. Autophagy also promotes the progression of early malignancies by supplying metabolic substrates to fuel their growth as well as managing various cellular stresses – including those incurred from anti-cancer treatments.
death \textsuperscript{321,322} (i.e., cell death with autophagy, as opposed to by autophagy\textsuperscript{323}). Although some examples of \textit{bona fide} cytotoxic autophagy have withstood scrutiny\textsuperscript{324,325}, it is now accepted that therapy-induced autophagy instead promotes cell survival in most instances, since its blockade leads to increased cell death\textsuperscript{307,308,319,320,326-328}. Therefore, autophagy predominantly serves as a cytoprotective mechanism in cancer cells exposed to therapeutics.

\subsection*{1.5.6 Autophagy in Ovarian Cancer}

Ovarian cancer typifies the paradoxical nature of autophagy’s role in cancer. As mentioned, the \textit{BECN1}-containing chromosomal locus exhibits monoallelic deletion in 65-87\% of ovarian tumours\textsuperscript{275-279}. Recent work has also revealed that Beclin1 protein expression is reduced in malignant tumours compared to normal ovarian tissue\textsuperscript{329}, though it remains to be determined whether protein expression is actually reduced in ovarian tumours as a result of heterozygous loss at the \textit{BECN1} locus.

Aside from Beclin1, a very recent study by Kenzelmann \textit{et al.} discovered that in response to DNA damage, p53 orchestrates an autophagy-stimulating transcriptional program that is important for p53-mediated tumour suppression\textsuperscript{330}. These findings are relevant to ovarian cancer, since early \textit{TP53} mutation is a near-universal phenomenon in high-grade serous tumours. It is thus tempting to speculate that ablating p53-mediated autophagy contributes to the high degree of genomic instability characteristic of high-grade serous ovarian tumours. Therefore, p53-driven, tumour-suppressive autophagy program in ovarian cancer remains a fascinating possibility that will require further investigation.

Despite the literature supporting a tumour-suppressive role for autophagy in ovarian cancer, numerous studies also report that ovarian tumour cells retain the capacity to upregulate autophagy (\textit{Table 1.3}). Most of these studies (66\%; 19/29) demonstrated
Table 1.3: Studies Reporting Autophagy Induction in Ovarian Cancer*

<table>
<thead>
<tr>
<th>Autophagy Inducer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Undetermined Effect of Autophagy Induction</strong></td>
<td></td>
</tr>
<tr>
<td>Mir-30d</td>
<td>335</td>
</tr>
<tr>
<td>siRNA-mediated depletion of spliceosome core machinery</td>
<td>336</td>
</tr>
<tr>
<td>L-asparaginase</td>
<td>337</td>
</tr>
<tr>
<td>Src-inhibitor Saracarinib and estrogen receptor antagonist Fulvestrant</td>
<td>338</td>
</tr>
<tr>
<td>siRNA-mediated depletion of RAB25</td>
<td>339</td>
</tr>
<tr>
<td>mTORC1-inhibitor Everolimus and Arsenic Trioxide</td>
<td>340</td>
</tr>
<tr>
<td>Proteasome inhibitors</td>
<td>341</td>
</tr>
<tr>
<td>Vincristine and/or ectopic expression of mutant p53 (175H)</td>
<td>342</td>
</tr>
<tr>
<td>Dindolylmethane, via ER-stress and AMPK</td>
<td>343</td>
</tr>
<tr>
<td>Human umbilical cord Wharton’s jelly stem cell extracts</td>
<td>344</td>
</tr>
<tr>
<td>Doxorubicin and the steroidal lactone Withaferin A, via ROS</td>
<td>345</td>
</tr>
<tr>
<td>p38 mitogen activated protein kinase inhibitor SB20190</td>
<td>346</td>
</tr>
<tr>
<td>Demethylating agents and/or HDAC inhibitors</td>
<td>347</td>
</tr>
<tr>
<td>Physialis minima L. chloroform extract</td>
<td>348</td>
</tr>
<tr>
<td>HIV protease inhibitor Saquinivir</td>
<td>349</td>
</tr>
<tr>
<td>Anti-Her2/Neu Immunotoxins</td>
<td>350</td>
</tr>
<tr>
<td>Phospho-enriched protein in astrocytes (PEA-15)</td>
<td>351</td>
</tr>
<tr>
<td>Soy-derived isoflavonoid Genestein</td>
<td>352</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>353</td>
</tr>
<tr>
<td><strong>Cytoprotective Effect of Autophagy Induction</strong></td>
<td></td>
</tr>
<tr>
<td>Sphingosine analog and immunosuppressant FTY720</td>
<td>354, 355</td>
</tr>
<tr>
<td>Cisplatin, via Protein Phosphatase 2A (PP2A)</td>
<td>356</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>357</td>
</tr>
<tr>
<td>Cisplatin, via Nucleus Accumbens-1 (NAC-1)</td>
<td>358</td>
</tr>
<tr>
<td>Radiation</td>
<td>359</td>
</tr>
<tr>
<td>Cisplatin via p62/SQSTM1</td>
<td>360</td>
</tr>
<tr>
<td>Arsenic Trioxide</td>
<td>361</td>
</tr>
<tr>
<td>Aplasia Ras homologue member I (ARHI/DIRAS3)</td>
<td>328</td>
</tr>
<tr>
<td><strong>Cytotoxic Effect of Autophagy Induction</strong></td>
<td></td>
</tr>
<tr>
<td>Monofunctional platinum (II) complex (Mono-Pt)</td>
<td>362</td>
</tr>
<tr>
<td>Src-/Abl-kinase inhibitor Dasatinib</td>
<td>363</td>
</tr>
</tbody>
</table>

*Search strategy: Medline search for “autophag* AND ovarian cancer” on April 26 2013 yielded 91 results, which were each examined for evidence of autophagy induction. If the study experimentally assessed the purpose of autophagy induction (e.g., blockade with autophagy inhibitors or siRNA depletion of ATG genes), articles were then classified based on whether autophagy hampered (2 articles) or promoted (8 articles) cell viability. If such experiments were not conducted, articles were classified as “undetermined effect” (19 articles).
autophagy upregulation, but did not assess its effects on cell viability. Of the ten that did, two described autophagy-mediated cell death and eight demonstrated pro-survival autophagy. Therefore, while autophagy clearly remains inducible in ovarian cancer, its function is relatively uncharacterized and would therefore benefit from further study.

1.5.7 Therapeutic Manipulation of Autophagy in Ovarian Cancer

The context-dependent nature of autophagy makes it difficult to modulate therapeutically in ovarian cancer. If acting as a tumour-promoting mechanism, blocking autophagy could deprive ovarian tumours of a key survival advantage, thereby enhancing the efficacy of anti-neoplastic agents. On the other hand, if autophagy is acting as a lethal self-cannibalization mechanism, blocking it could instead blunt therapeutic efficacy. The only autophagy inhibitors currently in clinical use are Chloroquine (CQ) and its derivative Hydroxychloroquine (HCQ), though targeted autophagy inhibitors are in pre-clinical development\textsuperscript{331,332}. One small trial of autophagy blockade (using CQ) in glioblastoma multiforme has yielded promising results\textsuperscript{333}, and additional trials in other tumour types are underway\textsuperscript{334} (Table 1.4). Notably, none of these trials specify the inclusion of ovarian tumours. This may be due, at least in part, to the lack of consensus on autophagy’s role in this cancer. Therefore, further pre-clinical studies are needed to more thoroughly understand autophagy’s role in tumorigenesis, and in doing so, justify autophagy modulation as a therapeutic paradigm. To start, it will be important to investigate autophagy separately in the histologic sub-types of epithelial ovarian cancer given the dramatic pathologic and molecular heterogeneity among them. Furthermore, autophagy will need to be studied using \textit{in vitro} and \textit{in vivo} models that attempt to recapitulate the disease in the most physiologically-relevant context possible. Through such investigations, it will be possible to develop rational strategies for modulating autophagy in hopes of achieving greater therapeutic efficacy and improving outcomes for women who suffer from this disease.
# Table 1.4: Clinical Trials of Autophagy Modulation in Cancer*

<table>
<thead>
<tr>
<th>Trial Identifier</th>
<th>Condition</th>
<th>Intervention(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCQ/CQ(^{A}) Combination Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT00933803</td>
<td>NSCLC(^{B})</td>
<td>Carboplatin, Paclitaxel, HCQ</td>
</tr>
<tr>
<td>NCT01649947</td>
<td>NSCLC(^{B})</td>
<td>Carboplatin, Paclitaxel, HCQ</td>
</tr>
<tr>
<td>NCT01480154</td>
<td>Advanced ST(^{C})</td>
<td>MK-2206, HCQ</td>
</tr>
<tr>
<td>NCT01506973</td>
<td>Pancreatic</td>
<td>Gemcitabine, HCQ</td>
</tr>
<tr>
<td>NCT01206530</td>
<td>CRC(^{D})</td>
<td>FOLFOX, Bevacizumab HCQ</td>
</tr>
<tr>
<td>NCT01006369</td>
<td>mCRC(^{D})</td>
<td>Capecitabine, Oxaliplatin, Bevacizumab, HCQ</td>
</tr>
<tr>
<td>NCT01266057</td>
<td>Advanced Cancers</td>
<td>Sirolimus, Vorinostat, HCQ</td>
</tr>
<tr>
<td>NCT01023737</td>
<td>Malignant ST(^{C})</td>
<td>Vorinostat, HCQ</td>
</tr>
<tr>
<td>NCT00909831</td>
<td>Metastatic ST(^{C})</td>
<td>Temsirolimus, HCQ</td>
</tr>
<tr>
<td>NCT01634893</td>
<td>Refractory/Relapsed ST(^{C})</td>
<td>Sorafenib, HCQ</td>
</tr>
<tr>
<td>NCT00813423</td>
<td>Refractory Advanced ST(^{C})</td>
<td>Sunitinitb, HCQ</td>
</tr>
<tr>
<td>NCT01748500</td>
<td>Prostate</td>
<td>Pantoprazole</td>
</tr>
<tr>
<td>NCT01828476</td>
<td>Prostate</td>
<td>ABT-263, HCQ</td>
</tr>
<tr>
<td>NCT01128296</td>
<td>Pancreatic</td>
<td>Gemcitabine, HCQ</td>
</tr>
<tr>
<td>NCT00568880</td>
<td>MM(^{E})</td>
<td>Bortezomb, HCQ</td>
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<tr>
<td>NCT01510119</td>
<td>RCC(^{F})</td>
<td>Everolimus, HCQ</td>
</tr>
<tr>
<td>NCT01144169</td>
<td>RCC(^{F})</td>
<td>Neo-adjuvant HCQ</td>
</tr>
<tr>
<td>NCT01687179</td>
<td>L(^{G})</td>
<td>Sirolimus, HCQ</td>
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<td>NCT01438177</td>
<td>Refractory/Relapsed MM(^{E})</td>
<td>VELCADE, Cyclophosphamide, CQ</td>
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<tr>
<td><strong>HCQ/CQ(^{A}) Single-Agent Treatment</strong></td>
<td></td>
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<td>NCT01292408</td>
<td>Breast</td>
<td>HCQ</td>
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<tr>
<td>NCT00969306</td>
<td>SCLC(^{H})</td>
<td>CQ</td>
</tr>
<tr>
<td>NCT01023477</td>
<td>DCIS(^{I})</td>
<td>Chloroquine</td>
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<tr>
<td>NCT00962845</td>
<td>Melanoma(^{J})</td>
<td>HCQ</td>
</tr>
<tr>
<td><strong>Assessment of Autophagy Biomarkers - No HCQ/CQ(^{A})</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01210274</td>
<td>MDS or AML(^{K})</td>
<td>Azacitidine</td>
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<td>NCT01092728</td>
<td>Melanoma</td>
<td>Dasatinib</td>
</tr>
<tr>
<td>NCT01358045</td>
<td>BCC(^{L})</td>
<td>Calcitrol, Diclofenac</td>
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<tr>
<td>NCT01497925</td>
<td>Prostate</td>
<td>ADI-PEG 20</td>
</tr>
<tr>
<td>NCT01009437</td>
<td>Breast</td>
<td>Ritonavir</td>
</tr>
<tr>
<td>NCT01594242</td>
<td>MM(^{E})</td>
<td>Bortezomib</td>
</tr>
</tbody>
</table>

*ClinicalTrials.gov search terms used: “cancer” and “autophagy”;
\(^{A}\)Hydroxychloroquine/Chloroquine; \(^{B}\)Non-small cell lung carcinoma; \(^{C}\)Solid Tumours; \(^{D}\)Colorectal Carcinoma (m: metastatic); \(^{E}\)Multiple Myeloma; \(^{F}\)Renal Cell Carcinoma; \(^{G}\)Lymphangioleiomyomatosis; \(^{H}\)Small Cell Lung Carcinoma; \(^{I}\)Ductal Carcinoma In Situ; \(^{J}\)Resectable Stage II/IV; \(^{K}\)Myelodysplastic Syndromes or Acute Myeloid Leukemia; \(^{L}\)Basal Cell Carcinoma
1.5.8 Summary

Autophagy is an evolutionarily-conserved cellular self-digestion mechanism that facilitates sequestration of cytoplasmic components and their delivery to lysosomes. This mechanism is orchestrated by dedicated molecular machinery and carefully regulated by nutrient- and energy-sensing complexes that integrate multiple upstream signals. Autophagy is important for maintaining cellular homeostasis, since disruption of its basal activity promotes neurodegeneration, myopathy, and tumorigenesis. It can also be upregulated beyond basal levels, serving to moderate cellular stresses and promote cell survival. Autophagy upregulation plays a cytoprotective role in tumours, supporting their deregulated metabolism, but also mitigating the cytotoxic effects of anti-cancer therapies. In ovarian cancer, there is interest in clarifying the context-specific functions of autophagy to determine how its therapeutic modulation may benefit patients.

1.6 Scope of Thesis

A process of cell exfoliation, survival in suspension, and re-implantation is critical in mediating the earliest stages of high-grade serous tumorigenesis. This sequence appears to be conserved over the course of tumour evolution, since it also defines intraperitoneal metastasis. Given the propensity of ovarian tumour cells to continually undergo cycles of cellular detachment and re-implantation, we hypothesized that they harbor important molecular adaptations enabling survival under such conditions. Our goal was therefore to identify and characterize some of these mechanisms, as doing so could uncover novel therapeutic paradigms for this disease.

To conduct our investigation, we first established an *in vitro* model of the exfoliation-survival-implantation process using patient-derived ovarian tumour cells in a non-adherent culture environment (Chapter 2). Using this model, we characterize the formation of *in vitro* spheroids and their resemblance to those routinely extracted from patient ascites. Intriguingly, we find that spheroid cells exist in a quiescent state based on
the upregulation of its defining molecular mediators. We also demonstrate autonomous down-regulation of AKT activity upon spheroid formation, and suggest that this plays a role in mediating quiescence. Finally, we demonstrate that upon re-attachment to an adherent surface, spheroids reactivate proliferative mechanisms in an AKT-dependent manner. Together, these studies uncover reversible alterations to the biology of metastatic ovarian cancer cells that occur in association with non-adherent spheroids.

In agreement with reduced AKT activity in spheroids, we also demonstrate an autonomous induction of autophagy (Chapter 3). Moreover, pharmacologic inhibition of any residual AKT activity in spheroids augments their upregulation of this process. In adherent cells as well, we show that pharmacologic AKT inhibition robustly induces autophagy. Interestingly, our work goes on to demonstrate that upregulation occurs independently of Beclin1 despite the canonical role for this protein as an autophagy-inducer. Thus, we uncover a non-canonical autophagy program in ovarian cancer cells that is stimulated by down-regulation of AKT activity.

Building upon our analysis of autophagy (Chapter 4), we next demonstrate that its upregulation can be blocked using three approaches: siRNA-mediated depletion of ATG genes, the Specific and potent autophagy inhibitor 1 (Spautin1), and the classical autophagy blocker Chloroquine (CQ). We then show that by any approach, blockade results in significantly decreased cell viability. Moreover, we find that the combination of AKT inhibition and autophagy blockade reduces viability in a synergistic manner. Taken together, our findings establish autophagy a cytoprotective mechanism upregulated in metastatic ovarian cancer cells cultured as spheroids and/or subjected to AKT inhibition.

1.7 References


126. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev 2002;16:1472-87.


Cancer hallmark of cell signaling that correlates with survival in ovarian cancer.


Chapter 2

2 Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids

2.1 Introduction

Metastasis of epithelial ovarian cancer (EOC) is most often the cause of disease-associated mortality. In the majority of cases, disease has spread beyond the ovaries at the time of diagnosis. Cytoreductive surgery and adjuvant chemotherapy (platinum-taxane combination regimens) may achieve optimal tumor debulking, but the persistence of microscopic disease often results in recurrence in the form of additional metastatic lesions. Disseminated disease is likely a major factor in lowering the average five-year relative survival rate for EOC to only 43.8%, making this cancer the fifth most lethal among women and the most lethal of the gynaecologic malignancies. Consequently, an improved understanding of the cellular biology of EOC metastasis and its underlying molecular mechanisms will shed light on novel therapeutic options.

At the cellular level, EOC metastasis begins when tumor cells are shed into the peritoneal cavity where they exist in suspension as single cells or multi-cellular aggregates/spheroids. The presence of EOC cells and spheroids contributes to the accumulation of large quantities of fluid in the peritoneal cavity (malignant ascites), a common feature of advanced-stage EOC. Once suspended in ascites, solitary EOC cells and spheroids diffuse throughout the peritoneal cavity and are believed to seed the formation of secondary lesions.

It has been suggested that small pockets of “dormant” cells somehow persist during and after treatment of metastatic EOC, remaining undetectable to current screening methodologies and shielded from front-line chemotherapeutics. These dormant
cells may be capable of reactivation when growth conditions are favorable, going on to seed the formation of secondary lesions.

An important characteristic of dormancy is cellular quiescence, or exit from the cell cycle into a G0 or non-dividing state. Quiescence can be defined as a reversible cell cycle arrest accompanied by the accumulation of regulatory proteins necessary for the maintenance of this state: specifically, p130/RBL2 (a pRB-related pocket protein) and p27kip1 (a cyclin-CDK inhibitor), both of which increase in expression during G0. Although quiescence is an emerging concept in the study of ovarian cancer, it has never been formally characterized in EOC cells cultured under non-adherent conditions. This is surprising given that spheroids are thought to comprise a key phase of the metastatic cascade. Consequently, it is of great interest to determine if quiescence is prevalent in spheroid cells. Such a finding would implicate spheroids as pockets of "dormant" cells that – by virtue of their non-cycling state – could elude chemotherapeutics and contribute to metastasis.

In addition, the regulation of quiescence in ovarian cancer remains even less characterized. One study has centered around the role of Mirk/dyrk1b in directly regulating p130; however, the regulatory mechanisms controlling this cellular state are no doubt multifaceted, including activation of quiescence-promoting factors (e.g., Mirk/dyrk1B) as well as de-activation of proliferation-promoting pathways. One such pathway may be phosphatidylinositol 3-kinase (PI3K)/AKT, a canonical promoter of cell cycle entry, and as such, a negative regulator of quiescence. It is well established that PI3K/AKT signaling is important in ovarian cancer pathogenesis and can promote proliferation, survival, as well as migration and invasion in ovarian cancer cells. However, these studies have not examined the possibility of endogenous de-activation of AKT activity at some point during the metastatic process, a change that could potentiate dormancy characterized by quiescence.

Herein, we sought to (i) determine whether spheroids undergo reversible quiescence, (ii) explore the association of this phenomenon with PI3K/AKT signaling,
and (iii) perform our studies in a disease-relevant manner using clinical specimens. We initiated our studies on native spheroids filtered directly from patient ascites, observing no increase in proliferation over time. To extend these findings, we obtained and utilized primary EOC cells from patient ascites samples and cultured them in suspension at sufficient density to facilitate aggregation of bulk tumor cells. Under these conditions, EOC cells rapidly clustered to form quiescent spheroids with downregulated AKT signaling, yet retained their capacity to reattach to a favourable substratum and proliferate in an AKT-dependent manner.

2.2 Materials and Methods

2.2.1 Establishment of primary cultures from ascites

Ascitic fluid collected from chemotherapy-naive patients at time of paracentesis or debulking surgery was used to generate primary ascites cell cultures as described previously\textsuperscript{31}. Patient samples used in this study were high-grade carcinomas of the serous subtype and at least stage III disease (Appendix B). The presence of epithelial cancer cells in these cultures was confirmed by expression of epithelial cell adhesion molecule (EpCAM) and lack of CD45 expression, as assessed by flow cytometry. All work with patient materials has been approved by The Univeristy of Western Ontario Health Sciences Research Ethics Board (Protocol number 12668E).

2.2.2 Isolation of spheroids from ascites fluid

Native spheroids were isolated by filtration through a 40µm cell-strainer (Becton Dickinson, Franklin Lakes, NJ), washed with PBS into a collection tube containing a 10% formalin solution for immediate fixation, or resuspended in growth medium and cultured \textit{in vitro}. The presence of epithelial cancer cells was confirmed by flow-cytometric detection of EpCAM.
2.2.3 Cell culture

The human ovarian cancer cell line OVCAR3 was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI-1640 (Wisent, St. Bruno, Canada) supplemented with 5% fetal bovine serum (FBS; Wisent). Primary cultures of cells or spheroids were maintained in a 1:1 mixture of MCDB 105: M199 (Sigma, St. Lewis, MO) supplemented with 10% FBS (Wisent) and 50µg/ml penicillin–streptomycin (Wisent). Cells were maintained in a 37°C humidified atmosphere of 95% air and 5% CO₂.

Adherent cells were maintained on tissue culture-treated polystyrene (Sarstedt, Newton, NC). Non-adherent cultures (native and in vitro spheroids) were maintained on Ultra-low Attachment plates (ULA; Corning, Corning, NY), which are coated with a neutral hydrophilic gel to prevent cell attachment. To form in vitro aggregates, single-cell suspensions of $1 \times 10^5$ cells/mL were seeded directly to ULA plates. This methodology follows techniques of aggregate formation previously described in the EOC spheroid literature\textsuperscript{12,16,32,33}.

2.2.4 Cell number & viability assays

Adherent cells were trypsinized and resuspended in FBS-containing medium to first generate a single-cell suspension. Non-adherent spheroids were exposed to trypsin-EDTA for 10-20 minutes with vortexing and trituration to disaggregate cells. Trypsin was inactivated using a small volume of FBS. To enumerate viable cells, Trypan Blue reagent (Gibco/Invitrogen, Carlsbad, CA) was applied (1:1 dilution) and cells counted in a hemacytometer (triplicates, two counts per replicate). To determine whether cells seeded to non-adherent culture maintained viability as clusters or as solitary cells, they were allowed to re-attach to tissue culture plastic and stained with HEMA3 (Fisher, Kalamazoo, MI) following 2, 4, 8, 12, and 24h periods in non-adherent culture. HEMA3 staining was performed 24h after cells/spheroids were allowed to re-attach to tissue
culture plastic (i.e., re-attachment was allowed to proceed for 24h following re-seeding to tissue culture plastic, following which, HEMA3 staining was done). Wells were imaged using an Olympus IX70 inverted microscope with ImagePro software to generate random fields of view across the plate. Mean single-cells/field and mean spheroids/well were quantified at 2h and 12h using ImageJ software (NIH, Bethesda, MD).

2.2.5 Ki67 immunohistochemical staining

OVCAR3 intact spheroids or single-cell suspensions of adherent cells were embedded in 1% agarose (w/v). Agarose pellets then underwent paraffin embedding and sectioning (Molecular Pathology, Robarts Research Institute, London, ON), followed by Ki-67 immunohistochemistry (Dept. of Pathology, University Hospital, London, ON). Images were captured using an Olympus AX70 upright microscope ImagePro software and Ki-67-positive nuclei counted (min. 1,000 nuclei per culture condition).

2.2.6 Flow cytometry

2.2.6.1 Propidium Iodide (PI) Staining

Single-cell suspensions were generated as described above, fixed in 95% ethanol, and stored at 4°C. For staining, cells were pelleted and resuspended in PI Staining Solution (PBS with 2% FBS, 0.25µg/µL RNase, 10µg/mL PI), incubated at 37°C for 30 min, then overnight at 4°C. The following day, labeled cells were passed through 40µm cell-strainers to remove large particles and flow cytometry performed on a Beckman Coulter Epics XL-MCL to quantify cell cycle proportions (experiments in triplicate, 10,000 events per replicate). Histograms depicting cell count over PI intensity were generated using the FlowJo software (Ashland, OR).

2.2.6.2 BrdU/PI Staining
Adherent or non-adherent cells were pulse-labeled with 10µM bromodeoxyuridine (BrdU; GE Healthcare, Buckinghamshire, UK) for 2h. Single-cell suspensions were fixed in 95% ethanol and stored at 4°C. For staining, cells were sequentially resuspended and pelleted in the following solutions: 2N HCl/0.5% TritonX-100, 0.1M NaB₄O₄ pH 8.5, mouse anti-BrdU primary antibody (1:50; Becton Dickinson), anti-Mouse FITC-conjugated secondary antibody (1:250; Vector Laboratories, Burlingame, CA), and PI Staining Solution [PBS with 2% FBS, 0.25µg/µL RNase, 10µg/mL PI]. Following this, subsequent steps were carried out as described for ‘PI Staining’ above.

2.2.6.3 **EpCAM and CD45 Staining**

Single-cell suspensions were incubated in a 2% FBS solution containing anti-EpCAM PE-conjugated antibody (1:50; Becton Dickinson) or anti-CD45, PC5-conjugated antibody (1:50; Beckman Coulter). EpCAM or CD45 expression was quantified using a Beckman Coulter Epics XL-MCL flow cytometer with at least 10,000 events counted per test.

2.2.7 **Immunoblotting**

Cell lysates were obtained using a modified radioimmunoprecipitation assay (RIPA) buffer [50mM HEPES pH7.4, 150mM NaCl, 10% glycerol, 1.5mM MgCl₂, 1mM EGTA, 1mM sodium orthovanadate, 10mM sodium pyrophosphate, 10mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 1X protease inhibitor cocktail (Roche, Laval, QC)]. Cells were washed once in PBS followed by addition of lysis buffer. Protein concentration of each lysate was determined using Bradford’s method³⁴ and 25-30µg of protein resolved by 8% or 14% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PVDF; Roche), and blocked with 5% skim milk in TBST (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% Tween 20). Membranes were washed in TBST and incubated overnight at 4°C with antibodies (1:1000 in 5% skim
milk/TBST or 5% BSA/TBST). Immunoreactive bands were visualized by incubating for 1h at room temperature with a peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10,000 in 1% skim milk/TBST; GE Healthcare) followed by exposure to enhanced chemiluminescence reagent (ECL Plus; GE Healthcare).

2.2.8 Antibodies and other reagents

Antibodies were obtained against pAkt-Ser473 (#9271; Cell Signaling Technology, Danvers, MA), total AKT1/2/3, p27, p130 (H-136 sc-8312; C-19 cs-528; C-20 sc-317; Santa Cruz Biotechnology, Santa Cruz, CA), p45SKP2 (REF 323300; Invitrogen, Carlsbad, CA), and actin (A2066; Sigma, Saint Louis, MO). Akt inhibitor VIII (Akti-1/2) was purchased from EMD/Calbiochem (Merck, Darmstadt, Germany).

2.2.9 Spheroid dispersion assay

Day 3 spheroids were re-attached to 48-well tissue culture-treated plates (one spheroid/well) and treated with DMSO or Akti-1/2 (5µM) at 24h post-attachment. Phase contrast images were captured at 72h using an Olympus IX70 inverted microscope and ImagePro software and dispersion area quantified using ImageJ (NIH, Bethesda, MD).

2.2.10 BrdU cytochemistry on dispersing spheroids

Day 3 spheroids were re-plated to glass coverslips in 24-well plates and treated with DMSO or Akti-1/2 (5µM) at 24h post-attachment. At 72h, dispersing spheroids were labeled with BrdU (OVCAR3 pulse-labeled for 2h; EOC samples labeled overnight), fixed in a buffered 10% formalin solution, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS. This was followed by sequential incubations and washes in 2N HCl/0.5% Triton X-100, 0.1M NaB₄O₄ pH 8.5, mouse anti-BrdU primary antibody (1:100; Becton Dickinson), anti-Mouse FITC-conjugated secondary antibody (1:250; Vector Laboratories), and 4',6-diamidino-2-phenylindole (DAPI; 1:5000; Sigma). Stained
coverslips were inverted and mounted on glass slides with VectaShield mounting medium (Vector Laboratories). Fluorescence images were captured using an Olympus AX70 upright microscope and ImagePro software.

2.2.11 Statistical analysis

Data were expressed as mean ± SEM. All statistical analysis was performed using two-tailed Student $t$-tests. All tests of significance were set at $p < 0.05$.

2.3 Results

2.3.1 Primary EOC cells form non-adherent spheroids resembling those found in patient ascites

To perform controlled analyses of spheroid biology, we utilized a reproducible in vitro method of non-adherent culture similar to established methods of aggregate formation described in the EOC spheroid literature$^{12,16,32,33}$. Our objective for this study was not to culture ovarian cancer-initiating cells (i.e., use stem cell selection medium or limiting dilutions to generate cancer-initiating cell-derived spheroids$^{35}$). Instead, primary EOC cells isolated and cultured from patient ascites were seeded to Ultra low-Attachment (ULA) cultureware in full growth medium where they rapidly clustered to form multicellular aggregates or spheroids (Fig. 2.1A). OVCAR3 cells and all patient EOC samples tested thus far were able to form these structures, shown in brightfield images (Fig. 2.1B) and histological sections stained with hematoxylin & eosin (Fig. 2.1C). Mean size and number were calculated for five EOC samples (Fig. 2.1D), revealing both properties to be reproducible within EOC samples. Additionally, the number of spheroids decreased over time in all samples tested (Fig. 2.1D; upper panel), though there was no consistent trend in size (Fig. 2.1D; lower panel). Histological analysis of spheroids generated in vitro, compared to native spheroids from patient ascites, did not reveal significant differences in morphology (Fig. 2.1E).
Figure 2.1: EOC cells in suspension culture form multicellular spheroids that resemble native spheroids in patient ascites.

(A) Schematic diagram of in vitro spheroid formation. (B) Brightfield images of spheroids at day 3 of formation. (C) Images of day 3 spheroids embedded in paraffin, sectioned, and H&E-stained. (D) Mean spheroid number and size were quantified per patient sample by pooling replicates from three independent experiments. (E) Brightfield image of native EOC98 spheroids filtered directly from ascites, and of three patient samples that were paraffin-embedded, sectioned, and H&E-stained.
2.3.2 Both native and in vitro spheroids experience decreased cell proliferation

To initiate our studies of cell proliferation, we analyzed native spheroids filtered directly from patient ascites fluid. These were maintained in non-adherent culture over seven days during which time cell counting was performed (Fig. 2.2A). EOC98 and EOC114 viable cell number remained unchanged, whereas EOC100, following an initial decrease in cell number, reached a plateau and did not significantly increase over subsequent days. Although EOC101 exhibited an initial 1.40-fold increase in cell number, there was no further increase observed between days three and seven. Therefore, native spheroids in non-adherent culture did not experience a significant change in cell number over time.

To evaluate whether in vitro EOC spheroids exhibited a similar trend, single-cell suspensions of OVCAR3 and primary EOC cells were seeded to non-adherent culture and cell number subsequently assessed over a seven-day time course. In most EOC samples, an initial decrease in cell number was followed by a general plateau or reduced rate of decline following day three (Fig. 2.2B). Although OVCAR3 cells and EOC42 appeared to initially increase in number, this was soon followed by a decline and subsequent plateau that was similar to other EOC samples (Fig. 2.2B). This is in stark contrast to what is seen in adherent EOC cell lines and primary cells\(^{36}\), which increase in cell number over time. A closer examination of cell viability during spheroid formation was performed by collecting non-adherent cultures at 2, 4, 8, 12, and 24h time points and re-seeding them to tissue culture plastic, thereby assessing the capacity of cells to re-attach as a measure of viability. This assay revealed that by approximately 8-12h in non-adherent culture, the number of single, unclustered cells had dropped dramatically, presumably as they underwent detachment-mediated apoptosis (i.e., anoikis\(^{37}\)). All remaining viable cells were those that had clustered to form spheroids (Fig. 2.2C).

To assess whether the remaining viable cells were experiencing decreased proliferation, Ki67 immunostaining was performed on OVCAR3 spheroids, revealing a
Figure 2.2: Both native and in vitro spheroids experience decreased cell proliferation.

Cell viability was assessed over seven days by Trypan Blue exclusion for (A) native spheroids and (B) in vitro spheroids. Viability is depicted as a proportion of viable cells relative to day 1 and 0, respectively. (C) Left: EOC primary cells seeded to non-adherent culture were removed at indicated time points and re-attached to 6-well tissue culture dishes. Re-attached cells were stained with HEMA3 and imaged. Right: Graphs were generated from counts in random fields of view (single cells) or complete wells (clusters). (D) PI analysis of DNA content was performed in non-adherent and adherent (sub-confluent) cells by flow cytometry. Graphs were generated using FlowJo software (Ashland, OR). Cell cycle proportions are reported as Mean ± SEM.
dramatic reduction in Ki67-positive nuclei to 13.6±0.5% compared to 24±0.5% Ki67-positive nuclei in actively proliferating monolayer cells (p < 0.001; Fig. S2.1)

To further characterize reduced proliferation, we assessed whether cells were undergoing arrest in a particular phase of the cell cycle. Using PI staining for DNA content and quantifying by flow cytometry, we compared cells in spheroids to proliferating adherent cultures. EOC39 cells, as early as 6h in non-adherent culture, demonstrated a significant decrease in S-phase proportion (2.5±0.6% S-phase compared to 11.6±0.4% in adherent cells) and in G2/M-phase proportion (12.9±1.8% G2/M-phase compared to 22.7±0.7% in adherent cells; p < 0.05; Fig. 2D). This was accompanied by a notable accumulation of cells in G1/G0 (83.4 ± 2.6% G1/G0-phase compared to 63.8±0.8% in adherent cells; p < 0.05). Similar trends were consistently observed in another seven EOC primary cell samples (Table 2.1). Therefore, EOC cells in spheroids experience decreased proliferation, having either transiently arrested at the G1/S-phase transition or exited the cell cycle into G0.

2.3.3 Spheroids exhibit growth arrest in G0

We next set out to determine whether spheroids are comprised of quiescent (G0) cells. To provide a molecular description of quiescence, the expression of p130/RBL2 and p27Kip1 were assessed by Western blot. p130 is essential for maintenance of G0, forming repressive complexes on E2F target-gene promoters to suppress cell cycle-dependent transcription 8. p27Kip1, a cyclin-CDK inhibitor, has also been implicated as an essential mediator of quiescence: its deletion can abrogate cell cycle exit10, while its over-expression can induce quiescence and growth-factor insensitivity in cancer cells 9. In OVCAR3, we observed significant increases in p130 and p27Kip1 expression in spheroids (Fig. 2.3A). In multiple EOC samples, spheroids exhibited 2.1±0.18-fold increase in p130 (p < 0.001; n = 17) and a 4.5±0.85-fold increase in p27Kip1 levels (p < 0.01; n = 12) compared to their adherent counterparts (Fig. 2.3B). Therefore, elevated expression of quiescence-associated proteins suggests that spheroid cells exist in a quiescent state.
Table 2.1: EOC primary cells experience reduced cell cycle kinetics when cultured as spheroids.

<table>
<thead>
<tr>
<th></th>
<th>Adherent/Proliferating</th>
<th>Day 3 Spheroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G₁/G₀</td>
<td>S</td>
</tr>
<tr>
<td>EOC39</td>
<td>63.8 ± 0.8</td>
<td>11.6 ± 0.4</td>
</tr>
<tr>
<td>EOC27</td>
<td>69.3 ± 0.5</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>EOC33</td>
<td>69.7 ± 2.3</td>
<td>11.2 ± 0.8</td>
</tr>
<tr>
<td>EOC50</td>
<td>63.9 ± 0.6</td>
<td>18.6 ± 0.3</td>
</tr>
<tr>
<td>EOC75</td>
<td>63.9 ± 1.1</td>
<td>13.9 ± 0.3</td>
</tr>
<tr>
<td>EOC68</td>
<td>71.7 ± 0.5</td>
<td>15.9 ± 0.7</td>
</tr>
<tr>
<td>EOC65</td>
<td>71.7 ± 0.7</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>EOC104</td>
<td>65.2 ± 0.5</td>
<td>21.2 ± 2.3</td>
</tr>
</tbody>
</table>

*Statistically significant differences between spheroid and adherent cells (p < 0.05).
Figure 2.3: EOC spheroids express markers of quiescence that correlate with reduced AKT phosphorylation.

(A) OVCAR3 and primary EOC cells were seeded to parallel adherent (A) and non-adherent (S) cultures. After 72 hours, spheroids or monolayers were lysed and 25-30 µg of protein subjected to 8% (pAKT-Ser473 and total AKT) or 12% (p130, SKP2, and p27Kip1) SDS-PAGE and Western blot. Blots were stripped and probed for total AKT. Actin was used as a loading control. (B) Western blots of primary EOC cells were quantified by densitometry and results displayed in bar graphs (spheroid as a proportion of adherent). Statistical significance is indicated by ** p<0.01 and ***p<0.001 (pAKT, n = 12; SKP2, n = 10; p130, n = 17; p27Kip1, n = 12)
2.3.4 Quiescence in spheroids is associated with reduced AKT phosphorylation

The PI3K/AKT pathway is well-established as a key player in all forms of cancer including EOC\(^2\). Active AKT forms an important signaling node in this pathway, exerting broad regulation over cell survival and proliferation (reviewed in Ref. \(^17\)). To assess the role of AKT, we monitored its phosphorylation at serine 473 (pAKT) and discovered that it was reduced in spheroids. Levels of pAKT-Ser473 were reduced to 35.2±4.3% of that seen in parallel adherent cells in OVCAR3 cells (Fig. 2.3A) and in twelve patient samples (\(p < 0.001\); Fig. 2.3B).

AKT kinases possess several mechanisms by which they can stimulate proliferation\(^3^8\), and as a result, negatively regulate quiescence. We focused on AKT regulation of the F-box protein p45\(^{SKP2}\) (SKP2), as SKP2 can exert strong post-translational regulation over both p130 and p27\(^{Kip1}\). AKT stabilizes SKP2\(^{39,40}\), preventing its degradation. SKP2 in turn facilitates SCF-mediated ubiquitination and degradation of p130 and p27\(^{Kip1}\), allowing cells to escape quiescence and re-enter the cell cycle\(^41,42\). However, in the absence of active AKT signaling, SKP2 is less stable and thus is expressed at a lower level, allowing accumulation of quiescence-promoting proteins (e.g., p130 and p27\(^{Kip1}\)). We found that SKP2 levels in spheroids of OVCAR3 and ten EOC primary cells were significantly decreased to 61.5±7.7% of the level in parallel adherent cultures (\(p < 0.001\); Fig. 2.3A, B).

Given that reduced pAKT in spheroids correlated with decreased proliferation, decreased SKP2, and increased p130, and p27\(^{Kip1}\) protein levels, we wanted to more directly determine the role of AKT in mediating these changes. Using a chemical inhibitor of AKT kinases (Akti-1/2; EMD Biosciences), dose-dependent inhibition of AKT phosphorylation was achieved in adherent monolayers of OVCAR3 (Fig. 2.4A) and four EOC samples (data not shown). Treatment of adherent OVCAR3 cells with 5µM Akti-1/2 for 72h significantly reduced the number of viable cells to 41.6±0.05% of
**Figure 2.4: Inhibition of AKT activity induces quiescence in adherent OVCAR3 cells.**

(A) OVCAR3 adherent cells were treated with increasing Akti-1/2 concentration (0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 µM). Complete loss of pAKT was achieved at a dose > 2.5 µM. OVCAR3 adherent cells were treated with DMSO or Akti-1/2 (5 µM) at 24h post-seeding. Following 72h treatment: (B) viability was assessed by Trypan Blue exclusion counting and depicted as a proportion of viable cells relative to control; (C) cells were pulse-labelled for 2h with BrdU and BrdU/PI cell cycle analysis performed by flow cytometry; and (D) cells were lysed and Western blot performed to assess p130, SKP2, and p27Kip1 levels. Actin was used as a loading control. Statistical significance is indicated by *p < 0.05 and **p < 0.01.
DMSO controls \((p < 0.001; \text{Fig. 2.4B})\). This decrease was not due to cell death since the number of dead cells was not significantly increased with Akti-1/2 treatment \((p < 0.05)\). Therefore, a reduction in cell proliferation, rather than increased cell death, likely contributed to the decreased cell number observed. To more directly assess proliferation, BrdU/PI analysis was performed. This revealed a significant shift in cell cycle distribution when cells were treated with Akti-1/2 (Fig. 2.4C): specifically, a significant accumulation of cells in G\(_1\)/G\(_0\) and G\(_2\)/M-phases \((p < 0.05)\), and a 60.7% decrease in S-phase \((p < 0.001)\). Finally, Western blot of lysates obtained from Akti-1/2-treated cells revealed decreased SKP2 and increased p130 and p27\(^{kip1}\) protein levels upon inhibition of AKT phosphorylation (Fig. 2.4D). Therefore, the quiescent phenotype of EOC spheroids with low pAKT is recapitulated in adherent EOC cells when subjected to chemical inhibition of AKT activity.

### 2.3.5 Spheroids initiate AKT-dependent cell proliferation upon re-attachment

Given that the final phase of EOC metastasis involves attachment and growth of spheroids on peritoneal surfaces, we next asked whether \textit{in vitro} spheroids, which have become quiescent, are capable of re-initiating growth as well. We performed re-attachment experiments and found that EOC spheroids readily adhere to surfaces 1-2h post-seeding, followed by rapid dispersion to form an expanding monolayer (Fig. 2.5A). To test whether AKT phosphorylation is important for dispersion, we inhibited AKT activity during this process, which significantly reduced cell dispersion by 25.1 – 58.7% in five patient samples \((p < 0.05; \text{Fig. 2.5B})\).

Therefore, spheroid dispersion was at least partly dependent upon AKT activity; however, it remained unclear whether this was due to the role of AKT-mediated proliferation or motility. To address this, we used BrdU incorporation to label cells in dispersion areas. Immunocytochemical staining showed that dispersing cells had incorporated BrdU, implying re-entry to the cell cycle (Fig. 2.5C). Furthermore, Akti-1/2 treatment significantly reduced incorporation by 74.1% in OVCAR3 \((p < 0.001)\) and by
Figure 2.5: Spheroids re-attach and form proliferative ‘dispersion zones’ in an AKT-dependent manner.

(A) EOC10 day 3 spheroids were seeded to adherent culture and dispersion was imaged at 4, 24, and 72h post-attachment (left to right). (B) Dispersing spheroids ± Akti-1/2 [5µM] were imaged and dispersion area quantified (at least 10 dispersion zones per treatment group). Dispersing spheroids of (C) OVCAR3 and primary EOC cells or (D) native EOC98 dispersing spheroids were labeled with BrdU and immunofluorescence was performed to detect BrdU-positive nuclei. At least 4,000 nuclei were counted per treatment group. Statistical significance is indicated by *p < 0.05), **p < 0.01 and ***p < 0.001.
29.2 – 76.8% in primary EOC spheroids (p < 0.05; Fig. 2.5C). To determine if AKT activity is required for re-attachment of native spheroids, these were collected directly from fresh EOC patient ascites, seeded to standard tissue culture plastic, and treated with Akti-1/2. AKT inhibition of native EOC98 spheroids produced a 63.5% reduction in dispersion zone proliferation (p < 0.001; Fig. 2.5D). Therefore, cells emerging from in vitro and native spheroids following re-attachment depend upon AKT activity to re-establish proliferation.

2.4 Discussion

Multicellular spheroids are commonly observed in the malignant ascites of EOC patients and an appreciation for their role in the metastatic program has been developing over recent years. We hypothesized that these structures may represent an important example of the “dormant pockets” of cells theorized to promote metastasis and recurrence. To address this concept, we conducted an in-depth investigation of spheroid pathobiology by establishing a disease-relevant biological model. Using this model, we have demonstrated quiescence in spheroids that is characterized most notably by reduced AKT activity, as well as increased p130/RBL2 and p27\(^{kip1}\) expression and decreased SKP2 levels. Quiescence is reversed, however, upon re-attachment to an adherent substratum, allowing ‘resting’ cells to emerge, disperse, and proliferate in an AKT-dependent manner. Importantly, native spheroids filtered from patient ascites were analyzed directly and were also found to consist of non-proliferating cells that are capable of re-initiating AKT-dependent proliferation.

These data suggest a mechanism in which reduced AKT activity in spheroids destabilizes SKP2, limiting SKP2-mediated degradation of p130 and p27\(^{kip1}\) to promote quiescence. This is reversed upon spheroid dispersion, however, involving re-activation of AKT to drive cell cycle re-entry. Therefore, EOC cells may utilize the AKT pathway and its downstream effectors to dynamically regulate what has been referred to as a “dormant-to-proliferative metastatic switch”\(^1,43\).
To our knowledge, this work is the first to describe spheroid formation-induced dormancy in EOC cells. We have specifically defined quiescence, a necessary characteristic of dormancy, by increased expression of p130 and p27\(^{\text{Kip1}}\). Accumulation of these proteins to mediate cell cycle exit can be invoked under acute conditions in ovarian cancer cells, such as serum starvation\(^{11,44}\). However, we have demonstrated an identical response in EOC spheroids even when cultured in full-serum, thus confirming quiescence as a robust phenomenon within these structures. The induction of quiescence with a concomitant reduction in AKT phosphorylation is also a novel finding in ovarian cancer, which is supported by related observations in other cancer types such as pleural mesothelioma\(^{45}\) as well as studies of anoikis in EOC cell lines\(^{46}\).

The literature contains some examples of ovarian cancer cell lines that maintain proliferation when cultured as spheroids\(^{32}\), apparently contradicting our findings. However, the use of such highly proliferative lines may artificially “drown out” dynamics in proliferation occurring during the metastatic process. By utilizing ascites-derived primary cells and native spheroids formed within the peritoneal cavity, we are thus able to observe alterations in proliferation that we would not have otherwise had the opportunity to study. Additionally, we have extended our findings to OVCAR3 cells so as to demonstrate the reproducibility of reversible dormancy in a cell line model that harbors less extensive abrogation of proliferative control than others.

Importantly, we have also demonstrated that spheroids can re-attach and disperse on adherent surfaces. Whereas prior studies have focused on the importance of cell adhesion during reattachment\(^{32,47,48}\), including recent work from the Brugge lab\(^{33}\), our interest in quiescence led us to characterize cell proliferation during spheroid cell dispersion. Our data reveals for the first time that dispersion is a proliferative process that occurs in an AKT-dependent manner, thereby characterizing an important yet unstudied aspect of this phenomenon. Furthermore, given that AKT has previously been shown to increase cell attachment, migration, and invasion in ovarian cancer cells\(^{29,30}\), it is possible that in addition to proliferation, these processes may also contribute to AKT-dependent dispersion.
We have used multiple clinical specimens in our study to ensure the consistency of these findings. Regardless of inherent heterogeneity across EOC patient samples\textsuperscript{21,49,50} and despite the fact that independent samples are known to exhibit divergent genotypic and phenotypic profiles\textsuperscript{51,52}, we have demonstrated notable reproducibility in our results, further emphasizing their physiological relevance. That is, we have found reversible dormancy to be a conserved property of metastatic EOC spheroids. When viewed in an evolutionary context, one could speculate that spheroid formation and dormancy may represent broadly advantageous adaptations that equip EOC cells to withstand the assortment of selection pressures inherent to patient ascites, mediating their survival during the metastatic process.

In summary, we have shown that EOC cells withdraw to a temporary state of dormancy by aggregating to form multicellular spheroids, yet retain their capacity to rapidly re-enter the cell cycle upon re-attachment. We conclude that spheroids present in patient ascites exist as dormant structures that retain their ability to seed and grow as secondary metastatic lesions. Further studies elucidating the events by which AKT and its effectors contribute to EOC metastasis in a biologically-relevant and tractable model system as highlighted here should reveal new therapeutic targets for marked advancements in the treatment of this insidious disease.

2.5 References


Figure S2.1: OVCAR3 spheroid cells exhibit a decrease in Ki67-positive nuclei compared to adherent counterparts.
Intact spheroids and single-cells suspensions of adherent cells were embedded, sectioned, and subjected to Ki67 immunohistochemical staining. At least 1,000 nuclei were counted per culture condition to determine the Ki-67 positive percentage. ***p < 0.001
3 Metastatic ovarian cancer cells induce autophagy in a Beclin1-independent manner upon AKT inhibition or spheroid formation

3.1 Introduction

Metastatic disease represents the lethal majority of ovarian cancers, thus an understanding of the mechanisms underlying this process is essential to properly treat it. In the U.S. alone, an estimated 22,280 new cases of ovarian and 15,500 deaths were reported last year\(^1\). Although the 5-year relative survival for localized disease confined to the ovary is 92.5%, the majority of women (61%) are diagnosed with metastatic disease (Stage III-IV), for which survival is only 27.3\(^\%\)\(^2\). Metastasis in ovarian cancer is characterized by disease dissemination within the intra-peritoneal compartment\(^3\), mediated by single cells and multicellular aggregates (spheroids) present in ascites fluid\(^4\). Spheroids are thought to serve as important vehicles of dissemination that ultimately form secondary tumors\(^5\). Prior studies have demonstrated that spheroid formation protects ovarian cancer cells from anoikis (detachment-mediated apoptosis)\(^6\), suppresses PI3K/AKT signaling to promote quiescence\(^7\), and impart chemotherapeutic resistance\(^8\)\(^\text{-}\)\(^10\). Yet spheroids retain the ability to re-attach and displace cells lining the peritoneal cavity\(^11\), disaggregate\(^12\)\(^\text{-}\)\(^15\), and re-enter the cell cycle in an AKT-dependent manner\(^7\).

Delineating the mechanisms that afford ovarian cancer cell spheroids resistance to the challenges of metastasis provide platforms for novel therapeutic development. We have uncovered macroautophagy (autophagy) as one potential survival mechanism utilized by spheroid-bound cancer cells. It is a highly-conserved mechanism that operates as basal to maintain homeostasis. Autophagy orchestrates the sequestration of cytoplasmic contents in membrane-bound vesicles and their subsequent lysosome-mediated degradation. It also facilitates turnover of damaged or defective organelles,
removal of misfolded or aggregated proteins, and destruction of intracellular pathogens, ridding the cell of harmful components and liberating constituent biomolecules to fuel metabolic and biosynthetic pathways. Autophagy is negatively regulated by the PI3K/AKT pathway – specifically by the downstream activity of mechanistic target of Rapamycin complex 1 (mTORC1). If mTORC1 activity is suppressed, however, an autophagy-inducing complex is allowed to form and convey activating signals to downstream effectors.

Autophagy effectors are mammalian homologues of autophagy-related, or “atg,” proteins originally discovered in yeast. Among these, the yeast Vps30/atg6 homologue Beclin1 has been studied extensively. It functions in a core complex with Class III PI3K (PI3K C3) and p150 and is required to initiate autophagy, although several autophagy-independent functions have also been described. Mouse models of Beclin1 disruption have revealed its importance in normal physiology and development, since mice with homozygous Beclin1 gene disruption die by embryonic day 7.5. Heterozygous mice (Beclin1+/−) are viable but they develop malignancies at higher rates than wildtype littermates. Analogously, the human chromosomal locus containing the BECN1 gene (17q21) exhibits single-copy loss in prostate, breast, and ovarian cancers, thus implying a tumor suppressive function for Beclin1 – and for autophagy by extension.

Although it is accepted that Beclin1 and autophagy are suppressed during tumorigenesis, autophagy upregulation in established tumors has been also described. To integrate this seemingly opposing evidence, it has been suggested that while homeostatic autophagy initially curtails tumorigenesis in normal cells, the process can be induced in tumor cells as an adaptive response. Autophagy in this context can mitigate intrinsic stresses that typify tumor cell pathobiology (e.g., high metabolic demands, ROS accumulation, ER-stress), as well as extrinsic stresses imposed by anti-neoplastic agents or by the tumor microenvironment (e.g., hypoxia, reduced nutrient and growth factor supply, and poor clearance of metabolic waste). What remains unclear, however, is by what mechanism autophagy would be upregulated in tumors whose formation was enabled by its initial downregulation: in other words, would tumor-
associated autophagy still depend upon an effector gene (e.g., \textit{BECNI}) that was partially deleted during tumorigenesis? We suspect that in such cases, autophagy induction mechanisms undergo adaptation, circumventing otherwise-indispensible genetic requirements and now drive autophagy by an alternate (i.e., Beclin1-independent) mechanism.

Given the inherent cellular stresses associated with metastasis, particularly with intraperitoneal dissemination, we hypothesized that ovarian tumor cells would retain the capacity to upregulate autophagy despite prevalent \textit{BECNI} mono-allelic deletion. Here we show that autophagy was upregulated simply by spheroid formation in metastatic ovarian cancer cells as well as by pharmacologic inhibition of AKT/mTORC1 signaling. Assessing Beclin1 protein abundance, we were surprised to discover that it was expressed at comparable levels in all samples. Even using data obtained from The Cancer Genome Atlas project, we found Beclin1 protein expression to be maintained regardless of gene copy-number. To test its requirement for autophagy induction, we performed siRNA-mediated knockdown of Beclin1 in ovarian cancer cell lines. Strikingly, this had no effect on autophagy in any cell line tested. In contrast, efficient knockdown of other effectors successfully inhibited both basal autophagy and its upregulation. Taken together, these data demonstrate that spheroid formation and AKT/mTORC1 inhibition stimulate Beclin1-independent autophagy upregulation in metastatic ovarian cancer cells.

3.2 Materials & Methods

3.2.1 Isolation of solid tumor tissue and spheroids from ascites fluid

All work with patient materials has been approved by The University of Western Ontario Health Sciences Research Ethics Board (Protocol numbers 12668E and 16391E). Solid tumor tissue from metastatic lesions was obtained from consenting patients at time of debulking surgery and processed as described in Appendix C. Briefly, tissue was
quickly dissected into cubes ~2-5 mm\(^2\) in size, wrapped in aluminum foil, frozen on dry ice, and stored at -80°C. To generate lysates, samples taken out of -80°C were mixed with dry ice pellets (to prevent thawing) and ground using a mortar and pestle. The powdered sample was then added to an appropriate volume of lysis buffer (see below for preparation) to generate protein lysates. Spheroids present in ascitic fluid at time of collection were isolated by filtration through a 40µm cell-strainer (Becton Dickinson, Franklin Lakes, NJ) then gently rinsed off the membrane with PBS and into a collection tube. Spheroids were embedded in cryo-matrix (Fisher, Ottawa, ON) and frozen on dry ice and stored at -80°C. The presence of epithelial cancer cells in solid tumor or ascites samples was confirmed by flow cytometry detection of epithelial cell adhesion molecule (EpCAM). Briefly, solid tumor was processed as described in Appendix C and ascites spheroids were disaggregated as described below. Resultant single-cell suspensions were immediately incubated in 2% FBS containing anti-EpCAM, PE-conjugated antibody (1:50; Becton Dickinson). EpCAM expression was quantified using a Beckman Coulter Epics XL-MCL flow cytometer with at least 10,000 events counted per test.

3.2.2 Establishment of primary cultures from ascites

Ascitic fluid collected from consenting patients at time of paracentesis or debulking surgery was used to generate primary ascites cell cultures as described previously\(^41\). Primary cultures of ascites cells were maintained in a 1:1 mixture of MCDB 105 medium: medium 199 medium (Sigma, St. Lewis, MO) supplemented with 10% FBS (Wisent) and 50 µg/ml penicillin–streptomycin (Wisent). These cultures could be expanded and passaged ~4-5 times following thawing from an initial cryopreservation.

3.2.3 Culture of ovarian cancer cell lines

Human ovarian cancer cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; CaOV3, SKOV3), RPMI-1640 (OVCAR3, OVCAR8, HeyA8), or Alpha
modified Eagle’s medium (AMEM; OVCA429) (Wisent, St. Bruno, Canada) supplemented with 5% fetal bovine serum (FBS; Wisent). The cell line EOC147-E2 is a clone derived from the EOC147 primary sample that has been continuously cultured for 2 years. EOC147-E2 cells were maintained in DMEM/F12 medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% FBS. To establish stable expression of eGFP-LC3B, sub-confluent (~60-70%) OVCAR8 cells were transfected with the pBMN-ires-puro-eGFP-LC3B construct and transferred to Puromycin-containing selection medium (1µg/mL). Following a period of selection, emerging clones with robust eGFP expression were isolated, expanded, tested for starvation-induced puncta formation, and cryo-preserved until needed. All cells were maintained in a 37°C humidified atmosphere of 95% air and 5% CO₂. 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 specific concentrations were seeded to ULA plates to form spheroids over time.

### 3.2.4 Immunofluorescence

Analysis of LC3 protein localization by immunofluorescent staining was performed on sections of cryo-embedded ascites spheroids or cells on coverslips. To generate sections of ascites spheroids, frozen blocks were sectioned on a Shandon cryostate at 6µm, mounted on slides, and stored at -20°C until needed. Cultured cells were seeded on coverslips and allowed to attach overnight before treatment with Akti-1/2. Both cells on coverslips (following 24h of treatment) and spheroid sections were then fixed in a buffered 10% formalin solution, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS. This was followed by overnight incubation with anti-LC3 primary antibody (1:250). The next day, after PBS washes, cells/sections were incubated with anti-rabbit FITC-conjugated secondary antibody (1:250; Vector Laboratories) for 1h, followed by PBS washes and a 5 min. incubation with 4',6-
diamidino-2-phenylindole (DAPI; 1:5000; Sigma, St. Louis, MO). Stained coverslips were inverted and mounted on glass slides with VectaShield mounting medium (Vector Laboratories). Fluorescence images were captured using an Olympus AX70 upright microscope and ImagePro software.

3.2.5 Immunoblotting

Cell and tissue lysates were obtained using a modified radioimmunoprecipitation assay (RIPA) 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, QC), 250 mM β-glycerophosphate]. Cells were washed once in PBS followed by addition of lysis buffer. Protein concentration of each lysate was determined using the method described by Bradford⁴². Total protein (25-30 µg) was resolved by 8% or 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PVDF; Roche, Laval, QC), and blocked with 5% skim milk in 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 specific antibodies (1:1000 in 5% BSA/TBST). Immunoreactive bands were visualized by incubating (1 hr, room temperature) with a peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10,000 in 1% skim milk/TBST; GE Healthcare) followed by exposure to enhanced chemiluminescence substrate reagent (ECL Plus; GE Healthcare). Immunoblots were imaged using radiographic film or the Chemidoc MP Imaging System (BioRad, Mississauga, ON) via the ImageLab image acquisition software (v4.1; BioRad, Mississauga, ON). Quantifications were performed on acquired images using in-built analysis features (‘Volume Tools’) of the software.

3.2.6 Antibodies and other reagents

Antibodies against pAkt-Ser473 (#9271), Akt (#, p-p70S6K(#9234), LC3B (#2775), Beclin1 (#3738S), Atg5 (#2855S), Atg7 (D12B11; #8558S), p4EBP1-Thr37/46
(236B4; #2855S), 4EBP1 (#9452) were obtained from Cell Signaling Technology (Danvers, MA). Anti-p70S6K antibody (S-04; sc-100423) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-actin antibody (A 2066) came from Sigma. Akt inhibitor VIII (Akti-1/2) was purchased from EMD/Calbiochem (#12408; SanDiego, CA) and Chloroquine was purchased from Sigma (C-6628). The pBMN-ires-puro-eGFP-LC3B construct was a generous gift from Craig McCormick (Dalhousie University, Halifax, NS).

3.2.7  Transmission Electron Microscopy

Adherent cells or spheroids treated with Akti-1/2 or vehicle control for 24h were harvested in cold fixative comprised of 2.5% glutaraldehyde in PBS pH 7.2-7.4. Adherent cells were washed once in PBS then scraped in fixative for collection, whereas spheroids were washed in PBS, pelleted, then re-suspended in fixative. Cells were left in fixative no longer than 16h, washed four times in PBS, and stored at 4°C in their final wash. Samples were incubated for 1h in 1% osmium tetroxide in buffer, then embedded in 2% agarose plugs for ease of handling. Following dehydration in a graded series of acetone solutions (20, 50, 70, 90, 95, 100, 100%), samples were transferred to liquid Epon 812 epoxy resin. Samples were poured into moulds and baked overnight at 60°C, solidifying into blocks that were trimmed for sectioning. At the Biotron Facility (University of Western Ontario), sections were cut on an Ultramicrotome (60nm thickness), picked up on copper mesh grids, and stained with uranyl acetate and lead citrate before examination on a Phillips CM10 Transmission Electron Microscope with digital imaging system.

3.2.8  Acquisition of The Cancer Genome Atlas (TCGA) data

TCGA datasets for ovarian serous cystadenocarcinoma samples were downloaded from the University of California Santa Cruz Cancer Genomics Browser (https://genome-cancer.ucsc.edu) and from the Memorial Sloan-Kettering Cancer Center’s cBio Portal for Cancer Genomics (http://www.cbioportal.org). 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 either as either thresholded copy-number calls or as log₂-transformed CNV values. mRNA expression data was acquired by the University of North Carolina TCGA genomic characterization center using Agilent 244K custom gene expression G4502A_07_3 microarrays. Raw data that was processed to yield gene-level transcription estimates that were downloaded as log₂ ratios normalized to a reference signal or as z-scores. Finally, 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 and downloaded either natural log-transformed values or as z-scores.

3.2.9 siRNA Transfection

For RNAi-mediated knockdown of gene expression, we utilized Dharmacon siGENOME SMARTpool reagents (Thermo Scientific, Waltham, MA), each containing a mixture of 4 unique siRNAs [Non-Targeting Control Pool #2 (D-001206-14-05), BECN1 (M-010552-01), ATG5 (M-004374-04), and ATG7 (M-020112-01)]. Cells were seeded to 6-well plates at a density of 200,000 cells/well in antibiotic-free medium and allowed to attach overnight. The next day, siRNA and transfection reagent (DharmaFect #1, T-2001-02) were each diluted in a separate tubes containing 200μL of serum-free medium. Following 5 min incubation, siRNA-containing medium was added to DharmaFect-containing medium. This mixture was allowed to incubate for 20 min to allow liposome formation and siRNA loading. Antibiotic-free complete medium was then added to a final volume of 2mL and plated onto cells. Transfected cells were split 1:2 into new 6-well plates 48h later and allowed to attach overnight. The next day, cells were re-transfected exactly as before, so as to achieve more efficient and sustained knockdown for an extended period of time. Cells were re-seeded for subsequent experimentation 24h later.
3.2.10 Graphing and statistical analysis

All graphs were generated using GraphPad Prism 5 (GraphPad Software, San Diego, CA) or Microsoft Excel 2011 for Macintosh (Microsoft Corporation, Redmond, WA). Data were expressed as Mean ± SD or Mean ± SEM, as indicated. All statistical analyses [Student’s t-test, Analysis of Variance (ANOVA) with Tukey’s Multiple Comparison Test, as well as Correlation and Linear Regression Analysis] were performed using GraphPad Prism 5 (GraphPad, San Diego, CA). Tests of significance were set at \( p < 0.05 \).

3.3 Results

3.3.1 Spheroid formation is sufficient to upregulate autophagy in metastatic ovarian cancer cells.

We hypothesized that one important stimulus for autophagy induction may be the process of spheroid, or multicellular aggregate, formation. To assess this, we collected tumor tissue and ascites fluid from consenting patients with advanced-stage (metastatic) epithelial ovarian cancer. We assessed autophagy first in spheroids filtered directly from patient ascites and embedded in cryo-matrix. Immunofluorescence staining for microtubule-associated light-chain 3 (LC3) protein was performed on frozen sections of these spheroids, revealing a cytoplasmic localization that was often concentrated into discrete puncta, indicative of autophagosomes (Fig. 3.1A). To study spheroid-mediated autophagy in greater detail, ascites-derived primary tumor cells (EOCs) from multiple patients were cultured under non-adherent conditions to form spheroids \textit{in vitro}, thus simulating the fluid-suspension phase of ovarian cancer metastasis. We obtained protein lysates from cells cultured in this way and performed immunoblots for LC3, since the cleaved and lipidated form of this protein (LC3-II) is identifiable as a differentially migrating species and indicates the presence of autophagosomes. As early as 4h following detachment and seeding to non-adherent culture, a significantly increased
Figure 3.1: Autophagy is induced during spheroid formation in metastatic ovarian cancer cells.

(A) Spheroids filtered from patient ascites fluid for immunofluorescence analysis. Nuclei (blue) and cytoplasmic LC3 staining (green) are visible. Orange arrowheads indicate LC3 puncta. Scale Bar: 20 µm. (B) EOC cells were seeded to non-adherent 6-well plates and lysates obtained at indicated time points. Immunoblot data from EOC67 (top) and quantification of LC3-I and LC3-II expression relative to Actin (bottom; n = 3 EOCs). (C) Immunoblot quantifications of parallel adherent (ADH) and (SPH) cultures of multiple EOCs (n = 10) at 24h (left) and 72h (right). Bars: Mean ± SEM. LC3-I and LC3-II levels were compared using the Student’s t-test (*p<0.05; **p<0.01)
abundance of LC3–II was observed; by approximately 24h (once aggregates/spheroids have formed), the ratio of LC3–II to LC3–I was greatest (p<0.01; Fig. 3.1B). This increased LC3–II:LC3–I ratio was maintained through 72h of non-adherent culture, despite a decrease in the absolute amounts of these species over that time (Fig. 3.1B). In addition to time-course analysis, we also assessed paired adherent/spheroid samples from 10 EOCs, verifying significantly increased LC3–II relative to LC3–I at both the 24h and 72h timepoints (p<0.05; Fig. 3.1C). Taken together, these data describe an increase in autophagy as a result of cell detachment and non-adherent culture to form spheroids.

3.3.2 Further upregulation of autophagy in spheroids and adherent cells is achieved with allosteric AKT inhibition.

Another way that autophagy can be induced is with the use of inhibitors targeting PI3K/AKT/mTOR pathway; since PI3K/AKT/mTORC1 activity negatively regulates autophagy, inhibition serves to de-repress and permit its induction above basal levels. Agents inhibiting PI3K, AKT, and/or mTOR kinases have been developed for the clinic and are currently being tried in multiple disease sites, including gynaecologic cancers such as ovarian (ClinicalTrials.gov). We have previously shown that the AKT kinase 1 and 2 inhibitor (Akti-1/2), an allosteric inhibitor of AKT activation, yields dose-dependent inhibition of AKT phosphorylation in NIH:OVCAR3 cells to cause cell-cycle arrest without increasing cell death. We have seen similar dose-dependent inhibition (as evidenced by loss of phosphorylation at Ser473 of AKT) in multiple primary EOC cells and EOC147-E2, a cell line derived from the EOC105 primary culture. Here we demonstrate that in adherent cultures subjected to AKT inhibition, de-phosphorylation of AKT was accompanied by reduced phosphorylation of p70S6K and 4EBP1 (downstream targets of mTORC1 that indicate its level of activity; Fig. 3.2A). This correlated with an increase in LC3 processing (i.e., increased LC3-II:LC3-I ratio), indicating an increase in autophagosomes. In spheroids, however, even without AKT inhibition we observed autonomous suppression of mTORC1 activity that correlated with increased LC3 processing relative to adherent cultures. Treatment of spheroids with Akti-1/2 further
Figure 3.2: AKT inhibition upregulates autophagy in adherent cells and augments autophagy upregulation in spheroids.

Subconfluent (~80%) adherent (ADH) cultures and spheroid cultures (SPH) were subjected to 24h of Akti-1/2 treatment (EOCs: 5µM; EOC147-E2 ADH: 3.5µM; EOC147-E2 SPH: 1µM). SPH treatment was initiated at time of seeding until 24h post-seeding. Lysates were then obtained and immunoblot performed for indicated proteins (ppp = multi-phosphorylated species; p = less phosphorylated species). (B) Sub-confluent (~60%) adherent cells were treated (24h) with Akti-1/2 (5µM) and subjected to immunofluorescence analysis. Nuclei (blue) and cytoplasmic LC3 staining (green) are visible. Orange arrowheads indicate LC3 puncta. Scale Bar: 20µm.
suppressed AKT/mTORC1 signaling beyond that achieved by spheroid formation alone, resulting in a further increase in LC3-II:LC3-I. These trends were observed in EOC147-E2 and multiple primary EOCs, four of which are depicted (Fig. 3.2A). To confirm that increased LC3 processing corresponded with an increase in autophagosomes, we also conducted immunofluorescence staining for LC3, revealing an increase in cytoplasmic, LC3-associated puncta that were apparent in both adherent and spheroid cells upon AKT inhibition (Fig. 3.2B).

3.3.3 Autophagy in spheroids and adherent cells is in flux and proceeds to completion

We also performed autophagic flux experiments to distinguish autophagy induction from late-stage blockade, since both can result in an increase in autophagosome abundance. If autophagy has truly been induced, an agent that blocks clearance of autophagosomes should result in a further increase in LC3-II, whereas if autophagy were already inhibited, any further increase could not occur. We used the antimalarial lysomotropic agent chloroquine (CQ) to halt the clearance of autophagosomes in adherent cultures and spheroids. A short (4h) treatment with CQ resulted in a buildup of LC3-II in untreated adherent cells and spheroids and further accumulation in cells treated with Akt-1/2 (Fig. 3.3A), thus indicating autophagic degradation was underway. Additionally, to verify that autophagy was not only being induced and in flux, but that autophagic degradation was being carried to completion, transmission electron microscopy (TEM) was performed. We examined ultra-thin sections of fixed cells for cytoplasmic vesicles bounded by double-membranes, the contents of which were undergoing degradation (i.e., autophagolysosomes or APLs). By TEM, we observed APLs in untreated spheroids, and to greater extent in spheroids and adherent cells treated with Akti-1/2. APLs were nearly undetectable in untreated adherent cultures (Fig. 3.3B; APLs in Akti-1/2-treated cells were identified by A. M. Cuervo, Albert Einstein College of Medicine, New York, personal communication).
Figure 3.3: Autophagy upregulation is in flux and proceeds to completion in both adherent cells and spheroids.
Subconfluent (~80%) adherent (ADH) cultures and spheroid (SPH) cultures were subjected to 24h treatment with Akti-1/2 (EOCs: 5µM; EOC147-E2 ADH: 3.5µM; EOC147-E2 SPH: 1µM). SPH cultures were treated at time of seeding until 24h post-seeding. (A) CQ pulse (50µM) was administered for final 4-5h immediately before cell lysis. Immunoblot and quantification (relative to actin) was performed for indicated proteins. (B) EOC67 was treated as described, then subjected to TEM sample prep. and analysis; Orange arrowheads indicate Autolysosomes (autophagosomes fused with lysosomes). Scale bars: 500nm
Therefore, based on experiments revealing enhanced LC3 processing, increased autophagosomes that are in flux, and abundant late-stage autophagolysosomes (APLs) containing degraded cytoplasmic material, we conclude that autophagy is upregulated and proceeds to completion in metastatic ovarian cancer cells. This occurs autonomously during spheroid formation as well as when adherent cells or spheroids are subjected to AKT inhibition.

3.3.4 Although single-copy loss of the BECN1 gene is common in ovarian cancer, tumors retain similar levels of Beclin1 protein expression

Following our observation of robust autophagy induction in metastatic ovarian cancer cells, we wished to assess expression of Beclin1 in these samples given its role as a canonical autophagy-effector. In order to quantitatively assess the expression of Beclin1 in a large number of serous ovarian tumors (91.1% of which are from metastatic, Stage III-IV cases), we interrogated level 3 array comparative genomic hybridization (aCGH), mRNA expression microarray, and reverse phase protein array (RPPA) data generated by The Cancer Genome Atlas (TCGA) project. Starting with aCGH data that was processed to yield gene-level copy-number calls, we found that 425/559 ovarian tumours (76%) possessed hemizygous deletion of the BECN1 gene (Fig. 3.4A). This confirms earlier reports of single-copy loss in ~70% of ovarian tumors\(^{23,27-29}\). To determine whether BECN1 copy-number correlated with expression, we plotted mRNA microarray data against copy-number calls and found that single-copy loss of BECN1 yielded a significant reduction in mRNA expression (Fig. 3.4B). To assess expression at the protein level, we plotted TCGA RPPA data in a similar manner, yet were surprised to find only a modest reduction in Beclin1 protein abundance that was not statistically significant; in fact, all tumors exhibited a similar range of Beclin-1 protein expression seemingly irrespective of copy-number (Fig. 3.4C). Using log\(_2\)-transformed copy-number data, we also performed regression analysis to measure the correlation between BECN1 copy number and gene expression. This analysis revealed a positive correlation between copy-number and mRNA level (Fig. 3.4D) but no correlation between copy-number and protein level (Fig. 3.4E).
Figure 3.4: Beclin1 protein expression is not significantly decreased in ovarian tumors despite prevalent single-allele loss.

(A) Gene copy-number calls at the BECN1 locus are depicted for 559 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) BECN1 mRNA (microarray; n = 482) and 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<0.01; ***p<0.001). (D,E) Original log2-transformed mRNA expression (n = 527) and ln-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.
Similarly, metastatic tumor samples and ascites-derived primary cultures obtained by our lab exhibited similar levels of Beclin1 protein expression: samples identified as the highest- and lowest-expressers were assessed on the same immunoblot (Fig. 3.5A), demonstrating only modest differences in protein abundance that were analogous to the narrow range of expression observed in TCGA protein data (Fig 3.4B,C). We also probed these blots for LC3 and found that processing varied across all samples tested. Interestingly, we noted that elevated LC3-II:LC3-I was observable even in those samples with the lowest Beclin1 expression (Fig. 3.5A). Therefore, despite prevalent hemizygous loss of the BECN1 gene in ovarian cancer, expression of its protein product was not correspondingly decreased. Instead, all tumors exhibited detectable levels of Beclin1 protein and a narrow range of expression.

3.3.5 Knockdown of Beclin-1 has no effect on autophagy upregulation in ovarian cancer cells

Thus far, we have shown that metastatic ovarian cancer cells retain not only the capacity to upregulate autophagy, but also Beclin1 protein expression. Consequently, we wondered whether their maintenance of Beclin1 was important in enabling autophagy upregulation. We first re-examined our findings (Figs. 3.1, 3.2, 3.3), particularly in EOCs found to express relatively low levels of Beclin1 protein. If Beclin1 remained important for autophagy, we reasoned that such samples may be less able to undergo the process. However, we were surprised to find that basal LC3 processing was no less extensive than in other samples with more abundant Beclin1 (Fig. 3.5A). Likewise, we have already demonstrated that samples such as EOC67 and EOC129 (Beclin1 low-expressers) exhibited robust induction of autophagy similar to that observed in all other EOCs tested (Fig. 3.1, 3.2, 3.3).

It is possible, however, that cells with reduced Beclin1 expression have adapted such that they can upregulate autophagy using only a limited amount of this protein. To address this possibility, we first sought to identify ovarian cancer cell lines with low (versus high) BECN1 expression. Using datasets from the Cancer Cell Line Encyclopedia
Figure 3.5: In metastatic ovarian cancer cells and cell lines, Beclin1 exhibits only subtle decreases in protein expression.

(A) Lysates were obtained from fresh-frozen samples of metastatic tumor samples and early-passage cultures of ascites-derived cells. Immunoblot was performed for indicated proteins and Beclin1 expression quantified. (C) Log2-transformed mRNA expression data for 52 ovarian cancer cell lines [Cancer Cell Line Encyclopedia (CCLE) datasets] displayed as a function of BECN1 copy number (red ‘x’ = ovarian lines used in our lab. (C) Immunoblot for Beclin1 was performed on indicated lines. Quantification performed on two independent lysates (adherent cultures, ~80-100% confluent). Bars: Mean ± SD.
(CCLE) project\textsuperscript{48}, BECN1 copy-number and mRNA expression level was plotted for 52 ovarian cancer cell lines (Fig 3.5B). We then selected commonly-used lines with two copies (HEYA8, SKOV3, OVCAR8) or mono-allelic deletion (OVCAR3, CAOV3) of BECN1 for further analysis. Immunoblot for Beclin1 identified relatively low- and high-expressing lines, in general agreement with their mRNA expression levels from the CCLE (Fig. 3.5C). EOC147-E2 cells were also identified as a relatively low-expressor (Fig. 3.5C), with comparable expression to CAOV3 cells (which is heterozygous for BECN1).

To determine whether even low levels of Beclin1 protein remained essential for autophagy, we performed siRNA-mediated knockdown of BECN1 expression on the EOC147-E2 cell line, given its identification as a low-expressor. As a comparator, we also performed knockdown in HEYA8 cells to test whether this cell line’s high Beclin1 expression was indicative of increased dependence on this protein for autophagy. Transfection with pooled siRNAs against BECN1 efficiently reduced Beclin1 protein compared to pooled non-targeting (NT) siRNAs in both EOC147-E2 (Fig. 3.6A) and HEYA8 cells (Fig. 3.6B). Efficient reduction of Beclin1 protein did not affect basal LC3 processing, and strikingly, nor could it ablate autophagy upregulation mediated by AKT inhibition (Fig. 3.6A,B). Additionally, Beclin1 depletion was unable to prevent the formation of eGFP-LC3 puncta in OVCAR8 cells expressing eGFP-labeled LC3B (Fig. 3.6C). In stark contrast, knockdown of essential autophagy effectors ATG5, ATG7, or both was able to reduce autophagic degradation, as evidenced by diminished LC3 processing (Fig. 3.6A,B) and formation of eGFP-LC3B puncta (Fig. 3.6C). Therefore, although autophagy in metastatic ovarian cancer cells requires other key effector proteins, we found Beclin1 to be dispensable for the process.

3.4 Discussion

Although loss of a BECN1 allele is thought to promote tumorigenesis through impairment of autophagy, established tumors are nonetheless able to upregulate
Figure 3.6: Beclin1 depletion has no effect on autophagy induction.
Cells were transfected with Control siRNAs or siRNA pools targeting BECN1, ATG5, ATG7, or ATG5+7. Transfection was repeated 72h later, and following overnight incubation, cells were seeded to adherent 6-well plates. (A) EOC147-E2 cells were treated for 24h with either DMSO or Akti-1/2 (3.5µM) at 10-12 days post-transfection (n = 4 experiments). (B) Hey A8 cells were treated for 24h with DMSO/Akti-1/2 (5µM) at 72h post-transfection (n = 2 experiments). Lysates were collected and immunoblot performed for indicated proteins. (C) OVCAR8-eGFP-LC3 cells were subjected to identical transfections, and 72h post-transfection, treated with DMSO/Akti-1/2 (5µM). 24h post-treatment, images were captured using an Olympus IX70 inverted microscope and ImagePro software. Scale bar: 10µm.
autophagy in response to cellular stress. Indeed, the ovarian cancer metastatic process necessarily involves numerous cellular stresses, many of which are known autophagy inducers (e.g., detachment, nutrient depletion, hypoxia). We therefore wondered whether metastatic ovarian cancer cells retained the capacity to induce autophagy despite prevalent BECN1 mono-allelic deletion, and if so, how essential Beclin-1 was for this induction. Here we show that cells are indeed capable of robust autophagy induction, but this occurs independently of Beclin1. Specifically, we demonstrate autophagy upregulation by culturing cells under non-adherent conditions to form spheroids or by subjecting them to AKT inhibition. In assessing the contribution of Beclin1, we found that autophagy induction was robust in all primary cultures and cell lines tested, irrespective of Beclin1 expression level. Moreover, we were unable to ablate autophagy despite efficient siRNA-mediated knockdown of Beclin1 protein.

To our knowledge, this report is the first to describe autonomous autophagy upregulation during tumor-sphere formation. Previous work by Fung et al. described autophagy induction in luminal cells of mammary acini as they underwent extracellular matrix (ECM) detachment. Our model, though different in many ways, is analogous to ECM-detachment since it also involves disengagement of cells from a substratum followed by fluid suspension. Thus, our work is in agreement with the findings of Fung et al., and extends their findings in non-tumorigenic epithelial cells to epithelial ovarian cancer cells. Since Fung et al. also showed that detachment-initiated autophagy promoted cell survival, we are interested in assessing this during ovarian cancer spheroid formation. It will be important to conduct viability assays in which cells are subjected to autophagy blockade during spheroid formation or upon AKT inhibition. Given that autophagy is known to promote survival in established tumor cells, we propose that metastatic ovarian cancer cells upregulate this mechanism for a similar purpose.

A comparison of spheroid-associated autophagy with that stimulated by pharmacologic means has provided important insights into the mechanism by which autophagy is induced during spheroid formation. To stimulate autophagy pharmacologically, we inhibited the PI3K/AKT/mTOR pathway at the level of AKT,
resulting in mTORC1 suppression and de-repression of the autophagy machinery. Our previous work revealed autonomous downregulation of AKT activity in spheroids\(^7\), thus we initially postulated that this was causally linked to spheroid-mediated autophagy. However, our current observations revealed discordance in the timing of these two events: specifically, we observed that while autophagy was robustly induced by the first 24h of spheroid formation, AKT phosphorylation was retained in several EOC samples until 48-72h. In contrast, the timing of mTORC1 de-activation was always tightly correlated with increased LC3 processing, even in the presence of undiminished AKT phosphorylation. Taken together, these observations suggest that the mechanism of autophagy induction in spheroids is not necessarily AKT-dependent, instead an intervening mechanism downstream of AKT appears to be suppressing mTORC1 activity to induce autophagy. This mechanism is an active area of investigation in our lab. Thus far, we have learned that the liver kinase B1/AMP-activated protein kinase (LKB1/AMPK) pathway is activated early in spheroid formation (Peart \textit{et al.}, unpublished). AMPK activation can promote autophagy by de-activating mTORC1 though phosphorylation of TSC1/2\(^{53}\) and Raptor\(^{54}\), as well as by phosphorylation and activating the autophagy-inducer kinase ULK1\(^{32}\). Therefore, we suspect involvement of this pathway in spheroid-mediated autophagy. Moreover, recent work by Avivar-Valderas \textit{et al.} showed that LKB1/AMPK activation during ECM-detachment promoted autophagy in mammary cells and mouse embryonic fibroblasts\(^{55}\). Thus, it will be important to modulate LKB1/AMPK signaling in our model of spheroid formation to evaluate its contribution to autophagy induction.

A key finding of this study is the apparent dispensability of Beclin1 for autophagy upregulation in ovarian cancer cells. Our analysis of Beclin1 abundance revealed that its range of expression was unexpectedly similar across all ovarian tumors, even those with loss of one \textit{BECN1} allele. Based on this, we initially hypothesized that maintenance of Beclin1 expression in ovarian tumor cells implied its importance for autophagy. However, we were surprised to discover that knockdown of Beclin1 had no effect on autophagy, suggesting that these cells possess Beclin1-independent mechanisms to upregulate the process. Despite its canonical role as an essential autophagy effector,
evidence of Beclin1-independent autophagy has been accumulating in recent years: in neuronal, macrophage, breast cancer, and ovarian cancer cell lines, autophagy was induced independently of Beclin1 by exposure to staurosporine, hydrogen peroxide, resveratrol, or arsenic trioxide, respectively. Conversely, an alternate form of autophagy has been described that occurs independently of the autophagy-effectors Atg5, Atg7, and LC3 – but retains dependence on Beclin1. These reports highlight redundancies in autophagy machinery, demonstrating that the absence of certain key effectors (e.g., Beclin1) is not sufficient to cripple the process. Given the importance and evolutionary-conservation of autophagy, it is conceivable that ovarian tumor cells – while still requiring other effector proteins – have adapted their autophagy machinery so as to not rely on Beclin1.

Nonetheless, the sustained expression of Beclin1 might imply its continued importance in ovarian tumor cells, perhaps in serving other autophagy-unrelated functions. Such functions have been suspected of Beclin1 since mouse models of its disruption were generated. While mice with homozygous Beclin1 disruption (Becn1−/−) die by embryonic day 7.5, Atg5−/− and Atg7−/− mice, in contrast, are able to complete embryonic development. However, they die within 24h due to autophagy insufficiency during the neonatal starvation period. Furthermore, the tumor spectrum of Becn1−/− mice also differs from other mouse models of autophagy disruption: mono-allelic Beclin1 deletion in mice gives rise to hepatocellular carcinomas, lung carcinomas, and lymphomas. In contrast, despite effectively ablating autophagy, tissue-specific deletion of Atg7 or mosaic deletion of Atg5 was less tumorigenic: both developed benign liver adenomas, but never the hepatocellular carcinomas seen in Becn1−/− mice. This discordance in both knockout phenotype as well as tumor spectrum suggests additional functions of Beclin1 separate from its role in autophagy. Indeed, evidence for specific autophagy-independent functions continue to be described for Beclin1, including its role in Toll-Like Receptor-mediated phagocytosis in macrophages and in cytokinesis and endocytic degradation of the Epidermal Growth Factor Receptor (EGFR) in HeLa cells. These examples represent alternative functions for Beclin1 when in complex with Class III PI3K, p150 and other co-factors. Most recently, however, Fremont et al. have
demonstrated a critical role for Beclin1 in kinetochore assembly and chromosome congression that is not only autophagy-independent, but also independent of the PI3K C3 and p150 core complex. Therefore, strong in vitro and in vivo evidence describes Beclin1 as a multi-functional protein, and although we have demonstrated its dispensability for autophagy upregulation, its expression may be retained for alternate autophagy-independent functions.

This study describes autophagy upregulation in metastatic ovarian cancer cells that occurs irrespective of Beclin1 expression and is unaltered by siRNA-mediated Beclin1 knockdown. We propose that while Beclin1 and autophagy may initially be downregulated to promote ovarian tumorigenesis, metastasizing tumor cells later gain the ability to upregulate autophagy – however, these cells no longer require Beclin1 to do so. These findings not only illustrate the redundancy of autophagic degradation mechanisms, but also demonstrate the ability of tumor cells to relentlessly adapt and evolve, circumventing any molecular barriers to their continued growth and survival.

3.5 References


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Chapter 4

4 Autophagy upregulation promotes the viability of metastatic ovarian cancer cells treated with an AKT inhibitor.

4.1 Introduction

The need for new and more effective therapeutics in ovarian cancer is highlighted by the low rate of survival experienced by patients. Although women with localized disease experience a 5-year survival rate of 92.5%, they comprise the minority of patients. Unfortunately, the vast majority of women with ovarian cancer are diagnosed with metastatic disease, which dramatically decreases their survival probability to only 27.3%. Standard treatment of ovarian cancer, which involves cytoreductive surgery and chemotherapy, can be very effective initially at reducing tumor burden and prolonging life. However, these and other advances have achieved only an 8% increase in 5-year survival over a span of approximately 32 years.

Clearly there is a need for therapeutic strategies that provide greater survival benefit. To this end, numerous targeted therapies are being developed and are currently undergoing clinical trials in ovarian cancer. Agents such as Bevacizumab and Olaparib that exploit alterations in angiogenesis and DNA damage responses pathways, respectively, have both demonstrated promising improvements in progression-free survival. Inhibitors of PI3K/AKT/mTOR signaling are also of great interest, since this pathway exhibits activating alterations in a large proportion of ovarian tumors. However, clinical trials of these agents have thus far proved disappointing. For example, inhibition of Epidermal Growth Factor Receptor (EGFR) family members such as EGFR and ErbB2/HER2 (receptor tyrosine kinases that lie upstream of PI3K/AKT/mTOR signaling) has yielded response rates of only 0%-7% in ovarian cancer. Likewise, a phase II trial of the mTORC1 inhibitor Temsirolimus showed insufficient benefit in
progression-free survival to warrant subsequent phase III study\textsuperscript{12}. The failure of such agents is likely a complex phenomenon, but might involve upregulation of a cellular survival mechanism known as autophagy.

Macroautophagy (autophagy) is a conserved self-digestion mechanism that functions at basal levels in eukaryotic cells to maintain homeostasis and promote survival under conditions of cellular stress\textsuperscript{13-16}. It can be upregulated by a variety of stimuli, including anti-cancer therapeutics. Inhibitors of the PI3K/AKT/mTOR pathway specifically are known to potently induce autophagy through their inhibition of mTORC1, a canonical autophagy repressor\textsuperscript{17-19}. Therapy-induced autophagy has been shown to also promote tumor cell survival by a variety of mechanisms\textsuperscript{20-23}, thereby blunting the effectiveness of such therapies. Given that phase I/II clinical trials of PI3K/AKT/mTOR pathway inhibitors are currently underway in ovarian cancer (clinicaltrials.gov), it is essential to determine whether ovarian tumor cells upregulate autophagy as a response to these agents, and if so, whether it promotes tumor cell survival. If this is the case, a novel strategy may involve combinatorial autophagy blockade to maximize therapeutic efficacy.

To address these questions, we first generated cultures of metastatic ovarian cancer cells using patient-derived ascites (fluid in the peritoneal cavity that accumulates as a result of metastatic disease and contains malignant cells\textsuperscript{24}). Importantly, ascites contains readily-observable multi-cellular aggregates or spheroids\textsuperscript{25-28} and their dissemination throughout the peritoneal cavity is considered critical for seeding the formation of secondary metastases\textsuperscript{25-27,29,30}. Therefore, using both adherent and spheroid cultures, we subjected metastatic ovarian cancer cells to PI3K/AKT/mTOR pathway inhibition achieved by the allosteric AKT kinase inhibitor Akti-1/2. Our previous work demonstrated that autophagy is indeed upregulated by AKT inhibition in both adherent cells and spheroids. Moreover, in spheroids, AKT inhibition served to augment an already-elevated level of autophagy. Given these findings, we were interested to determine what impact autophagy upregulation was having on ovarian cancer cell viability.
In the this study, our aim was to determine the consequences of blocking autophagy upregulation, hypothesizing that this would deprive ovarian cancer cells of a key survival mechanism and lead to a loss of viability. To achieve autophagy blockade in ovarian cancer cells, three approaches were taken: siRNA-mediated depletion of autophagy-related (ATG) proteins as well as treatment with the novel Specific and Potent Autophagy Inhibitor 1 (Spautin1) and the classical autophagy-inhibitor Chloroquine (CQ). In both adherent and spheroid cultures, blocking autophagy using any of these approaches led to decreased cell viability. Moreover, we found that combined AKT inhibition and autophagy blockade reduced viability in a synergistic manner. Autophagy upregulation therefore represents a cell survival mechanism in metastatic ovarian cancer cells, the blockade of which augments the effectiveness of AKT inhibition at decreasing cell survival.

4.2 Materials & Methods

4.2.1 Culture of ovarian cancer cell lines and ascites-derived cells

All work with patient materials has been approved by The University of Western Ontario Health Sciences Research Ethics Board (Protocol # 12668E and 16391E). Primary cultures of ascites cells were established as described in Chapter 3. Human ovarian cancer cell lines OVCAR8 and Hey A8 were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and the OVCA429 cell line was a generous gift from Barb Vanderhyden (Ottawa Hospital Research Institute, Ottawa, ON). The cell line EOC147-E2 is a clone derived from the EOC147 primary sample that has been in continuous culture for 2 years. All cell lines and ascites-derived primary cultures were grown as described in Chapter 3.
4.2.2 Cell Viability Assays

4.2.2.1 alamarBlue

Adherent cells were seeded to 96-well flat bottom tissue culture plates (EOCs: 2,500-5,000/well; EOC147-E2 5,000/well) and treated following overnight incubation. At least 72h post-treatment, AlamarBlue reagent (Invitrogen, Carlsbad, CA) was mixed with fresh complete medium according to manufacturer’s instructions and added to each well. Using a microplate spectrophotometer (Wallac 1420 Victor 2; Perkin-Elmer, Waltham, MA), fluorescent signal was generated using 560nm excitation wavelength and recorded at 590nm. Experiments were performed in triplicate or greater and fluorescence readings were normalized to cells treated with vehicle control.

4.2.2.2 CellTiter-Glo

Cells were seeded to 24-well ultra-low attachment plates (ULA) at a density of 5.0x10^4 per well to form spheroids as previously described and treatment was initiated at time of seeding. At 72h post-treatment, spheroids were collected, pelleted, and briefly loosened/disaggregated by trypsinization (~5 min.). CellTiter-Glo reagent (Promega, Madison, WI) was prepared according to manufacturer’s instructions and added to spheroids in trypsin (1:1 volume ratio). 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). Experiments were performed in triplicate or greater and luminescence readings were normalized to cells treated with vehicle control.

4.2.2.3 Cell Counting
Cells were seeded to adherent 6-well tissue culture plates (1.5x10^4/well) and to 24-well ultra-low attachment plates (ULA) at a density of 5.0x10^4 per well to form adherent and spheroid culture, respectively. Treatment was initiated immediately in spheroid cultures and following an overnight incubation in adherent cells. Following treatment incubation time (72h), adherent cells were trypsinized and resuspended in FBS-containing medium to first generate a single-cell suspension. Spheroids were exposed to trypsin-EDTA for 10-20 minutes with vortexing and trituration to disaggregate cells. Trypsin was inactivated using a small volume of FBS. To enumerate viable cells, Trypan Blue reagent (Gibco/Invitrogen, Carlsbad, CA) was applied (1:1 dilution) and cells counted in a hemacytometer (two counts per replicate of experimental triplicates).

4.2.3 Immunoblotting, antibodies and other reagents

Whole-cell protein lysates were obtained and immunoblots performed as described in Chapters 2 and 3. Antibodies against LC3B (#2775) and Beclin1 (#3738S), were obtained from Cell Signaling Technology (Danvers, MA). Anti-Actin antibody (A2066) and Chloroquine (C-6628) were obtained from Sigma. Akt inhibitor VIII (Akti-1/2) was purchased from EMD/Calbiochem (#12408; SanDiego, CA). Spautin1 was obtained from Cellagen Technology (#C3430-2s; San Diego, CA). For RNAi-mediated knockdown of gene expression, we utilized Dhharmacon siGENOME SMARTpool reagents (Thermo Scientific, Waltham, MA), each containing a mixture of 4 unique siRNAs [Non-Targeting Control Pool #2 (D-001206-14-05), ATG5 (M-004374-04), and ATG7 (M-020112-01)]. Cells were transfected

4.2.4 Combination Index Analysis

Interaction effects of drug combinations (CQ+Akti-1/2 or Spautin1+Akti1-1/2) were assessed using combination index (CI) and isobologram analysis, as described previously. EC50 values for each were determined empirically using the alamarBlue viability assay (described above) and non-linear curve fit analysis (described below).
Based on their individual EC50s, serial dilutions of either drug alone and their combination were generated such that a 1:1 equipotent ratio was maintained at all dilutions. Viability assays were conducted as described above, and the readings analyzed using the CalcuSyn 2.0 Software (BioSoft, Cambridge, UK) to generate isobolograms and CI scores. The isobologram is generated by plotting EC50 values for each drug as x- and y-intercepts that are connected by a line segment. A single point that represents the CI score is also plotted. A CI value to the left, overlapping, or to the right of the line segment indicates synergistic, additive, or antagonistic effects, respectively. Correspondingly, CI values of <1, =1, and >1 indicate synergistic, additive, or antagonistic effects, respectively.

4.2.5 Graphing and statistical analysis

Graphs were generated using GraphPad Prism 5 (GraphPad Software, San Diego, CA) or Microsoft Excel 2011 for Macintosh (Microsoft Corporation, Redmond, WA). Data were expressed as Mean±SD or Mean±SEM as indicated. All statistical analyses [Student’s t-test, Analysis of Variance (ANOVA) with Tukey’s Multiple Comparison Test, and Non-Linear Curve-Fit Analysis to generate and compare EC50 values] were performed using GraphPad Prism 5. Tests of significance were set at $p < 0.05$.

4.3 Results

4.3.1 Knockdown of ATG5 and ATG7 reduces ovarian cancer cell survival upon AKT inhibition

It is well established that autophagy is a pro-survival mechanism. Therefore, we hypothesized that metastatic ovarian cancer cells subjected to AKT inhibition were inducing autophagy to preserve their viability. To test this, we knocked down the expression of critical autophagy-related proteins using RNAi, thereby disabling
autophagy upregulation. Our previous studies demonstrated that \textit{ATG6/BECN1} knockdown was insufficient to ablate autophagy upregulation in ovarian cancer cells, whereas reducing of \textit{ATG5} and/or \textit{ATG7} expression resulted in efficient downregulation of autophagy based on processing of the canonical autophagy marker LC3 (assessed by immunoblot and fluorescence microscopy; see Chapter 3, Fig. 3.6). Thus, for the present study, we again transfected the EOC147-E2 cell line (a clone of the ascites-derived primary sample EOC147) with negative control siRNA (siNT) or with siRNAs targeting \textit{ATG5} and/or \textit{ATG7} and assessed the effect that this had on cell viability.

In adherent cells and spheroids that were not subjected to AKT inhibition (DMSO vehicle control), decreasing basal autophagy via \textit{ATG} gene knockdown had modest effects on viability. In fact, we observed that autophagy blockade by single-gene knockdown of \textit{ATG5} or \textit{ATG7} generally did not affect cell viability in untreated adherent cells or spheroids (Fig. 4.1A,C). However, the combined knockdown of \textit{ATG5} and \textit{ATG7} significantly reduced cell viability: adherent cells exhibited a 25% decrease (Fig. 4.1A), and spheroids suffered a more drastic ~50% decrease (Fig. 4.1C). These experiments were conducted in FBS-containing culture media. Therefore, even at basal levels, autophagy appears important for the viability of metastatic ovarian cancer cells, in both adherent and spheroid cultures.

In addition to blocking basal autophagy, we also assessed the effect of blocking autophagy upregulation. Autophagy-competent and -deficient cells (made so by siRNA transfection) were treated with Akti-1/2, an allosteric AKT inhibitor that we have previously characterized as an autophagy inducer in metastatic ovarian cancer cells (Fig. 3.2-3.3). In adherent cultures subjected to AKT inhibition, we found that knockdown of both \textit{ATG5} and \textit{ATG7}, as well as of \textit{ATG5} alone, led to a 35% and 25% drop in cell viability, respectively (Fig. 4.1B). Likewise, in spheroids exposed to Akti-1/2, knockdown of \textit{ATG7} or both \textit{ATG5} and \textit{ATG7} reduced viability by ~25% and ~55%, respectively (Fig. 4.1D). Therefore, blocking Akti-1/2-mediated upregulation of autophagy led to a dramatic decrease in cell viability, implying cytoprotective functions for this process in the context of AKT inhibition.
Figure 4.1: AKT inhibition combined with siRNA-mediated autophagy blockade reduces cell viability.

EOC147-E2 cells were transfected with Non-Targeting siRNAs or siRNA pools targeting ATG5, ATG7, or both. Transfection was repeated 72h later, and following overnight incubation, cells were seeded to their respective culture condition. (A,B) Adherent cultures were maintained on tissue culture-treated plates. Quadruplicate wells were treated with (A) DMSO vehicle control or (B) Akti-1/2 (3.5µM) and viability assessed by alamarBlue assay at 72h post-treatment (n = 4 experiments). (C,D) Spheroids were formed in Ultra Low-Attachment plates. Triplicate wells were treated with (C) DMSO vehicle control or (D) Akti-1/2 (1µM) and viability assessed by CellTiter-Glo (n = 2 experiments). Bars: Mean ± SEM relative to Control siRNA. One-way ANOVA performed with Tukey’s Test. Different letters denote statistically significant differences among treatment conditions (p<0.01).
4.3.2 Autophagy blockade mediated by the Specific and Potent Autophagy Inhibitor 1 (Spautin1) reduces the viability of ovarian cancer cells subjected to AKT inhibition

In addition to depletion of ATG proteins, we also tested chemical means of blocking autophagy in metastatic ovarian cancer cells. The Specific and potent autophagy inhibitor 1 (Spautin1) was the first targeted autophagy inhibitor to be published. It is a small molecule that inhibits USP10 and USP13, two ubiquitin-specific peptidases responsible for stabilizing members of the autophagy-inducing Class III PI3K (PI3K C3) complex\textsuperscript{34}. By inhibiting USP10/13, Spautin1 causes the degradation of multiple complex members (Beclin1, ATG14L, p150, and C3 PI3K), and in turn, blocks autophagy induction (Fig. S4.1).

Here we utilized Spautin1 for the first time in ovarian cancer cells, demonstrating that it can effectively block autophagy upregulation in EOC147-E2 cells and ascites-derived epithelial ovarian cancer cells (EOCs), cultured under both adherent and non-adherent conditions (Fig. 4.2A). Densitometry of LC3 immunoblots from five EOCs was used to quantify these changes, revealing that Spautin1 co-treatment significantly reduced levels of LC3-II (the autophagosome-associated LC3 species) to near basal levels (Fig. 4.2B). Additionally, in a clone of the OVCAR8 ovarian cancer cell line expressing an eGFP-LC3B construct, Spautin1 co-treatment prevented the shift from diffuse to punctate eGFP-LC3B cellular localization that occurs upon AKT inhibition (Fig. 4.2C).

Interestingly, despite its published mechanism of action\textsuperscript{34}, Spautin1-mediated autophagy blockade did not correlate with a reduction in Beclin1 protein expression (Fig. 4.2A,B), suggesting that Beclin1 protein level was not critical for Spautin1’s mechanism of action. This may be a common feature of ovarian cancer cells, since we made similar observations in the ovarian cancer cell lines OVCA429, HEY, and HEYA8 (Fig. S4.2A). We have confirmed that HeLa cervical carcinoma cells did exhibit reduced Beclin1 levels with Spautin1 co-treatment (Fig. S4.2B), which is in agreement with the original characterization of Spautin1 by Liu et al.\textsuperscript{34}. To test the contribution of Beclin1 to Spautin1-mediated autophagy blockade, we also compared the effects of Spautin1
Figure 4.2: Autophagy upregulation is blocked by the Specific and Potent Autophagy Inhibitor 1 (Spautin1).

(A,B) Cells were seeded to 6-well adherent (ADH) or non-adherent (SPH) culture dishes. Non-adherent cells were treated at time of seeding and adherent cells treated 12h after seeding to allow adhesion (Akti-1/2 5µM; Spautin1 10µM). Lysates were obtained 24h post-treatment and immunoblot performed for indicated proteins. (B) Representative blots of EOC147-E2 and EOC67 cells are depicted. (B) Quantification of band intensity of LC3-II or Beclin1 normalized to Actin for n = 5 EOC samples. Bars: Mean ± SEM. One-way ANOVA with Tukey’s Multiple Comparison Test compared means (*p<0.05; **p<0.01). (C) OVCAR8-eGFP-LC3 cells were seeded (3.0x10⁵ cells/well) to 6-well plates and treated following overnight incubation (Akti-1/2 5µM, Spautin1 10µM). Images captured 24h post-treatment, images were captured using an Olympus IX70 inverted microscope and ImagePro software. Scale bar: 10µm
treatment on Beclin1-expressing and -depleted cells. We found that comparable autophagy blockade was achieved by Spautin1 irrespective of Beclin1 expression, since at least a ~50% reduction in LC3-II was observed in either case (Fig. S4.2C). Taken together, these data demonstrate that Spautin1 efficiently blocks Akti-1/2-mediated autophagy upregulation and that this appears to occur in a Beclin1-independent manner.

Given that Spautin1 efficiently inhibited autophagy upregulation, we then asked what effect simultaneous treatment with Akti-1/2 and Spautin1 would have on cell viability. Multiple EOCs were treated with Akti-1/2, Spautin1, or both, and cell viability was assessed. Single-agent Akti-1/2 was used at a previously-determined concentration that: (i) inhibited AKT phosphorylation, (ii) induced autophagy, and (iii) achieved a 50% reduction in cell viability (EC50). Spautin1 was used at a concentration that consistently blocked autophagy induction (Fig. 4.2). Treatment with single-agent Spautin1 had a modest effect on all EOCs tested, resulting in no greater than a 30% loss of viability, while single-agent Akti-1/2 (EC50) led to an approximate 50% drop in viability (Fig. 4.3A). When cells were treated with both agents in combination, a decrease in viability of ≥75% was observed in all EOCs, which was greater than that of either agent alone (Fig. 4.3A). These trends were found to be highly statistically significant when data from individual EOCs were pooled (p<0.001; Fig. 4.3B). Identical experiments were conducted with the EOC147-E2 cell line, also revealing that combination treatment reduced cell viability by a significantly greater amount than either agent alone (p<0.001; Fig. 4.3C). Therefore, our results indicate that in metastatic ovarian cancer cells, suppression of autophagy induction by Spautin1 compromises cell viability.

4.3.3  AKT inhibition combined with Chloroquine (CQ)-mediated autophagy blockade reduces ovarian cancer cell viability

In addition to the novel autophagy-inhibitor Spautin1, we also used the lysomotrophic agent Chloroquine (CQ) to block autophagy. CQ is a small molecule that disrupts lysosome function by altering intra-lysosomal pH. Since lysosomes are essential for the degradation of autophagic cargo, CQ-mediated lysosomal dysfunction thus
Figure 4.3: AKT inhibition combined with Spautin-1 treatment reduces cell viability.

Cells were seeded to 96-well adherent plates the day before treatment. Triplicate wells were treated (Akti-1/2 EC50, Spautin1 10µM) and following 72h incubation, viability measured by alamarBlue. (A) For each EOC samples, viability readings were normalized to DMSO vehicle control. Bars represent Mean ± SD. (B) Normalized viability data from all EOCs were pooled (n = 10 EOC samples) and one-way ANOVA performed. (C) EOC147-E2 cells were treated identically, repeated experiments (n = 3) pooled and one-way ANOVA with Tukey’s Multiple Comparison Test performed. Bars represent Mean ± SEM. Different letters denote statistically significant differences (p<0.001).
inhibits autophagy. Unlike early-stage autophagy blockade that limits the formation of autophagosomes and thus decreases LC3-II (e.g., Spautin1), CQ blocks autophagy in its final stages, instead preventing the clearance of autophagosomes (Fig. S4.1). As a result, un-degraded autophagosomes accumulate within the cell, manifesting as an over-abundance of LC3-II.

We have previously demonstrated LC3-II accumulation upon CQ treatment of adherent cells and spheroids (see Chapter 3, Fig. 3.3A). Here cells were treated with CQ (50µM), Akti-1/2 (EC50), or both, and their viability assessed. In multiple EOCs, treatment with CQ or Akti-1/2 alone led to reductions in viability of 35-50% and ~50%, respectively (Fig. 4.4A). Their combination, however, yielded a more pronounced loss of viability (70-95%) (Fig. 4.4A). When data from all 8 EOCs were subsequently pooled, these trends were found to be statistically significant ($p<0.001$; Fig. 4.4B). Similar results were also obtained from identical experiments using the EOC147-E2 cell line ($p<0.001$; Fig. 4.4C). Therefore, CQ-mediated autophagy blockade, on its own or in combination with AKT inhibition, significantly decreases cell viability.

Although LC3-II accumulation is observed upon CQ treatment (see Chapter 3, Fig. 3.3A), this observation is not sufficient to infer the completeness of autophagy blockade. This is due to a lack of “reference points” against which to compare autophagy inhibition. For example, whereas relative effectiveness of autophagy inhibition can be inferred by measuring a decrease in LC3-II from a reference level, such comparisons cannot be made with drugs like CQ that increase LC3-II abundance. Therefore, as we were unable to empirically determine the CQ dose at which autophagy is abolished, we opted to treat cells with a range of CQ concentrations. CQ was titrated alone or in combination with fixed-dose AKT inhibition so as to test both the effect of blocking basal autophagy (DMSO vehicle control) as well as blocking autophagy upregulation (Akti-1/2). Readings for either condition were normalized to their individual controls and non-linear curve-fit analysis performed to generate CQ EC50 values (example curve-fit depicted in Fig. 4.4D). In EOC147-E2 cells, comparison of cells treated with DMSO vs. Akti-1/2 revealed a significant difference in their CQ EC50s: specifically, Akti-1/2 co-
Figure 4.4: AKT inhibition combined with Chloroquine (CQ) treatment reduces cell viability.

96-well adherent plates seeded the day before treatment. (A,B,C) Triplicate wells treated for 72h with Akti-1/2 (EC50) and CQ (50µM), and viability assessed by alamarBlue. (A) Viability readings normalized to vehicle control (DMSO) for individual EOC samples. Bars: Mean ± SD. (B,C) Normalized viability data pooled from (B) all EOC samples (n = 8) and (C) all experiments with EOC147-E2 cells (n = 3). Bars: Mean ± SEM. Different letters denote statistically significant differences, based on one-way ANOVA with Tukey’s Test (p<0.001). (D, E) Triplicate wells treated with a range of CQ doses (0, 2.5, 5.0, 10, 25, 50, 75, 100, 150, 250µM) combined with either DMSO or Akti-1/2 (EC50) and viability assessed at 72h. (D) Non-linear curve-fit analysis and EC50 comparisons performed for EOC147-E2 cells (p<0.001). (E) CQ EC50 comparisons for EOC147-E2 cells and all EOCs. Bars: Mean ± 95% confidence interval.
treatment caused a 67% decrease from 68.4µM to 22.7µM, suggesting that AKT inhibition sensitized these cells to autophagy blockade by CQ (Fig. 4.4D). Similar results were observed across multiple EOCs: although they exhibited a broad range of sensitivity to CQ (EC50s of 25µM–150µM) in the absence of autophagy induction (i.e., DMSO vehicle control), significant decreases of 32-78% were observed in the presence of Akti-1/2 in nearly all cases (Fig. 4.4E). Taken together, these findings demonstrate that AKT inhibition sensitizes metastatic ovarian cancer cells to the deleterious effects of CQ-mediated autophagy blockade.

4.3.4 Spheroids are more resistant to single-agent treatment than adherent cells, but remain highly sensitive to the combination of AKT inhibition and autophagy blockade

Given our finding that autophagy blockade led to decreases in adherent cell viability, we wished to determine whether similar effects were observed in spheroid cells. To test this, we treated EOCs in both culture conditions with fixed doses of Akti-1/2 alone, an autophagy inhibitor alone (Spautin1 or CQ), or their combination. Data from individual EOCs were pooled and statistical analysis conducted to directly compare these treatments between adherent cells and spheroids. We observed a similar response to Spautin1 in spheroid and adherent cells, as both exhibited minimal decreases in viability (Fig. 4.5A). In the case of Akti-1/2 treatment, however, spheroid-based cells were dramatically less sensitive than their adherent counterparts, exhibiting only a 10% reduction in viability (as compared to 50%). Yet despite their lack of response to single-agent Akti-1/2, spheroid cells remained highly-sensitive to its combination with Spautin1, as co-treatment was just as potent as it was in adherent cells (#p<0.001; Fig. 4.5A).

Similar observations were made upon CQ treatment. Whereas spheroids exhibited de-sensitization to both single-agent CQ and Akti-1/2 treatment compared to adherent cells (*p<0.001), they retained equivalent sensitivity to combination treatment (Fig. 4.5B). We also conducted Trypan Blue exclusion cell counting (identifies viable cells) in a large number of adherent and spheroid EOC cultures subjected to similar CQ ± Akti-1/2
Figure 4.5: Spheroids are more sensitive to combined AKT and autophagy inhibition than either modality alone.

Cells were seeded to parallel adherent (ADH) and non-adherent (SPH) cultures and treated following overnight incubation or at time of seeding, respectively. Cell viability was quantified by alamarBlue (ADH) or CellTiter-Glo (SPH) and normalized to DMSO vehicle control (not depicted). Normalized viability data from individual EOC samples (n = 7) treated with (A) Akti-1/2 (EC50) ± Spautin1 (10µM) or (B) Akti-1/2 (EC50) ± CQ (25µM) were pooled (n = 7 EOC samples) and depicted as Mean ± SEM. One-way ANOVA with Tukey’s Multiple Comparison Test was performed (*p<0.001). Drug combination more effectively decreased viability compared to single-agent treatment (#p<0.01)
combination treatments and observed that spheroids were slightly less sensitive than adherent cells to CQ alone, but exhibited a much more pronounced drop in viability when CQ and Akti-1/2 were combined (Fig. S4.3). Taken together, these data imply that while spheroids are able to resist the effects of either AKT inhibition or autophagy blockade alone, their combination yields dramatic reductions in cell viability.

4.3.5 AKT inhibition and autophagy blockade work in a synergistic manner to reduce ovarian cancer cell viability

Thus far we have demonstrated that co-treatment of ovarian cancer cells with AKT inhibition and autophagy blockade reduces cell viability to a greater extent than either alone. However, it remained to be determined whether these treatments were acting merely in an additive (i.e., their combined effect was simply the sum of either effect alone) or synergistic manner (i.e, the combined effect is greater than additive). Synergy implies that one agent enhances the effect of the other. It is valued over an additive effect since either agent can be used at lower doses (thus reducing toxicity), while still achieving greater therapeutic efficacy. To assess synergy, we performed isobologram analysis, which provides a quantitative combination index (CI) value as a measure of drug interaction\(^\text{36}\). Briefly, using EC50s as a starting point (Table S4.1), serial dilutions of either drug alone and their combination were generated with a 1:1 ratio of equipotency at all dilutions (see X-axis in Fig. 4.6A). Cell viability data were obtained for each condition (EOC129 data depicted in Fig. 4.6A) and CI analysis performed using these data (isobologram and CI value depicted in Fig. 4.6B). A CI score <1, =1, or >1 indicates synergistic, additive, or antagonistic effects, respectively. For EOC129 and all other EOCs tested, the combination of Akti-1/2 and Spautin1 was found to be synergistic as all values were <1 (Fig. 4.6C). Synergy was also observed for the Akti-1/2 CQ combination (in all but one EOC), albeit to a lesser degree (Fig. 4.6D). CI analyses were also performed in EOC147-E2 cells, revealing a synergistic-to-additive effect of Spautin1 co-treatment and an additive effect of CQ co-treatment (Fig. 4.6E). In conclusion, combination treatment with AKT inhibition and autophagy blockade synergistically reduces the viability of the majority of metastatic ovarian cancer cells.
Figure 4.6: The combination of AKT inhibition and autophagy blockade synergistically reduces cell viability of metastatic ovarian cancer cells.

(A) Triplicate wells of adherent cells were treated with either drug alone or in combination (A = Akti-1/2, S = Spautin1) and viability assessed by alamarBlue 72h post-treatment. EOC129 data are depicted. Bars: Mean ± SD. (B) Isobologram of Combination Index data for EOC129. X- and Y-intercepts represent the calculated Akti-1/2 and Spautin1 EC50s, respectively. CI score coordinates represent interaction effect of two drugs when used at their EC50s. CI scores for (C) Akti-1/2 and Spautin1 treatment or (D) Akti-1/2 and CQ at their EC50 doses are depicted as horizontal bars. (E) EOC147-E2 cells were subjected to identical analysis and CI scores (at EC50 doses) from 3 repeated experiments are depicted as horizontal bars.
4.4 Discussion

Combinatorial autophagy blockade is an emerging paradigm in cancer treatment, providing a means of sensitizing tumor cells to existing or investigational therapeutics. It is based on the premise that tumor cells upregulate autophagy to mitigate the deleterious effects of anti-cancer agents. Consequently, blocking therapy-induced autophagy deprives them of an important survival mechanism. In agreement with this concept, our present work demonstrates that metastatic ovarian cancer cells treated with an AKT inhibitor upregulate autophagy to promote their survival, since its blockade resulted in significantly decreased cell viability. Our work therefore supports a pro-survival role for autophagy upregulation in this context and suggests that its therapeutic inhibition may be a treatment strategy worth exploring in ovarian cancer.

This study assessed the role of autophagy upregulation not only in adherent cultures, but also in multicellular aggregates (i.e., spheroid cultures), as these are considered important for ovarian cancer metastasis. Given our previous finding of elevated autophagy simply due to spheroid formation, and its further upregulation upon AKT inhibition (see Chapter 3, Fig. 3.1-3.3), we expected spheroids to be acutely sensitive to autophagy blockade. Indeed, spheroids subjected to non-pharmacologic means of autophagy blockade appeared more sensitive to $ATG5 + ATG7$ knockdown than their adherent counterparts (Fig. 4.1C,D). However, autophagy blockade by pharmacologic means did not yield similar results: Spautin1 treatment was equally ineffective in both culture conditions and CQ was significantly less effective in adherent cultures.

We suspect that these results may be attributable to the general drug-resistant phenotype that is inherent to multicellular aggregates (reviewed in Ref. 37), rather than a specific insensitivity to autophagy blockade. Indeed, it is well established that three-dimensional or “tumor-sphere” culture of ovarian cancer cells endows them with resistance to chemotherapeutics 38-43. Furthermore, our results presented in Figure 4.5 also demonstrate reduced sensitivity to Akti-1/2 treatment, indicating that spheroid cells can
also resist targeted agents. Thus, spheroids may be less sensitive to autophagy-blocking drugs by virtue of general drug resistance mechanisms, rather than being specifically unresponsive to autophagy blockade. This general drug-resistant state could explain why spheroids were sensitive to non-pharmacologic means of autophagy blockade (i.e., RNA interference; Fig. 4.1C,D), but not autophagy-blocking drugs that can be circumvented by spheroid-associated drug-resistance mechanisms. Overall, we conclude that autophagy blockade in spheroid cells is in fact detrimental to their survival, but it is unclear why this effect is less potent than would be predicted based on their high levels of autophagy.

As a clinical agent, Chloroquine (CQ) has been used as an anti-malarial for decades and is well-tolerated with minimal toxicity in patients. CQ (or its derivative HCQ) is currently being tried clinically as an autophagy inhibitor in combination with targeted anti-cancer agents (clinicaltrials.gov). For example, although trials of the mTOR inhibitor Temsirolimus have demonstrated a 0% stable disease rate in patients with metastatic melanoma, its combination with HCQ achieved stable disease in 4/5 (74%) of patients with this cancer in a phase I trial (AACR 2011 Abstract #4500)44. Likewise, two controlled, randomized trials in glioblastoma multiforme revealed that CQ administration in combination with standard therapy increased mean overall survival by at least two-fold compared to controls treated with standard therapy alone45,46. Although preliminary, these studies highlight the potential of CQ combination treatment as a new therapeutic paradigm in cancer. To date, no similar trials have been initiated specifically for ovarian cancer. However, our work indicates that further pre-clinical evaluations of autophagy blockade in ovarian cancer are warranted.

In addition to CQ, there is interest in developing alternate autophagy inhibitors with improved pharmacokinetic properties (such as the recently-published molecule Lys05, a dimeric form of CQ47) as well as alternate mechanisms of action (such as Spautin1, the first targeted autophagy inhibitor to be published34). In our hands, Spautin1 potently blocked autophagy upregulation by Akti-1/2, leading to synergistic decreases in viability in all patient-derived cultures tested. The original characterization of Spautin1 by Liu et al. determined that Class III PI3K complex members, but particularly the
autophagy regulator ATG6/Beclin1, are all de-stabilized by administration of this drug and that their proteasomal degradation subsequently mediates autophagy blockade. We have used HeLa cells (one of the cell lines used by Liu et al.) to assess this mechanism in our hands, finding that autophagy blockade does indeed correlate with decreased Beclin1 protein levels (Fig. S4.2B). In contrast, in all ovarian cancer cells we have tested, Spautin1 achieved efficient autophagy blockade despite Beclin1 levels remaining unchanged, suggesting that Spautin1 is functioning in a Beclin1-independent manner. This finding is not without precedent: of the 3 reports of Spautin1’s use since its original publication, one assessed Beclin1 expression, and they too noted that Beclin1 levels remain unchanged despite efficient autophagy blockade. Moreover, our data are in keeping with our previous demonstration that autophagy upregulation is a Beclin1-independent process in ovarian cancer cells (see Chapter 3). Taken together, these data call for further studies to fully elucidate Spautin1’s mechanism of action, but nonetheless establish this drug as a highly-efficacious autophagy inhibitor in metastatic ovarian cancer cells.

Our finding that autophagy mediates ovarian tumor cell survival raises important questions about the cellular mechanisms underlying this function. Specifically, it remains to be determined whether autophagy promotes cell survival by protecting cells from apoptotic or non-apoptotic cell death, as this has been demonstrated in other systems. To address this question, it will be important to conduct apoptotic or necrotic cell death assays to determine if either is occurring upon autophagy blockade. Aside from preventing cell death, autophagy upregulation in ovarian cancer cells may also be influencing cell proliferation. This possibility can be tested by performing BrdU-labeling and cell cycle analysis as was done in Chapter 2. Such techniques would also be useful to further investigate the unexpected increase in cell viability observed with ATG7 knockdown (Fig. 4.1A). Since ATG7 knockdown achieves only partial autophagy blockade (see Chapter 3, Fig. 3.6C and Ref. 51), and since it is established that partial autophagy disruption – rather than its complete ablation – is known to promote mammary hyperplasia and tumorigenesis, we hypothesize that ATG7 knockdown could prompt a subtle increase in cell proliferation which manifests as the apparent increase in cell
viability that we have observed. Overall, through further investigation of cell cycle kinetics as well as apoptotic and necrotic cell death mechanisms, the mechanism by which autophagy promotes cell survival in metastatic ovarian cancer cells can be elucidated.

Overall, our work represents an important pre-clinical investigation into the utility of autophagy blockade in combination with targeted therapies. Moreover, by demonstrating the sensitivity of ascites-derived metastatic tumour cells to autophagy inhibition, our work supports the application of this therapeutic paradigm to patients with metastatic high-grade serous carcinoma, representing the majority of women with ovarian cancer. Presently, the allosteric AKT inhibitor MK-2206 (an analog of Akti-1/2), is under investigation at the phase II level in advanced ovarian, Fallopian tube, and peritoneal carcinoma (NCT01283035). With the trial of this and other PI3K/AKT/mTOR inhibitors in ovarian cancer, it will be important to determine whether autophagy is being upregulated in tumor cells as a compensatory response. If this is the case, our study suggests that combinatorial autophagy blockade may significantly enhance cytotoxicity, providing a novel therapeutic strategy that will hopefully lead to improved survival for patients suffering from this devastating disease.

4.5 References

Table S4.1: Single-agent EC50* values (μM) for ovarian cancer cells used in Combination Index analysis

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</tr>
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<td>EOC147-E2</td>
<td>4.9</td>
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*EC50 determined using non-linear curve-fit analysis of amalarBlue viability for a range of drug concentrations as described in Materials & Methods (Section 4.2)
Figure S4.1: Autophagy blockade is achieved using multiple approaches in ovarian cancer cells.

The Specific and Potent Autophagy Inhibitor 1 (Spautin1) targets the Ubiquitin Specific Peptidase (USP) 10 and 13, preventing them from stabilizing members of the Class III PI3K complex. Consequently, complex degradation prevents the induction of autophagy at the isolation membrane. siRNA-mediated knockdown of ATG5 and ATG7 expression prevents the processing and localization of LC3-II to expanding autophagosomes, thus abrogating their formation. Chloroquine (CQ) is a lysomotropic agent that de-acidifies lysosomes, nullifying pH-dependent lysosomal hydrolases and leading to a buildup of LC3-II-positive autophagosomes.
Figure S4.2: Spautin1 blocks autophagy by a Beclin1-independent mechanism in ovarian cancer cells.

Cells were seeded to 6-well adherent plates and treated following overnight incubation (Akti-1/2 5µM; Spautin1 10µM). Lysates were obtained 24h post-treatment and immunoblots performed for indicated proteins. (A) Blots of ovarian cancer cell lines OVCA429, HEY and HEYA8 are depicted, as well as (B) the HeLa cervical carcinoma cell line. (C) EOC147-E2 cells were first transfected with a Non-Targeting siRNA, or an siRNA pool targeting BECN1. Either condition was treated as previously described and immunoblot performed. Levels of indicated proteins were quantified and depicted relative to Actin (loading control).
Figure S4.3: EOC spheroids exhibit greater sensitivity to combined Akti-1/2 and CQ treatment.

Cells were seeded to (A) adherent (n = 11 EOC samples) or (B) spheroid (n = 12 EOC samples) cultures. Treatment (concentrations as indicated) was initiated following overnight incubation or at time of seeding, respectively. Viability was quantified 72h post-treatment by Trypan Blue cell counting and normalized to DMSO vehicle control for each EOC. Normalized data were pooled and one-way ANOVA with Tukey’s Multiple Comparisons Test performed for each culture condition. Bars represent Mean ± SEM for pooled EOC samples. Different letters denote statistically significant differences (p<0.001).
Chapter 5

5  Discussion

5.1 Summary of Findings

The most prevalent subtype of ovarian cancer – high-grade serous carcinoma – is also the most lethal, since the majority of cases are characterized by advanced-stage (metastatic) presentation\(^1\). Given its origins in the distal Fallopian tube, this cancer is known to rely on a sequence of cellular exfoliation and re-implantation to achieve ovarian tumor formation\(^2\). The similarity of this sequence with the process of intra-peritoneal metastasis is striking, and suggested to us that its underlying molecular mechanisms are conserved over the course of disease progression.

To characterize these mechanisms, we established an \textit{in vitro} model of metastatic dissemination using ascites-derived epithelial ovarian cancer cells (EOCs). When cultured under non-adherent conditions, EOCs formed multicellular aggregates (spheroids) resembling those seen in patient ascites (Chapter 2). Spheroid cells existed in a low-proliferation, quiescent state and also exhibited autonomous down-regulation of AKT activity. We suggested that quiescence was maintained by this reduced AKT activity via de-stabilization of SKP2, a member of the SCF ubiquitin ligase complex. As quiescence is a reversible state by definition, we also demonstrated that spheroid attachment to an adherent surface prompted constituent cells to re-enter the cell cycle in an AKT-dependent manner.

In conjunction with quiescence, we discovered that spheroid formation also induced autophagy, a conserved cellular self-digestion mechanism (Chapters 3 & 4). Likewise, pharmacologic AKT inhibition, which induces cytostasis and quiescence, also induced autophagy in both spheroids and adherent cells. By blocking autophagy induction using multiple approaches, we demonstrated that this process was important in
sustaining the viability of quiescent cells. Furthermore, combining pharmacologic AKT inhibition and autophagy blockade synergistically reduced cell viability, further supporting a cytoprotective role for this process in ovarian cancer cells.

The data presented in this thesis describe quiescence and autophagy separately in metastatic ovarian cancer cells. However, they are induced by the same stimuli (specifically, non-adherent spheroid formation or AKT inhibition) and occur simultaneously. Therefore, when considered together, we propose that coordinate induction of quiescence and autophagy constitute a dormant phenotype in metastatic ovarian tumor cells (Fig. 5.1).

5.2 Dormancy as a Unifying Concept

5.2.1 Introduction to Cancer Dormancy

Cancer dormancy is clinically defined as the ‘pause’ in cancer progression where residual disease remains undetected, only to emerge later as metastatic lesions. Dormant disease is traditionally thought to exist as disseminated tumor cells that are found lodged at common sites of metastasis in patients (e.g., bone marrow). These cells are defined as dormant based on their lack of proliferative markers such as Ki67. Mouse models developed to study the phenomenon of dormancy have also demonstrated that a low- or non-proliferative cellular state is adopted by disseminated tumor cells. Therefore, a low-proliferation or quiescent state has been established as one of the defining features of dormant tumor cells.

Given the low abundance of these cells, characterizing the molecular mechanisms regulating dormancy has been a challenge. However, Aguirre-Ghiso’s group has discovered that arrival in a foreign microenvironment activates stress-signaling pathways, ushering these cells into dormancy. More specifically, the failure of cells to engage foreign extracellular matrix components reduced integrin-mediated intra-cellular
Both quiescence and autophagy are controlled by AKT kinases via regulators such as mTORC1, p130, and p27. The LKB1/AMPK pathway is positioned to control both quiescence and autophagy downstream of AKT by inhibiting mTORC1 and phosphorylating p27 (Thr198). PERK may be an upstream activator of LKB1/AMPK signaling in dormant cells. DYRK1B regulates quiescence by phosphorylating p27 and the DREAM complex member LIN52. Together, quiescence and autophagy constitute two major features of dormancy in metastatic ovarian cancer cells; they can promote metastasis, resistance, and recurrence – all of which contribute to poor patient outcome. Autophagy box: Autophagy can be divided into “basal” and “induced” branches. While basal autophagy depends upon Beclin1, we propose that autophagy induction can proceed in a Beclin1-independent manner.

**Figure 5.1: Proposed Model of Dormancy Regulation in Metastatic Ovarian Cancer.**
signaling while activating stress-associated p38α/β signaling. This pushed cells into a quiescent state characterized by upregulation of cyclin-dependent kinase inhibitors (CKI), particularly the quiescence-associated CKI p27<sup>Kip1</sup> <sup>3,10-12</sup>. Dormancy is also associated with activation of all three arms of the unfolded protein response (UPR), of which the PERK (pancreatic endoplasmic reticulum kinase) pathway was found to be particularly important<sup>13,14</sup>. PERK activation attenuated translation initiation, thereby suppressing the expression of Cyclin D1/D3 and CDK4 genes<sup>13</sup>. Therefore, dormancy in disseminated tumor cells is mediated by mechanisms involving the loss of integrin-mediated signaling and the simultaneous upregulation of p38 and PERK signaling.

While quiescence ensures minimal proliferation of dormant cells, emerging evidence suggests that autophagy is a key mechanism promoting their continued survival. Autophagy was initially associated with dormancy in studies of <i>C. elegans</i> larvae during dauer diapause (a protective whole-organism dormancy induced by environmental stress)<sup>15</sup>. Autophagy disruption in this model compromised survival, supporting its conserved role in maintaining viability. Analogous findings have been reported in human cancer cells. In gastrointestinal stromal tumors (GISTs), for example, autophagy was required for sustained viability during therapy-induced dormancy<sup>16</sup>. This phenomenon has been described in numerous other cancer cell types as well (reviewed by White<sup>17</sup>). Most relevant to this thesis, a seminal study from the Bast lab demonstrated the necessity of autophagy for dormancy in ovarian tumor xenografts. The authors showed that re-expressing the imprinted tumor suppressor <i>DIRAS3/ARHI</i> induced autophagy and tumor dormancy. When ARHI expression was down-regulated in these dormant tumors, growth was rapidly re-initiated. Strikingly, re-growth was abolished if autophagy was blocked by CQ administration during the dormant phase, thus elegantly demonstrating its importance for the survival of dormant ovarian cancer cells <i>in vivo</i><sup>18</sup>. Taken together, these reports indicate that in addition to quiescence, autophagy is emerging as another essential feature defining a dormant phenotype.
5.2.2 Dormancy and its Mechanisms in Metastatic Ovarian Cancer Cells

Based on the current molecular understanding of dormancy presented above, we propose that quiescence and autophagy are in fact two key elements of an overarching dormancy phenotype. Although traditionally associated with solitary disseminated tumor cells with extremely long latency periods, our findings indicate that clustered ovarian tumor cells also employ quiescence and autophagy to achieve a dormant state. More than just their conceptual unification as features of dormancy, however, we propose that the coordinate induction of quiescence and autophagy in metastatic ovarian cancer is interlinked at the molecular level by common regulatory mechanisms.

One obvious example of such a mechanism is the PI3K/AKT/mTOR pathway, since throughout this thesis, we have directly demonstrated that this pathway impinges upon both quiescence and autophagy (Fig. 5.1). Aside from the AKT kinases, however, another important molecular link is the LKB1/AMPK pathway. As previously discussed, AMPK activation can promote autophagy by de-activating mTORC1 via phosphorylation of TSC1/2 and Raptor, as well as activating the autophagy-inducing kinase ULK1. Furthermore, work by Liang et al. demonstrated that the LKB1/AMPK pathway could induce autophagy by phosphorylating and stabilizing p27, although the precise mechanism linking this protein to the autophagic machinery remains unclear. In keeping with its role as a cyclin-dependent kinase inhibitor, p27 stabilization by the LKB1/AMPK pathway also contributes to the induction of cell cycle arrest and quiescence that occurs in conjunction with autophagy upregulation. Furthermore, LKB1/AMPK inhibition of mTORC1 also prevents the translation of important cell cycle-dependent genes, thus preventing proliferation. Taken together, these findings suggest that LKB1/AMPK signaling – in cooperation with p27 – can simultaneously induce both autophagy and quiescence to impart a dormant state.

The ER-stress kinase PERK is another potential regulator of both quiescence and autophagy. As previously mentioned, integrin disengagement stimulates p38-mediated
PERK activation in dormant cells, promoting quiescence by blocking the translation of cell cycle-dependent genes\textsuperscript{10,13}. Interestingly, PERK also protected dormant cells from insults such as glucose-deprivation and chemotherapy treatment, though the mechanism mediating this protection was not described\textsuperscript{13,14}. As a possible explanation, recent work has demonstrated that PERK signaling is capable of inducing a cytoprotective autophagy response in mouse embryonic fibroblasts and mammary epithelial cells\textsuperscript{25,26}. Interestingly, the stimulus for this PERK-mediated autophagy was extracellular matrix detachment, which is analogous to our own model of non-adherent culture\textsuperscript{25,26}. Moreover, PERK activation promoted autophagy by a mechanism dependent upon LKB1/AMPK-mediated inhibition of mTORC1\textsuperscript{25}. Although mechanistic details have yet to be delineated, this emerging data ties the ER-stress kinase PERK into the induction of dormancy, upstream of the LKB1/AMPK pathway.

Based on these reports, and the findings presented in this thesis, we propose that a PERK/LKB1/AMPK regulatory mechanism (also involving p27 as an effector) could promote a dormant phenotype by coordinately regulating quiescence and autophagy (Fig. 5.1). In support of this mechanism, our lab has described the immediate and sustained activating phosphorylation of both LKB1 and AMPK during spheroid formation (Peart, DiMattia and Shepherd, unpublished). We are currently engaged in knockdown studies of these proteins to determine their contribution to the dormancy phenotype. Additionally, we assessed the expression of BiP, a PERK activator, and found that it was increased 48h following seeding to non-adherent culture (Fazio, Shepherd and DiMattia, unpublished). Although we predicted this elevation in BiP, its timing was unexpectedly late given that autophagy is induced immediately following seeding. It is possible that we are seeing a delayed transcriptional response resulting in BiP upregulation following its initial dissociation and activation of PERK. It will be important to clarify the status of UPR signaling downstream of BiP by more directly assessing PERK phosphorylation and activity. If activated during spheroid formation, its contribution to the dormancy phenotype can also be tested using RNAi-mediated PERK knockdown experiments. Finally, it will be important to also assess phosphorylation of p27 at Thr198, given that this residue is uniquely linked to regulating dormancy.
In summary, the regulation of dormancy is likely multi-faceted. Our work thus far has identified that AKT signaling is able to coordinately regulate its key features quiescence and autophagy by modulating its regulators mTORC1, p130, p27. Downstream of AKT, we postulate than an intervening PERK/LKB1/AMPK pathway can also coordinately control autophagy and quiescence through many of the same targets. AKT signaling and this hypothetical PERK/LKB1/AMPK axis may function simultaneously or distinctly in a temporally-regulated or context-dependent manner. By assessing and modulating the activity of key members of these pathways, we hope to elucidate the importance of each for coordinating dormancy in metastatic ovarian cancer cells.

5.3 Remaining Questions Regarding the Role of Beclin1 in Ovarian Cancer

In Chapters 3 and 4, we characterized robust upregulation of cyto-protective autophagy in ovarian cancer cells. This finding is seemingly at odds with the accepted notion that autophagy is a tumor-suppressive mechanism, which is based largely upon evidence that the 17q21 locus (encompassing BECN1, the gene encoding the autophagy-promoting protein Beclin1) exhibits heterozygous loss in a large proportion of ovarian tumors\textsuperscript{27-31}. Losses of 17q21 were taken to mean that BECN1 gene dosage is critically important for autophagy. Thus, we questioned how autophagy could still be induced in ovarian tumor cells. As a first step, we assessed protein expression of Beclin1 using datasets from The Cancer Genome Atlas (TCGA) project, and were intrigued to find that instead of being reduced in tumors with mono-allelic BECN1 loss, it was retained at near-diploid levels. Suspecting that this maintenance of protein level may be facilitating autophagy upregulation, we next knocked down Beclin1 expression using siRNAs. Surprisingly, this had no effect on autophagy induction in ovarian cancer cells, leading us to conclude that while a diploid level of Beclin1 is retained in the majority of ovarian cancer cells, this protein appears dispensable for their induction of autophagy.
5.3.1 Autophagy-independence

Our findings raise significant questions about the relationship between Beclin1 and autophagy in ovarian cancer. For instance, what function is Beclin1 serving in ovarian tumors if not mediating autophagy upregulation? Evidence of autophagy-independent functions for Beclin1 and the PI3K C3 complex have been emerging in recent years (discussed in Chapters 1 & 3). Extra-autophagy functions are also evident upon closer examination of the mouse models of autophagy disruption (see Chapter 1, Table 1.2). Specifically, the knockout phenotype of core PI3K C3 complex members (Ambra1, Beclin1, Pik3c3) is embryonic lethality32-35, whereas embryos harboring knockout of other Atg genes (Atg3, Atg5, Atg7) are viable at birth (though they die at post-natal day 1) 36-39. Additionally, while Becn1 heterozygotes develop malignant carcinomas and lymphomas32,33, complete knockout (tissue-specific) of Atg5 or Atg7 is required for tumor formation40 – however, tumors that do develop upon Atg5/7 knockout are only benign adenomas and never the spectrum of malignancies seen with heterozygous Becn1 disruption32,33,40. Interpreting these comparisons, one could conclude that the more severe phenotypes observed with abrogation of PI3K C3 complex members (most notably of Becn1), indicates important extra-autophagy functions.

We have begun to explore possible autophagy-independent functions of Beclin1 by performing viability assays on ovarian cancer cells subjected to Beclin1 knockdown. Although autophagy remained unaffected (as demonstrated in Chapter 3), we nonetheless observed a significant reduction in cell viability (Appendix A, Fig. 1). Therefore, Beclin1 appears to be serving a pro-survival function, though we currently do not have data clarifying the specific autophagy-independent mechanism by which this occurs. It will be important to first investigate how Beclin1 depletion reduces viability by performing cell cycle analysis as well as cell death (apoptotic and non-apoptotic) assays. These and other experiments can be used to direct further mechanistic studies into Beclin1’s autophagy-independent function(s).
5.3.2 \textit{BECN1} and \textit{BRCA1}

Our work also raises a fundamental question about the reason for \textit{BECN1} heterozygous loss in ovarian cancer: if Beclin1 is in fact dispensable for autophagy upregulation, does its mono-allelic loss actually promote ovarian tumorigenesis? Interestingly, upon closer examination of TCGA copy-number data from serous ovarian tumors, we have noted that \textit{BECN1} mono-allelic deletion results from large deletion events encompassing chromosomal region 17q21 (Appendix A, Fig. 2A), rather than focal loss of the \textit{BECN1} locus. It is therefore conceivable that loss of other tumor-suppressive genes in this region, and not \textit{BECN1}, are ‘driving’ these mono-allelic deletion events. In other words, \textit{BECN1} may simply be a ‘passenger’ gene that is inextricably linked to copy-number deletions on chromosome 17q.

One possible candidate ‘driver’ gene is the breast and ovarian cancer susceptibility gene \textit{BRCA1}. Although not mentioned in the Beclin1 and autophagy literature, \textit{BRCA1} is actually located only 218,000 bp telomeric to \textit{BECN1} (Appendix A, Fig. 2A). Moreover, there is a nearly perfect correlation between \textit{BECN1} and \textit{BRCA1} heterozygous loss (Appendix A, Fig. 2B).

\textit{BRCA1} is of obvious importance to ovarian tumorigenesis. Germline mutations of this gene are found in \textasciitilde10\% of ovarian cancer patients\textsuperscript{41} and are associated with the majority of hereditary tumors\textsuperscript{42}. Sporadic ovarian cancers also harbour somatic \textit{BRCA1} mutation in \textasciitilde4\% of cases (Ref.\textsuperscript{41} and Appendix A, Fig. A2B), as well as epigenetic silencing via hypermethylation of the \textit{BRCA1} promoter in 11\% of cases\textsuperscript{41}. Importantly, all of these \textit{BRCA1} alterations are often accompanied by loss of the single remaining wild-type allele (i.e., loss-of-heterozygosity, or LOH), resulting in decreased mRNA and protein expression\textsuperscript{43,44}. Furthermore, nearly all cases of hypermethylation occur in conjunction with single-allele loss\textsuperscript{45}. Therefore, \textit{BRCA1} single-allele loss is a prevalent feature of both hereditary and sporadic ovarian tumors. It is tempting to speculate that instead of \textit{BECN1}, loss of a \textit{BRCA1} allele may be an important driver of chromosome 17q deletions.
5.3.3 A Proposed Model of Beclin1 and Autophagy Regulation in Ovarian Cancer

It is presently unclear what \textit{BECN1} loss contributes to ovarian tumorigenesis, or if it is simply lost as a ‘passenger’ as discussed above. Therefore, in the absence of experimental evidence clearly delineating the role of \textit{BECN1} and autophagy in ovarian cancer, we propose a model that attempts to satisfy seemingly contradictory viewpoints on this subject while integrating our own findings of Beclin1-independent autophagy. This model separates basal autophagy from autophagy induction, and by considering each as a distinct process, attempts to accommodate both Beclin1-dependent and – independent mechanisms of regulation (Fig. 5.1).

Specifically, we envision that Beclin1-dependent basal autophagy runs at unstimulated, homeostatic levels – by definition, it is not dynamically modulated and therefore is not intertwined with complex regulatory mechanisms. In contrast, autophagy induction is stimulated by a diverse array of cellular stresses and thus may have evolved greater versatility and redundancy in its regulation. According to this model, \textit{BECN1} could undergo heterozygous loss in precursor lesions, partially ablating basal autophagy to promote ovarian tumorigenesis. Once a tumor has become established, however, microenvironmental factors (e.g., spheroid formation) or anti-cancer therapeutics (e.g., AKT inhibition) may stimulate cyto-protective autophagy induction by mechanisms that circumvent both Beclin1 and basal autophagy altogether.

Importantly, this model does not preclude autophagy-independent functions of Beclin1. In fact, we further speculate that such functions could be responsible for Beclin1’s sustained protein expression in advanced tumors. Given that its autophagy-independent function(s) appear important in promoting metastatic tumor cell viability (Appendix A, Fig. 1), this could drive its protein expression back to near-diploid levels. Thus, we speculate that following initial down-regulation to promote tumorigenesis, Beclin1’s autophagy-independent functions prompt its advantageous re-expression in established tumors.
It is clear that many questions remain regarding the role of Beclin1’s autophagy-dependent and –independent functions in ovarian cancer. These would be best addressed using a mouse model of HGS-OvCa pathogenesis in combination with Beclin1 disruption. For example, Kim et al. recently characterized such model based on conditional deletion of Dicer and Pten in the oviduct\textsuperscript{46}. By crossing these mice with Beclin1\textsuperscript{+/-} mice, we could study the effects of heterozygous Beclin1 loss on various paramaters of HGS-OvCa pathogenesis (e.g., tumorigenesis, time to ascites formation, survival, etc.). Such a model would also allow in vivo assessment of autophagy (e.g., LC3 immunoblot or immunofluorescence) as well as Beclin1 protein levels over the course of disease progression. Therefore, the combination of Beclin1 disruption with models of HGS-OvCa could help clarify the contribution of Beclin1 and autophagy to the pathogenesis of this disease.

5.4 Remaining Questions Regarding the Regulation of Quiescence

In Chapter 2, it was suggested that reduced AKT activity during spheroid formation promotes quiescence by destabilizing the essential SCF ubiquitin-ligase complex member SKP2, thus preventing SCF-mediated ubiquitination and degradation of p130 and p27. This mechanism was proposed based on clear evidence in the literature demonstrating that p130 and p27 are targets of the SCF complex\textsuperscript{47-49} and that AKT exerted control over the SCF complex through its phosphorylation and stabilization of SKP2\textsuperscript{50,51} (Fig. 5.1). To actually test this mechanism in our model, we have obtained adenoviral expression constructs of AKT1 and AKT2, each containing an N-terminal myristoylation sequence (a site of attachment of a myristoyl group, a covalent post-translational modification that ensures constitutive membrane localization and kinase activation). The establishment of constitutively-activate AKT using these constructs has been confirmed (Ramos-Valdes, Shepherd and DiMattia, unpublished). We would expect that this enforced AKT activity would suppress cell cycle exit to G0. If this occurs via SKP2, RNAi-mediated depletion of this protein should serve to rescue the quiescent state.
Through these experiments, we hope to delineate the contribution of AKT and SKP2 to mediating quiescence in our model.

We are also interested in assessing the contribution of the DYRK kinases to spheroid-mediated quiescence. As described in Chapter 1, DYRK1A/B directly phosphorylates and promotes the assembly of DREAM complex members at the promoters of E2F target genes, thereby facilitating exit from the cell cycle. DYRK1B also phosphorylates Cyclin D1 and p27 to decrease and increase their abundance, respectively, promoting quiescence as a consequence. Although it exhibits low expression in most normal tissues, early studies revealed that the DYRK1B gene locus is amplified in 30% of ovarian cancers. A more recent multi-platform genomic analysis of high-grade serous ovarian tumors also reported focal amplification of 19q13.2, a chromosomal region that includes DYRK1B. Using publicly available copy-number and mRNA expression data (including data from Ref.), we found that DYRK1B copy-number gain and high-level amplification occurred in 222/559 (39%) of high-grade serous carcinomas (Appendix A, Fig. 3A), appeared to be focal in nature (Appendix A, Fig. 3B), and correlated with DYRK1B mRNA expression (R²=0.32, r = 0.57, p<0.0001; Appendix A, Fig. 3C). Moreover, we found that amplification and/or elevated mRNA expression of this kinase significantly correlated with decreased overall survival (p<0.05; Appendix A, Fig. 3D).

Given that DYRK1B is amplified in high-grade serous ovarian tumors and that this correlates with survival, it is of great interest to determine whether its protein product regulates quiescence in our model. Friedman’s group has previously demonstrated that DYRK1B mediates quiescence in ovarian cancer cells subjected to serum starvation. We suspect that it may also drive quiescence during spheroid formation (despite full-serum conditions), perhaps most significantly in EOCs that exhibit DYRK1B amplification. This could be tested by simply depleting DYRK1B protein abundance in those samples exhibiting the highest levels of expression and subsequently determining how this affects quiescence during spheroid formation. Alternatively, specific DYRK1B small-molecule inhibitors can be used to modulate its kinase activity, as these have
recently been published\textsuperscript{57}. Such experiments would determine not only the status of DYRK1B in ascites-derived ovarian tumor cells, but also its contribution to spheroid-associated quiescence.

### 5.5 Remaining Questions Regarding the Effects of Autophagy Blockade

Concluding Chapter 4, we stated that autophagy promotes the survival of metastatic ovarian cancer cells undergoing spheroid formation and/or subjected to AKT inhibition. This was based on cell viability assays measuring mitochondrial activity (alamarBlue), ATP abundance (CellTiter Glo), or the number of cells with intact plasma membranes (Trypan Blue exclusion cell counting). However, a detailed investigation of autophagy’s function necessitates other assays that move beyond assessing cell viability. For one, it will be important to assay for cell death to determine whether blocking autophagy actually causes cells to undergo apoptosis or necrosis.

Additionally, as a functional assay of viability, we could also use an in vivo xenotransplantation model to test tumor-forming potential of cells and spheroids. This would involve intra-peritoneal (IP) injection of ovarian cancer cells or spheroids to establish tumors and/or ascites, as previously described\textsuperscript{58}. If cells or spheroids were pre-treated in vitro, this model would serve as an in vivo assay of their viability and tumorigenic potential, and the effects of autophagy-modulation during the pre-treatment phase could be determined. Alternatively, if treatments were conducted in vivo following IP injection and tumor formation, this model could also serve as a pre-clinical tool to explore autophagy blockade as a potential therapeutic modality for ovarian cancer metastasis.

### 5.6 Therapeutic Targeting of Dormancy in Metastatic Ovarian Cancer
Dormancy is associated with therapeutic resistance\textsuperscript{3,59}. This effect has traditionally been attributed to quiescence, which renders cells less vulnerable to chemotherapeutics by virtue of their non-cycling state. Moreover, upregulation of the quiescence-mediator p27 is associated with chemotherapeutic resistance\textsuperscript{60-62}. However, in addition to quiescence, other mechanisms are thought to contribute to dormancy-associated therapeutic resistance, such as p38-mediated inhibition of apoptosis, for example\textsuperscript{14}. Autophagy, which is emerging as a critical feature of dormancy, has also been implicated as a therapeutic resistance mechanism in various settings\textsuperscript{63-66}, specifically in the context dormant cells\textsuperscript{16}. Therefore, since dormancy is closely tied to therapeutic resistance, we propose that targeting it in metastatic ovarian tumor cells will result in greater therapeutic sensitivity.

5.6.1 Targeting Quiescence in Metastatic Ovarian Cancer

By targeting quiescence-promoting mechanisms, it may be possible to drive G0 cells into the cell cycle, and in doing so, re-sensitize them to chemotherapeutics. Recently, targeted inhibitors of DYRK1B have been published\textsuperscript{57}. Friedman’s group has demonstrated that inhibition of DYRK1B drove ovarian cancer cells (but not normal fibroblasts) into the cell cycle prematurely, leading to ROS accumulation and apoptosis\textsuperscript{67}. Additionally, citing our work (Chapter 2) as their rationale, this group has recently begun testing DYRK1B inhibition in ascites-derived cultures\textsuperscript{55} (AACR 2013: Abstract #2164). It will be interesting to determine the effect of combinatorial DYRK1B inhibition and chemotherapy treatment in our \textit{in vitro} metastasis model. Additionally, use of pre-clinical \textit{in vivo} models of metastasis (e.g., xenotransplantation via IP injection, as proposed above) would be expected to recapitulate our \textit{in vitro} results and provide insight into the utility of this therapeutic paradigm in patients.
5.6.2 Targeting Autophagy in Metastatic Ovarian Cancer

In addition to targeting quiescence, autophagy blockade may also hold promise for sensitizing dormant ovarian cancer cells to therapeutics. The rationale for targeting autophagy upregulation in metastatic ovarian cancer cells has already been discussed in Chapter 4 and data from this chapter demonstrate that combining Akti-1/2 (an allosteric AKT inhibitor) with autophagy blockade synergistically compromises cell survival. In this chapter, we have suggested further experiments to address the mechanism underlying these effects, as well as to test the potential of this therapeutic strategy \textit{in vivo}. These and further pre-clinical studies are highly relevant, since the AKT inhibitor MK-2206 (an analog of Akti-1/2), is currently undergoing clinical trials in patients with ovarian, Fallopian tube, and peritoneal cancers (NCT01283035). If patient tumors treated with this drug respond by inducing autophagy, as our \textit{in vitro} data suggests they would, it will be important to test whether combinatorial autophagy blockade can enhance its efficacy. Interestingly, an unrelated trial of MK-2206 and autophagy blockade has been initiated for advanced solid tumors – some of which might be ovarian tumors (NCT01480154). It is hoped that this and other trials of autophagy blockade (summarized in Chapter 1, Table 1.4) will not only demonstrate improved efficacy, but also encourage more extensive investigation into the potential of autophagy inhibition for cancer treatment.

5.7 Synthesis

This thesis began with the development of an \textit{in vitro} model of ovarian cancer metastasis and led to the discovery that quiescence and autophagy – key features of a dormant state – can readily be induced by metastatic ovarian cancer cells. The data presented in Chapters 2, 3, and 4 characterize these features and the molecular mechanisms governing their regulation. Taken together, this body of work has contributed to a deeper understanding of the ovarian cancer metastatic process as well as provided a rationale for targeting dormancy mechanisms as a novel therapeutic paradigm in ovarian cancer. To fully realize the potential of targeting dormancy, both basic and
pre-clinical investigations are currently underway in ours and other laboratories. By delineating the molecular underpinnings of dormancy as well as applying this understanding to the clinical realm, it may be possible to someday eradicate dormant tumor cells that are at the root of metastasis, therapeutic resistance, and disease recurrence.

5.8 References


25. Avivar-Valderas A, Bobrovnikova-Marjon E, Alan Diehl J, Bardeesy N, Debnath J, Aguirre-Ghiso JA. Regulation of autophagy during ECM detachment is linked to a selective inhibition of mTORC1 by PERK. Oncogene 2012.


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Appendix A: Additional Figures

**Figure 1: Depletion of Beclin1 significantly reduces cell viability.**
EOC147-E2 were transfected with Control siRNAs or siRNA pools targeting BECN1. Transfection was repeated 72h later, and following overnight incubation, cells were seeded to their respective culture condition. (A) Adherent cultures in quadroplicate wells were treated with DMSO vehicle control or Akti-1/2 (3.5µM) and viability assessed by Alamar Blue at 72h post-treatment (n = 4 repeated experiments). (B) Spheroids were formed in ultra-low attachment plates. Triplicate wells were treated with DMSO vehicle control or Akti-1/2 (1µM) and viability assessed by CellTiter Glo (n = 2 repeated experiments). Bars: Mean ± SEM relative to Control siRNA pool. One-way ANOVA with Tukey’s Test was performed. Different letters denote statistically significant differences (p<0.01).
A) chr 17:

23 Mb on chr 17q

TCGA Ovarian Tumors from 569 Patients

B) Chr 17:

BECN1 ∆ BRCA1

C) Altered in 244 (78%) of 311 cases

BECN1 & BRCA1

Copy number alterations are putative.
Figure 2: Broad deletions of chromosome 17q in high-grade serous ovarian carcinoma encompass both the \textit{BECNI} and \textit{BRCA1} genes.

(A) Log2-transformed copy number data from 569 high-grade serous ovarian cystadenocarcinomas were generated by The Cancer Genome Atlas (TGCA) Research Network and are depicted as a heat-map using the Integrative Genomics Viewer (Broad Institute). Samples sorted based on BECN1 copy-number (White: diploid copy-number; Red: gain/amplification; Blue: loss/deletion) (B) A ~500,000 bp region from chromosome 17q21.31 is expanded to reveal the \textit{BECNI} and \textit{BRCA1} genes (separation: 218,000 bp). Image generated using NextBio Genome Viewer (http://www.nextbio.com/) (C) Sequencing and copy-number data for the \textit{BECNI} and \textit{BRCA1} genes from 311 high-grade serous ovarian cystadenocarcinomas are depicted as an OncoPrint (cBio Portal for Cancer Genomics; http://www.cbioportal.org/; Memorial Sloan-Kettering Cancer Center). Vertical lines represent a single sample (blue = homozygous deletion; teal = heterozygous loss; green = mutation).
A) Altered in 222 (39%) of 569 Cases

DYRK1B

Gene Set Not Altered
Gene Set Altered

Logrank test p-value: 0.036101

B) chr 19:

9.6 Mb on chr 19q

TCGA Ovarian Tumors from 569 Patients

DYRK1B

C) R^2 = 0.32
r = 0.57
p < 0.0001

mRNA Expression (log2)

D) Logrank test p-value: 0.036101
Figure 3: *DYRK1B* amplification in high-grade serous ovarian carcinoma correlates with reduced overall survival.

(A) Low-level gains (pink) or high-level amplification (red) of the *DYRK1B* gene in 569 high-grade serous ovarian cystadenocarcinomas are depicted in an OncoPrint (cBioPortal for Cancer Genomics; [http://www.cbioportal.org/](http://www.cbioportal.org/); Memorial Sloan-Kettering Cancer Center). Tumour samples were analyzed by The Cancer Genome Atlas (TCGA) Research Network. (B) The Integrative Genomics Viewer (Broad Institute) was used to generate a heat-map representation of log2-transformed copy number values (sorted based on *DYRK1B* amplification; white = diploid copy-number, red = gain/amplification, blue = loss/deletion). (C) mRNA expression data from 538 of these samples were plotted as a function of log2 copy-number, and linear-regression analysis performed. (D) Overall survival was compared between patients with low-level gain, high-level amplification, and/or expression z-score >1.5 (*DYRK1B* High) and those without (*DYRK1B* Low) using cBioPortal’s survival analysis tools. Results of log-rank test are indicated.
Appendix B: Ethics Approval

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Gabriel DiMatia
Review Number: 12668E
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
Expiry Date: December 31, 2015
Documents Reviewed & Approved & Documents Received for Information:

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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 of Research Involving Humans and the Health Canada/JRH 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 the HSREB also complies with the membership requirements for REBs 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 00009540.

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Ethics Officer to Contact for Further Information:

- [Janice Telford](mailto:jtelford@uwyo.ca)
- [Grace Kelly](mailto:grace.kelly@uwyo.ca)
- [Shaela Walcott](mailto:shaela.walcott@uwyo.ca)

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Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. T.G. Shepherd
Review Number: 16391E
Review Date: August 12, 2009
Review Level: Expedited
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.

Chair of HSREB: Dr. Joseph Gilbert

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## Appendix C: Summary of Clinical Data for EOCs

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Appendix D: Protocol for Processing Solid Tumor Samples

Protocol for generating (A) fixed and (B) frozen specimens, as well as to (C) release tumor cells for *in vitro* culture.

**Equipment:**
- scalpel handle (autoclaved, sterile)
- scalpel blades (sterile)
- orange-capped (pee-bottle) container filled with 10% formalin
- tissue cassettes
- forceps (2 or more pairs; autoclaved, sterile)
- dry ice/ethanol bath
- dry ice pellets (small container)
- sheet of tin foil (approx. 15cm in length)

- Ensure that tumor tissue is submerged in sterile PBS as soon as possible to maintain viability and moisture. Ideally the sample is received already submerged in saline from the OR.
- Dissect the tumor tissue as symmetrically as possible (i.e., try to include all the different ‘regions’ of the tumor sample if you can grossly distinguish morphological differences). This way, all ‘regions’ can be represented in specimens that are frozen, fixed, and processed for *in vitro* culture.

(A) To fix the tissue:

1. Label the cassette (using a pencil) with patient number or some other distinguishing number.
2. Place individual pieces in tissue cassettes, close cassettes by snapping shut.
3. Drop cassettes into formalin container
4. Write patient sample number, description of sample (e.g., “omentum nodule”), and date on side of container
5. Store in 4°C fridge (“Rohann” shelf).
6. These samples should be rinsed in PBS twice the next day and subsequently stored in 70% EtOH.

(B) To freeze the tissue:

1. Tear tin foil into ~3cm² squares (for each piece of tissue)
2. Completely wrap each piece in its own tin foil square
3. Immediately bury wrapped pieces in dry ice pellets.
4. Once frozen, collect all wrapped pieces and place in a 50mL tube.
5. Write patient sample number, description of sample (e.g., “omentum nodule”), and date on side of tube.
6. Store @ -80°C in “Solid Tumor Samples” box (lowest shelf, stand-up -80°C freezer)
(C) To harvest tumor cells:

1. Dissect tumor pieces further into smaller (2-5mm²) pieces
2. Place each piece in its own 15mL tube
3. Pipet 1mL of Trypsin/EDTA into tubes with tissue
4. Place tubes in 37°C water bath for 30-45 min., vortexing every 10 min.
5. Collect 1mL of trypsin and tumor piece from each tube and pipette into individual wells of a 6-well plate
6. Pipette an additional 1mL of trypsin to each well
7. Using a sterile scalpel, chop and mince tumor pieces as thoroughly as possible
8. Using a pipette, tritrate trypsin (now 2mL) and minced tumor to attempt to break up clumps
9. Collect contents and return to 15mL tubes.
10. Return tubes to 37°C water bath for another 30-45 min., vortexing every 10 min.
11. Inactivate trypsin with 3mL of FBS-containing medium
12. Spin @ 1,500 rpm for 3 min.
13. Aspirate supernatant and resuspend pellet in 3mL medium (tritrate pellet several times)
14. Pass resuspended pellets through 40µm cell strainers (can use a single strainer several times) and into a common 50mL tube (i.e., pool all samples at this point)
15. Seed 6-well plates with filtered, single cell suspension (3mL/well)

Note: Tissue fragments collected in the cell strainer can be recovered by washing out the bottom of the strainer using PBS. Fragments can then be pelleted, resuspended in media, and cultured to obtain tumour cells.
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Curriculum Vitae

Rohann Jonathan Mark Correa

Education

2008 – Present  MD/PhD Degree
Schulich School of Medicine & Dentistry
University of Western Ontario, London, ON

2004 – 2008  Bachelor of Science Degree (Honours) – Biology with Thesis
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Research Experience

2008 – Present  PhD Student, Department of Biochemistry
Translational Ovarian Cancer Research Program
Supervised by: Dr. Gabriel E. DiMattia and Dr. Trevor G. Shepherd

2006 – 2008  BSc Student, Department of Biology
(Honours Thesis, Summer NSERC USRA, Summer Studentship)
Hubberstey Cytoskeleton Lab
Supervised by: Dr. Andrew V. Hubberstey

Publications


Conference Presentations


Renée Tousignant*, Jessica Cucullo, **Rohann Correa**, and Andrew Hubberstey (2008) Expression and effects of mammalian WDR1 isoforms on neurite extension and cell migration. 48th Annual Meeting of the American Society for Cell Biology, December 13-17, San Francisco, CA, USA *Presenter

**Awards & Achievements**

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<td>2012 – Present</td>
<td>PhD Award – Obstetrics &amp; Gynaecology Graduate Scholarship</td>
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<td>2010 – 2012</td>
<td>PhD Award – CIHR Strategic Training Program in Cancer Research and Technology Transfer</td>
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<td>2010</td>
<td>5th Canadian Conference on Ovarian Cancer Research – Teal Heart Award for best overall oral presentation</td>
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<td>2009, 2010</td>
<td>Margaret Moffat Research Day, University of Western Ontario – Best poster, cancer category</td>
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<td>2009 –2010</td>
<td>Masters Award – CIHR Institutes of Gender Health / Ontario Women’s Health Council</td>
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<td>2009, 2011</td>
<td>Paul Harding Research Day, Ob/Gyn Department, University of Western Ontario – Best Poster, Grad. Student Category (2009); 2nd Place Oral</td>
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Presentation, Grad. Student Category (2011)

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<td>U of Windsor President’s Medal</td>
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<td>2008</td>
<td>NSERC Undergraduate Student Research Award</td>
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<td>2008</td>
<td>President’s Roll/Dean’s List – all semesters of study</td>
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<td>2006</td>
<td>NSERC Undergraduate Student Research Award</td>
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<td>2006</td>
<td>U of Windsor In-Course Scholarship</td>
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<td>2004</td>
<td>U of Windsor Entrance Scholarship &amp; Community Scholars Award</td>
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<td>2004</td>
<td>Elected Valedictorian &amp; named Most Outstanding Graduate</td>
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**Community Activities**

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<td>Founding Member &amp; Presenter – Canadian Cancer Society Research Information Outreach Team (RIOT)</td>
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<td>Hospital Volunteer – Hotel-Dieu and Windsor Regional Hospital</td>
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<td>Selection Board Member – U of Windsor Volunteer Internship Program</td>
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<td>2005 - 2008</td>
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