Defining the Roles of Autophagy in Ovarian Carcinoma

by

Jaeline E. Spowart
B.Sc., University of Victoria, 2009

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in the Department of Biochemistry and Microbiology

Supervisory Committee

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Abstract

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Ovarian cancer is a significant concern for women's health as it is the most lethal of all gynaecological malignancies. One of the reasons for the high mortality of this disease is that traditionally used chemotherapeutic treatments tend to have poor initial or sustained efficacy against ovarian tumours. Resistance to such treatments may in part be mediated by autophagy, a cell survival process in which unnecessary or damaged components of the cytoplasm are engulfed within a double-membraned vesicle known as an autophagosome and ultimately degraded upon fusion of the autophagosome with a lysosome. Autophagy has been shown to be employed by cells to aid in their survival under stresses such as nutrient deprivation, hypoxia, chemotherapy treatment, and growth factor withdrawal. As these stresses are commonly encountered by ovarian cancer cells, it is possible that autophagy promotes ovarian cancer cell survival. This thesis aims to investigate which stimuli induce autophagy in ovarian cancer cells and whether or not this induction can promote cell survival. In addition, there is a particular focus on the comparison of autophagy utilization between subtypes of ovarian cancer, as the subtypes are in fact considered different diseases and may vary in their usage of autophagy.

The first chapter of this thesis provides relevant background information on autophagy as well as ovarian cancer and its subtypes. In the second chapter, I describe

studies in which tumours from a large cohort of patients with ovarian cancer are assessed for LC3A, a marker of autophagy, in addition to markers of other cellular processes including hypoxia. Here I found that LC3A was significantly associated with poor patient survival in patients with the clear cell subtype of ovarian cancer, but not other subtypes. I also found that LC3A expression was associated with markers of hypoxia in the clear cell patient tumours and that clear cell carcinoma cell lines preferentially induced autophagy in response to hypoxia *in vitro* as compared to cell lines of the high-grade serous subtype. These results indicate that clear cell ovarian tumours are uniquely dependent upon autophagy in response to hypoxia. In the third chapter, I investigated the autophagic response to treatment with the standard ovarian cancer chemotherapy drugs carboplatin and paclitaxel in a syngeneic mouse model of ovarian cancer. I found that these drugs did indeed induce autophagy and that the cancer cells utilized autophagy to promote resistance to these chemotherapeutics. In addition, when the tumour cells were grown in syngeneic mice, treatment with the autophagy inhibitor hydroxychloroquine resulted in a significant suppression of tumour growth. Together, my findings indicate that further investigation into the use of autophagy inhibitors in ovarian cancer patients is warranted and that different specific rational drug combinations for each subtype will likely yield optimal results.

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Acknowledgments

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Dedication

This thesis is dedicated to all of the women afflicted with ovarian cancer. May we find better ways to help you fight and conquer this disease.

Chapter 1: Introduction

1.1 Autophagy

1.1.1 The beginnings of the autophagy field

The term "autophagy" was first coined by Christian de Duve in 1963 at the Ciba Foundation Symposium on Lysosomes [1]. Though de Duve is perhaps best known for his work on lysosomes, for which he was awarded the Nobel Prize in Physiology or Medicine in 1974[1], he is widely considered to be the father of the field of autophagy. "Autophagy" literally means "self-eating" and was coined to describe the lysosomal degradation of cytoplasmic organelles and constituents as opposed to the breakdown of extracellular material, or heterophagy [1-3]. Early on, autophagy was observed in the presence of a variety of stimuli and in several different cell types. However, the first confirmed inducer of autophagy was glucagon, a link that was first reported in 1962 and confirmed in 1967 [2-4]. After another decade, the anticipated reverse function was described, namely insulin and amino acids were found to inhibit autophagy [5, 6]. This collection of results helped to establish the most well-known role of autophagy: a catabolic process activated in times of low nutrient levels to liberate the metabolites that cells need to survive such periods, but that is generally suppressed during times of ample nutrient availability.

Over the next two decades, a smattering of papers were published on autophagy. However, it was in the late 1990's that the field really expanded with the beginning of the elucidation of the molecular mechanisms of autophagy. A Japanese researcher, Yoshinori Ohsumi, started investigating the autophagy process in the genetically tractable system of

[1] Source: www.nobelprize.org/nobel_prizes/medicine/laureates/1974/press.html

yeast in the early 1990's and found that the morphology of the process in yeast was similar to that in mammals [7]. Ohsumi's lab continued to work out the molecular basis of autophagy in yeast by carrying out genetic screens for autophagy mutants, and in 1997 cloned the first autophagy-related (ATG) gene, *ATG1* [8, 9]. Within the next ten years, another 30 *ATG* genes were identified in yeast [10-14].

The knowledge of the molecular mechanisms of autophagy that was gained from yeast studies provided a valuable basis for autophagy research in other systems, particularly mammalian, and helped encourage and facilitate such research. Autophagy has now been implicated in a number of biological processes and pathologies, including immunology and inflammation, neurodegenerative diseases, and cancer, which has promoted further research in the field, motivated both by scientific curiosity and the potential to improve the outcomes of patients afflicted with such pathologies [15-17]. The profound growth of the autophagy field in recent years is illustrated by the fact that a decade ago there were less than 100 articles pertaining to autophagy published per year, whereas last year alone there were over 2200 autophagy articles published.[2] This field continues to grow rapidly and without doubt many new and exciting advances will be made in the near future.

1.1.2 The general process of autophagy

There are several different classes of autophagic processes including macroautophagy, chaperone-mediated autophagy, and microautophagy [18]. The latter two are not encompassed within the subject matter of this thesis and will not be discussed further. Macroautophagy will herein be referred to as "autophagy".

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[2] Source: www.ncbi.nlm.nih.gov/pubmed/

The mammalian target of rapamycin (mTOR) is a key regulator of autophagy. Many different stressors and activators converge on mTOR to dictate the promotion or inhibition of autophagy, respectively (Figure 1) [19]. mTOR complex 1 (mTORC1) accomplishes autophagy regulation through its interaction with the unc-51-like kinase 1/2 (ULK1/2)-Atg13-200 kDa focal adhesion kinase family-interacting protein (FIP200)-Atg101 complex. When mTORC1 is active, it phosphorylates this complex resulting in inhibition of ULK1/2 kinase activity. When mTORC1 activity is downregulated, ULK1/2 dissociates from mTORC1, resulting in dephosphorylation of ULK1/2, and activation of ULK1/2 kinase activity. Active ULK1/2 phosphorylates FIP200, Atg13, and itself. Once the ULK1/2 complex is activated, it localizes to the developing phagophore (or isolation membrane), the double-membraned autophagosome precursor [19].

At the site of the phagophore is the Beclin 1 complex which is composed of Beclin 1, vacuolar protein sorting (Vps) 34 (phosphatidylinositol 3-kinase (PI3K) class III), Vps15 (also known as p150), and activating molecule in Beclin 1-regulated autophagy 1 (AMBRA1) (Figure 1) [18]. The Beclin 1 complex produces phosphatidylinositol-3-phosphate (PI3P), a requirement for autophagosome formation [18]. The ULK1/2 complex also phosphorylates AMBRA1, releasing it from the microtubule-associated dynein motor complex, freeing the Beclin 1 complex to relocalize to the endoplasmic reticulum (ER) where it can facilitate autophagosome nucleation [20].

Downstream of the Beclin 1 complex are two ubiquitin-like systems: the Atg12-Atg5 conjugation system and the microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3) conjugation system (Figure 1). Atg12 conjugation to Atg5 is mediated by Atg7 (an E1-like enzyme) and Atg10 (an E2-like enzyme). Atg5 then interacts with Atg16L to form an Atg12- Atg5-Atg16L complex which localizes to the phagophore, promotes autophagosome

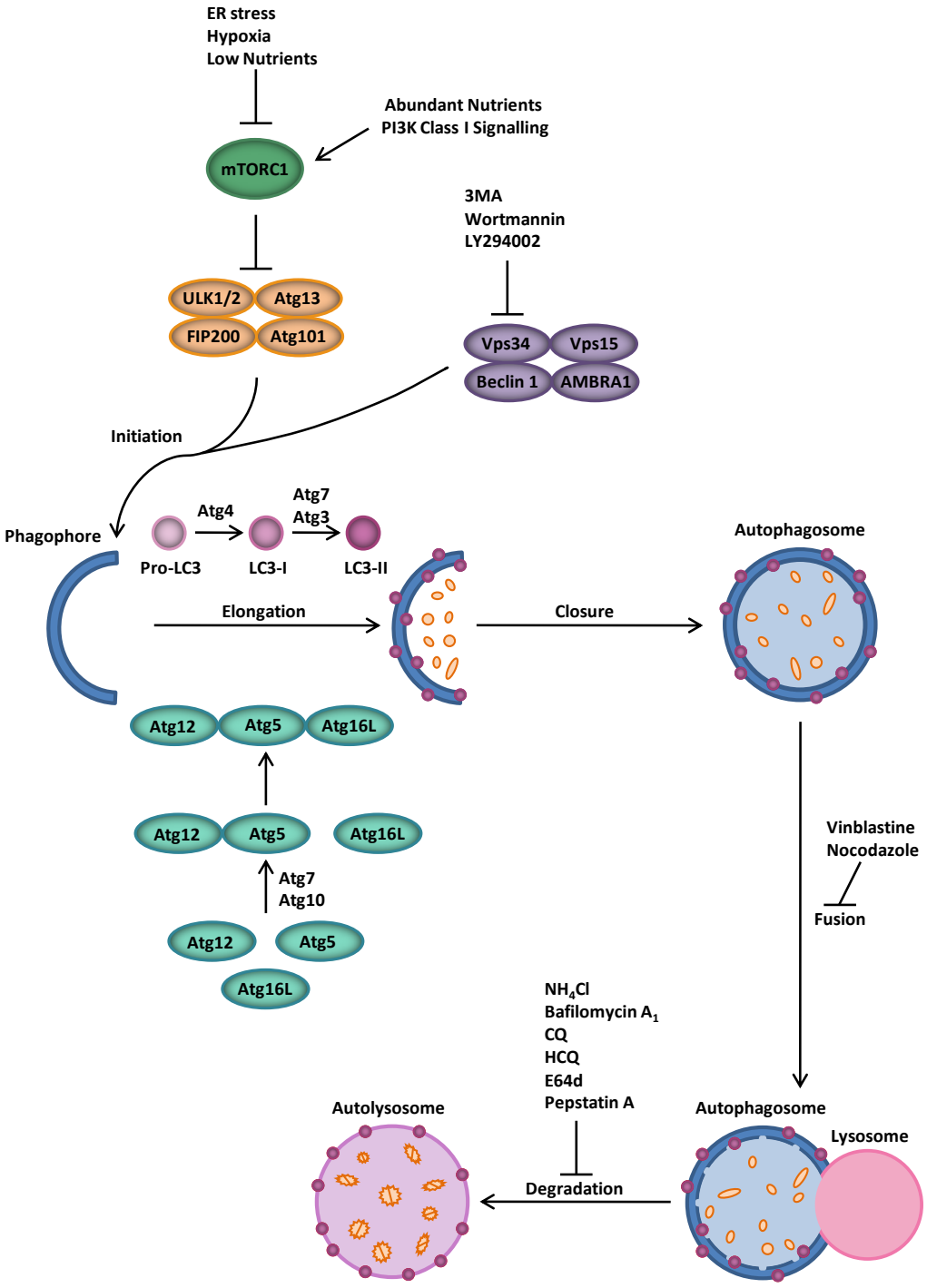

Figure 1. The general mechanism of the autophagy process and key targets of chemical autophagy inhibitors.

(caption on following page)

Figure 1. (Shown on previous page). Various anti- or pro-autophagic signals converge on mTORC1. When mTORC1 is active, it negatively regulates the ULK1/2 complex. When mTORC1 is suppressed, the ULK1/2 complex is activated and in turn promotes the activation of the Beclin 1 complex. These activations result in initiation of the autophagic process, beginning with the developing phagophore which sequesters cargo as it elongates. The formation of the Atg12-Atg5-Atg16L complex and the conversion of LC3-I to LC3-II are essential steps for the elongation and subsequent closure of the autophagosome. Once the autophagosome is completed, it then fuses with a lysosome and its contents are degraded and the resulting metabolites can be effluxed into the cytoplasm and used for other cellular processes. Commonly used chemical autophagy inhibitors and their targets are also shown. A further elaboration on the inhibitors is provided in section 1.1.3.

formation, and recruits and promotes the lipidation of LC3 at the phagophore [18].

Lipidation of LC3 is required for autophagosome formation and elongation. Newly synthesized LC3 (or pro-LC3) is immediately cleaved at its C-terminal end by Atg4, to form LC3-I (Figure 1). When autophagy is induced, LC3-I conjugation to phosphatidylethanolamine (PE) is mediated by Atg7 and Atg3 (an E2-like enzyme) to form LC3-PE (or LC3-II). Upon conjugation to PE, LC3-II is inserted into both the inner and outer autophagosomal membranes, promoting membrane elongation [21]. LC3 can also be involved in recruiting selective cargo to the autophagosome, for example by binding the ubiquitin-targeting molecules p62 and neighbour of BRCA1 gene 1 (Nbr1), or the mitochondria-targeting molecule BNIP3-like protein (BNIP3L or Nix) [22]. There are three isoforms of LC3 in humans: LC3A, LC3B, and LC3C. Both LC3A and B appear to function in autophagy in a similar manner, but it is not yet clear if LC3C is also involved in autophagy [23]. Upon closure of the autophagosomal membrane to form a complete autophagosome, the autophagosome fuses with a lysosome to form an autolysosome. In the autolysosome,

the autophagic cargo is degraded by acidic hydrolases. The degraded products are then released into the cytoplasm to be used by the cell as necessary [18].

1.1.3 Autophagy inhibitors

One of the most difficult obstacles in the field of autophagy research is the lack of specific autophagy inhibitors [24]. To date, there are no drugs that specifically inhibit autophagy. However, the use of genetic knockout or ribonucleic acid (RNA) knockdown models have allowed researchers to more accurately target autophagy and helped to determine the roles and relevance of the various autophagy-related proteins [25-27]. Unfortunately, these manipulations can only be used for *in vitro* work or in animal models and currently cannot be translated into a human clinical setting.

There are several classes of drugs that are currently in use to crudely target autophagy. One such class is the PI3K inhibitors, which target autophagosome formation [24]. This class includes wortmannin, LY294002, and 3-methyladenine (3-MA) (Figure 1) [28, 29]. These agents inhibit both class I and class III PI3K activity. As a result, the impact on autophagy can be somewhat conflicting as class I PI3K activity generally inhibits autophagy, whereas class III PI3K activity is required for autophagy [24]. As both classes of PI3K regulate a variety of cell signalling and membrane trafficking processes, treatment of cells with these agents can affect a multitude of processes in addition to autophagy [24].

Another class of inhibitors targets a later step of the pathway, namely the fusion of the autophagosome with the lysosome. As this step involves microtubules, it can be inhibited using microtubule-disrupting agents such as vinblastine and nocodazole (Figure 1) [30]. However, this class of autophagy inhibitors also affects processes other than autophagy, such as mitosis.

One additional group of inhibitors targets the lysosome itself. This group is comprised of agents that increase the pH of the lysosome such as ammonium chloride (NH_4Cl), bafilomycin A_1, and chloroquine (CQ) (and its derivative hydoxychloroquine (HCQ)), as well as drugs that act as lysosomal protease inhibitors such as E64d and pepstatin A (Figure 1) [24, 31, 32]. These drugs also present the problem of concurrently affecting other cellular process that require functional lysosomes while targeting autophagy, such as endocytosis.

Another problem with the above list of agents is that the majority of them are not approved for the treatment of human patients. As discussed in the following sections, there is a profound interest in treating humans with autophagy inhibiting drugs. Therefore, there is an acute need to develop specific, safe, and effectively potent autophagic inhibitors for use in human treatment as well as the research setting.

1.1.4 The roles and relevance of autophagy in cancer

It is postulated that autophagy plays different roles throughout tumourigenesis and established tumour growth and persistence. The current model proposes that autophagy promotes cellular and genomic integrity and therefore acts as a barrier to tumour initiation; however, once a tumour is established, autophagy promotes adaptation to stress and the ultimate survival of the tumour [33]. In non-cancerous cells, defects in autophagy can lead to tumourigenesis through the accumulation of damaged and defective organelles such as mitochondria, which can lead to production of reactive oxygen species (ROS), a potential deoxyribonucleic acid (DNA)-damaging agent. In addition, the cell's inability to dispose of its cellular "garbage" can lead to chronic inflammation, a pathology that is well-established to

promote the development of cancer, largely through the resulting production of additional DNA-damaging ROS [17, 34].

There is a growing field of research investigating the roles and relevance of autophagy in established tumours. While there has been some concern that autophagy could potentially act as a death mechanism instead of a survival mechanism in cancer cells, it is becoming increasingly accepted that autophagy is activated by cells in an attempt to adapt to stress, and the presence of autophagosomes in dying cells most often represents a failed survival attempt and not a mechanism to actively induce cell death [35]. That being said, there are no doubt certain scenarios in which a cell can induce high levels of autophagy for an extended period of time without respite and therefore, eventually "eat" itself to death. It should be noted though that these scenarios appear to be quite uncommon and often restricted to non-mammalian systems or mammalian cells cultured *in vitro* [35, 36].

Autophagy can be activated in response to stresses such as nutrient deprivation, hypoxia, chemotherapies, and growth factor withdrawal [37-48]. As these are stresses commonly faced by cancer cells, there is now a vast interest in treating cancer patients with autophagy modulating drugs in an effort to compromise tumour cell survival [17]. The role of autophagy as a cell survival mechanism in response to hypoxia and anti-cancer therapies will be further elaborated upon in the following sections as these are the autophagy inducers most relevant to the content of this thesis.

1.1.5 Autophagy in response to hypoxia

(Portions of this section have been modified from excerpts of the manuscript: Schlie K, Spowart JE, Hughson LR, Townsend KN, and Lum JJ. When Cells Suffocate: Autophagy in Cancer and Immune Cells under Low Oxygen. *Int J Cell Biol* 2011; **2011**: 470597)

In many tumours, cell growth and proliferation exceed the development of local vasculature supplying oxygen and nutrients. In response, tumours form disorganized angiogenic vessels, but these cannot adequately supply the tumour cells with oxygen, and as a result, the concentrations of oxygen within the tumour can span from physiological (2-9%), to hypoxic (≤ 2%), to severely hypoxic or "anoxic" (≤ 0.02%) [49-51]. Cancer cells in close proximity to vasculature contribute to tumour hypoxia by rapidly utilizing oxygen and nutrients that arrive at the tumour site. This can result in tumour cells experiencing chronic or cycling hypoxia depending on how quickly cancer cells consume oxygen once new vascular networks are formed and how far tumour cells are from existing vasculature [49, 50]. As a result of hypoxic stress, tumour cells in such a microenvironment can activate autophagy to help circumvent the effects of oxygen deprivation [38, 40, 41, 52-54].

One way in which tumour cells respond to hypoxia is through stabilization of hypoxia inducible factor-1α (HIF-1α). Along with HIF-1β, HIF-1α forms the transcription factor complex HIF-1. HIF-1β is always present in excess in cells whereas HIF-1α is constitutively targeted to the proteasome for degradation in the presence of oxygen, but is stabilized in hypoxic conditions. HIF-1 allows for adaptation to hypoxia by promoting a metabolic switch from oxidative phosphorylation to glycolysis and by initiating angiogenesis [55]. In addition, HIF-1 can help tumour cells adapt to hypoxia by inducing autophagy through transcription of target genes encoding for the autophagy regulatory proteins Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) and BNIP3L (Figure 2). BNIP3 and BNIP3L can displace B-cell CLL/lymphoma 2 (Bcl-2) or Bcl-2-like 1 protein (Bcl-X_L), from their inhibitory interactions with Beclin 1, freeing Beclin 1 to activate autophagy [38, 39]. Upregulation of BNIP3 and BNIP3L during hypoxia has been specifically shown to induce the selective degradation of mitochondria by autophagy (so-called mitophagy), a

process that promotes cell survival by reducing the generation of DNA-damaging ROS by dysfunctional mitochondria [39]. In addition, mitophagy may also promote cancer cell survival by eliminating a source of pro-apoptotic proteins [56]. A BNIP3/BNIP3L-

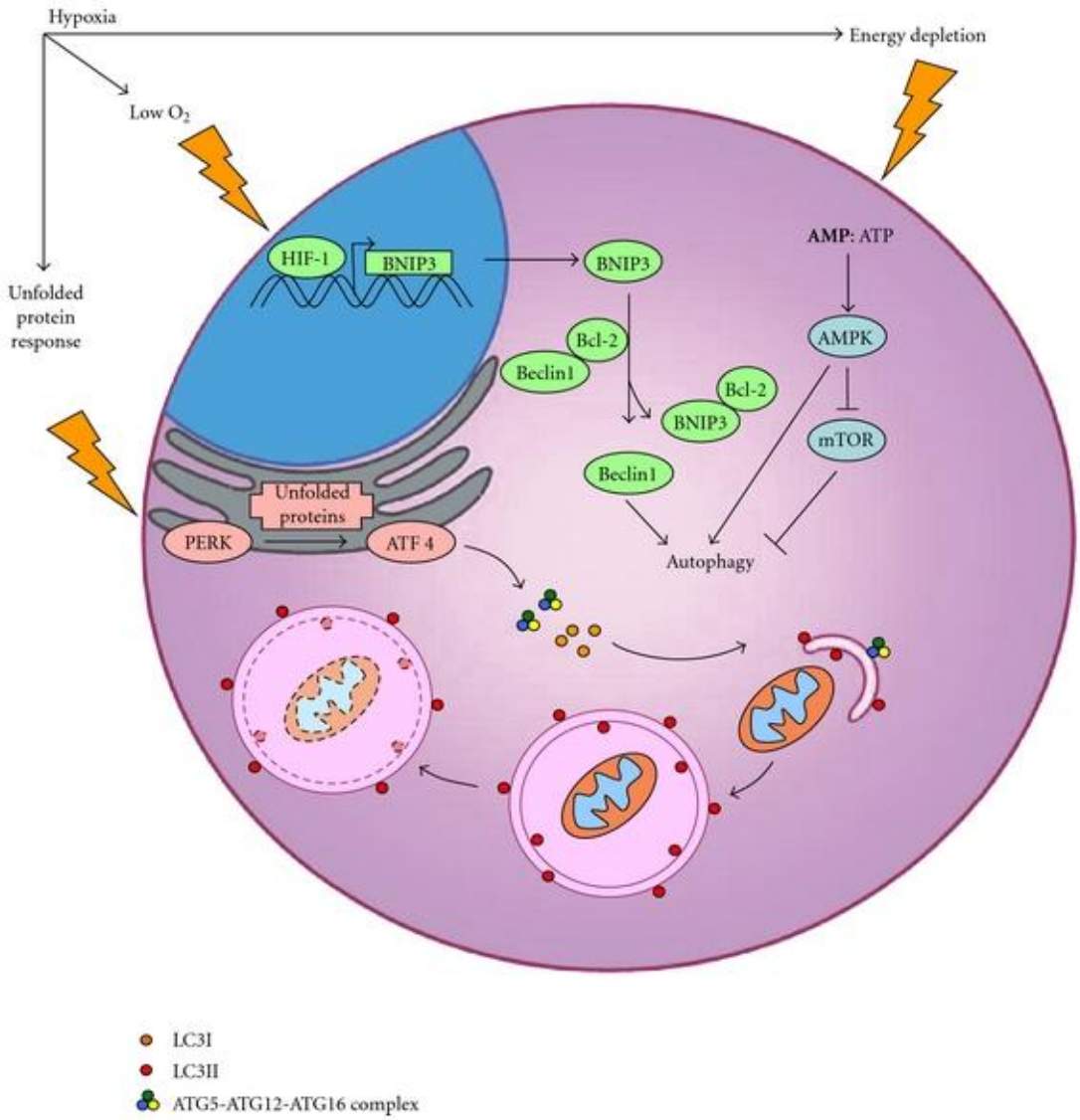

Figure 2. Pathways of autophagy induction by hypoxia.
During hypoxia, autophagy may be activated *via* the UPR, HIF-1, or AMPK. The mechanisms of activation are described in the text of section 1.1.4. (Figure taken from ref [42]).

independent form of HIF-1-induced autophagy has also been reported which was shown to initiate bulk degradation, but not mitophagy, under hypoxia in concert with platelet-derived growth factor receptor family signalling [52]. This latter form of HIF-1-induced autophagy is also important for tumour cell survival under hypoxia.

Autophagy is also believed to help sustain the energetic needs of the cell during hypoxia and nutrient deprivation by liberating metabolites that can be oxidized to generate adenosine triphosphate (ATP). One way that cells perceive and adapt to their energetic requirements is through the energy sensor adenosine monophosphate-activated protein kinase (AMPK). As the intracellular ratio of adenosine monophosphate (AMP) to ATP increases under hypoxia, AMPK activity promotes autophagy induction, serving as a means to prevent prolonged energy crisis and eventual cell death (Figure 2) [53, 54]. AMPK can induce autophagy either by inhibiting mTORC1 or by phosphorylating ULK1 [57].

During hypoxia, the unfolded protein response (UPR) is initiated because oxygen is required for the formation of disulfide bonds and the maturation of proteins destined to be secreted or incorporated into the plasma membrane (Figure 2) [58]. Another way in which autophagy can promote tumour cell survival during hypoxia is by degrading misfolded protein aggregates that accumulate in the ER. In hypoxic conditions, PKR-like ER kinase (PERK) detects unfolded or misfolded proteins and induces selective translation of activating transcription factor 4 (ATF4) messenger RNA (mRNA). ATF4 then acts to upregulate the expression of the essential autophagy genes *LC3* and *ATG5*. The resulting LC3 and Atg5 proteins then contribute to the elongation of the autophagosome and help to sustain the autophagic process under hypoxia, relieving the stress of the misfolded proteins on the tumour cells [40, 41, 58].

1.1.6 Autophagy in response to anti-cancer therapies

Preclinical data has shown autophagy to play a role in cell survival and treatment resistance to major classes of anti-cancer therapies including radiation, hormonal therapies, targeted therapies, and chemotherapies [40, 44, 59, 60]. These findings have led to the emergence of more than 30 clinical trials investigating the potential of adding autophagy inhibitors to cancer treatment protocols.[3]

Exactly how anti-cancer therapies induce autophagy is still under investigation. It is likely that some therapies, such as radiation and chemotherapy, do so by causing damage to DNA, cellular proteins, and organelles [17, 61]. The precise nature by which DNA damage induces autophagy is unclear, but DNA repair is an energetically costly process and it may be that autophagy helps to provide metabolites to provide the energy to fuel the process [61]. In addition, autophagy can degrade damaged proteins and organelles, thereby contributing to cellular integrity [17].

In contrast, hormonal therapies and some targeted therapies may act in a manner somewhat analogous to growth factor withdrawal by inhibiting pro-proliferative signalling [46]. Hormonal and targeted therapies often have cytostatic rather than cytotoxic effects on cancer cells, forcing the cells to become dormant or maintaining the cells in a dormant state [59, 60, 62]. Autophagy has been shown to be required by some types of cancer cells to maintain a dormant or quiescent state instead of succumbing to apoptosis in response to anti-proliferative stresses [62, 63].

Proteasome inhibitors are another class of targeted therapies, the most developed of which is bortezomib (Velcade®). These drugs inhibit the cell's ability to degrade misfolded and/or excessive proteins *via* the proteasomal pathway. Not surprisingly, there has been

[3] Source: www.clinicaltrials.gov

great interest in coupling autophagy inhibitors with proteasomal inhibitors as autophagy constitutes the other major pathway whereby cells can degrade proteins and could help cells compensate for the effects of a proteasome inhibitor. Preclinical studies have shown that treatment with bortezomib does indeed induce cell survival-promoting autophagy in cancer cells and clinical trials investigating the combination of bortezomib and autophagy inhibitors are now underway (NCT01438177, NCT00568880) [64].

One additional class of targeted therapies that triggers autophagy is angiogenesis inhibitors. It is known that tumours need to recruit blood vessels to supply oxygen and other nutrients to support the tumour cells. One strategy for compromising tumour cell survival is to inhibit their ability to recruit such vasculature. Unfortunately, such a strategy comes with the caveat of making the tumours even more hypoxic, and hypoxic tumour cells are known to be more resistant to radiation and chemotherapy [51]. Autophagy has recently been shown to be utilized by tumour cells to survive and adapt to hypoxia in response to anti-angiogenesis therapy and clinical trials investigating coupling autophagy inhibitors with anti-angiogenic drugs are also underway (NCT00813423, NCT01206530, NCT00933803, NCT01006369) [54].

In support of the role of autophagy as a cell survival adaptation in response to anti-cancer therapies in human cancer patients are the results of a clinical trial in glioblastoma multiforme patients. In this trial, 41 patients were administered daily doses of the autophagy inhibitor CQ for 12-18 months in addition to surgery, radiotherapy, and chemotherapy. The patients who received CQ treatment had a mean survival time of 25 $\pm$ 3.4 months as compared to 11.4 $\pm$ 1.3 months for 82 patients who did not receive CQ treatment (p = 0.000) [65]. These results are very promising and it is hoped that current clinical trials

underway will also find improvements in cancer patient survival with the addition of autophagy inhibitors.

It should be noted that in current trials investigating autophagy inhibition in combination with conventional cancer treatments, the most commonly used drug is HCQ, followed by CQ. While these are relatively safe and well-tolerated drugs, it takes five to six weeks for HCQ to reach steady state concentrations in human patients and even then, the achievable concentrations are likely not high enough to completely inhibit autophagy (Ravi Amaravadi, personal communication). As a result of the suboptimal effectiveness of HCQ and CQ as autophagy inhibitors, it is likely that results of current clinical trials will be somewhat promising, but not fully reflective of what could be accomplished with a completely effective autophagy inhibitor. Therefore, as previously mentioned, it is imperative that potent and safe autophagy inhibitors be developed for human treatment in order to maximally improve cancer patient outcomes using this treatment strategy.

The literature regarding autophagy in response to anti-cancer treatment is abundant and cannot be thoroughly covered within this thesis. However, a more detailed look at this literature as it pertains to ovarian cancer will be presented in section 1.3 (page 28).

1.2 Ovarian cancer

1.2.1 General disease characteristics, classification, and clinical management

Ovarian cancer is the leading cause of death among gynaecological malignancies, and the fifth leading cause of cancer death in women [66]. Current five-year survival rates are estimated at only 44%. Though this is a slight improvement over the survival rates three decades ago (38%) due to advances in surgery and platinum-taxane-based chemotherapies, the rates are still dismal and improved treatments are desperately needed [66]. One of the

reasons for such poor survival rates is that most cases of ovarian cancer are diagnosed when they are in advanced stage disease, when patient prognosis is much poorer than for early stage disease [67].

Ovarian cancer is broadly divided into two categories: epithelial ovarian cancer (EOC) (also known as ovarian carcinoma) and non-epithelial ovarian cancer. Non-epithelial ovarian cancers account for approximately 10% of all ovarian cancer cases and include the subtypes of germ cell tumours and sex cord stromal tumours [68]. This category of ovarian cancer is not encompassed within the subject of this thesis and will not be discussed further. The majority of EOC cases (>95%) are one of five subtypes, which are (in decreasing order of prevalence) high-grade serous, clear cell, endometrioid, low-grade serous, and mucinous [67]. For many years, EOC was largely regarded as a single disease, and as tumours of all subtypes involved the ovary, it was originally thought that the cell of origin for each subtype was ovarian [69]. Recent evidence indicates that the majority of tumours of the five main subtypes actually do not arise from ovarian cells and rather involve the ovary secondarily [69]. The proposed cell types of origin for each subtype will be discussed in the following sections.

When an ovarian tumour is diagnosed, it is assigned a grade and a stage and these parameters are used to help plan the patient's course of treatment and are considered when determining the patient's prognosis. Grade is a measure of the degree of differentiation of the tumour cells [70]. One commonly used grading system for ovarian cancer is known as the Silverberg (or Shimizu-Silverberg) system. This system is based on three parameters: the degree of nuclear atypia, the mitotic count, and the architectural features [70]. This grading system was used to grade the tumours of the ovarian cancer patient cohort described in Chapter 2. The stage is used to describe the extent of the disease and the commonly used

staging system for ovarian cancer has been determined by the International Federation of Gynaecology and Obstetrics (FIGO) [71]. The main categories of this system are outlined in Table 1 [71]. Interestingly, there is an inverse relationship between stage and tumour size in ovarian cancer which is largely due to the fact that each subtype is more likely to be diagnosed at either early (stage I-II) or late (stage III-IV) stage, meaning that the biology and tumour characteristics tend to be very different between the different stages of ovarian cancer [67, 72]. This concept will be elaborated upon further in the discussions of each subtype.

Table 1. International Federation of Gynaecology and Obstetrics staging guidelines for carcinoma of the ovary

FIGO Stage	Definition
I	Tumour limited to the ovaries
II	Tumour involves one or both ovaries with pelvic extension
III	Tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis
IV	Distant metastasis (excludes peritoneal metastasis)

Notes: Liver capsule metastasis is Stage III, liver parenchymal metastasis is Stage IV, pleural effusion must have positive cytology for Stage IV.
Table adapted from [71]

Despite the heterogeneity between ovarian carcinoma subtypes, the same standard treatment is used for all subtypes. The current practice is to treat all EOC patients with surgery with the addition of platinum/taxane chemotherapy for advanced cases [73]. The most commonly used chemotherapeutic combination is carboplatin and paclitaxel [74]. Carboplatin is often used in preference to its parent drug, cisplatin, as the treatment efficacy between the drugs is comparable but carboplatin is generally better tolerated with less severe side-effects [75, 76]. The lack of different treatment options specific for each subtype is likely another contributing factor to the poor prognosis of patients with this disease and this

problem is now starting to be acknowledged with the recent emergence of subtype-specific clinical trials.

It has now become apparent that each subtype of EOC is itself a unique disease, and therefore researchers should assess subtypes individually when considering everything from molecular pathogenesis to the efficacy of new treatments [77]. In addition to the field's recent appreciation for the significant differences between the subtypes, the diagnostic criteria for the various subtypes have evolved over time, resulting in a change in the distribution of subtype frequencies reported [67]. For example, it now appears that the majority of tumours that were diagnosed in the past as high-grade endometrioid tumours were actually high-grade serous tumours, so we will now likely see an increase in the ratio of high-grade serous to endometrioid tumours reported [78]. Unfortunately, these realizations and recent changes can make the data from some older EOC studies difficult to interpret or gauge the significance of. However, these advances have also made the field very exciting and dynamic and should help facilitate additional meaningful progress in our understanding of the molecular basis of ovarian carcinomas as well as improved subtype-specific treatments.

1.2.2 High-grade serous ovarian carcinoma

High-grade serous ovarian carcinoma (HGSC) is the most common type of EOC, accounting for 68-71% of ovarian carcinoma cases in North American populations [67]. Though many past studies have grouped this subtype with low-grade serous ovarian carcinoma (LGSC), it is now apparent that these are distinct subtypes with unique molecular characteristics and courses of disease progression [79]. Some researchers and clinicians have been tempted to think of LGSC and HGSC as a continuum of serous tumours but research

shows that this is not the case and it is only in very rare incidences that a low-grade serous tumour progresses to become a high-grade serous tumour [67, 73]. LGSC and HGSC are usually separated on the basis of a two-tier grading system (as opposed to the three-tier Silverberg system described earlier) but high-grade tumours tend to correspond to grade 2 or 3 serous tumours as classified by the three-tier grading system [67, 80].

In contrast to many of the other subtypes, HGSCs actually have high initial response rates to the current treatment standard of platinum/taxane therapy [81]. However, prognosis for these patients remains poor as the majority suffer recurrence [82]. HGSC is much more likely to be diagnosed at stage III/IV disease than stage I/II, when the disease has begun to spread throughout the peritoneal cavity and is harder to surgically remove [67]. The reason for this high proportion of late stage disease diagnoses is tied to the biological behaviour of this subtype. Early stage HGSC is unlikely to be detected with current imaging techniques such as transvaginal ultrasound because these techniques are most likely to detect large tumour masses confined to the ovary (which is the manner in which some of the other subtypes present), whereas high-grade serous tumours tend to be small and disseminated [67].

Several studies have now provided evidence that the majority of HGSCs actually originate from the epithelium of the distal fimbrial portion of the fallopian tube as opposed to the surface epithelium of the ovary or the epithelium of cortical inclusion cysts as originally thought (reviewed in [69, 78]). The proposed precursor lesion is referred to as a serous tubal intraepithelial carcinoma (STIC). However, convincing evidence that high-grade serous tumours never arise from the ovarian surface epithelium or cortical inclusion cysts on the ovary has yet to be presented and it is still possible that some cases of HGSC do arise from these tissues [78, 83]. Further research into this question is needed as some clinicians

are eager to alter their prophylactic surgical practices for patients at high risk of ovarian cancer (for example, by removing just the fallopian tubes but not the ovaries) but enough evidence has not yet been provided to support modification of these practices [83]. The origin of HGSC from the fallopian tube may also contribute to the explanation for why HGSC tends to be diagnosed at advanced stage disease as the cancerous cells can detach from the fallopian tube and travel to the peritoneal cavity where they are able to implant and survive, and can ultimately result in widespread disseminated disease, even though the individual tumours themselves may be of small volume [67]. In further consideration of the origin of HGSC, it should be noted that no association has been found between endometriosis (the presence of endometrial glands and stroma outside the uterine cavity and musculature) and the development of HGSC as there has been for other subtypes, and therefore this pathological condition does not appear to contribute to the development of high-grade serous tumours [84].

Compared to the other subtypes, HGSC is considered to be very genomically unstable [81, 85]. The vast majority (>96%) of HGSC tumours have mutations in the tumour protein p53 (*TP53*) gene and this appears to be an early event in the development of this disease as mutations are also detected in early stage tumours [85, 86]. In addition, cases that do not have mutations within the actual *TP53* gene itself have been reported to show other characteristics which could contribute to p53 disregulation such as amplification of the *MDM2* gene which encodes a protein that negatively regulates p53 [86]. By immunohistochemistry (IHC) analysis, *TP53* mutations in HGSC can result in either complete absence of p53 protein expression or p53 overexpression, which are both considered aberrant forms of expression compared to normal wild-type p53 expression which is described as being focal in nature [87]. The association between *TP53* mutations

and survival in HGSC patients is still unclear as one study found no association between the type or frequency of *TP53* mutations and patient survival in advanced stage HGSC, whereas another study found that patients with no p53 protein expression (presumed to reflect truncating mutations) as assessed by IHC had poorer survival than patients with p53 overexpression (presumed to reflect null mutations) [86, 87].

The second most commonly mutated genes in HGSC are *BRCA1* (breast cancer 1, early onset) & *BRCA2* (breast cancer 2, early onset), occurring in approximately 22% of tumours, and these are a combination of germline and somatic mutations [85]. An additional 11% of high-grade serous tumours have been found to have lost *BRCA1* expression through DNA hypermethylation. Interestingly, DNA hypermethylation of *BRCA1* appears to be a mutually exclusive event from the genetic mutations as none of the tumours with this noted epigenetic suppression of *BRCA1* expression had *BRCA1* or *BRCA2* mutations [85]. HGSCs also commonly have alterations in other genes involved in the homologous recombination pathway, which is employed to repair double-stranded breaks in DNA, and when these alterations are considered along with those affecting *BRCA1/2*, 51% of high-grade serous tumours have alterations that affect this pathway [85]. These combinations of aberrant p53 expression and defects in homologous recombination are likely largely responsible for the genomic instability characteristic of this subtype.

1.2.3 Clear cell ovarian carcinoma

The prevalence of clear cell ovarian carcinoma (CCC) is estimated to be 9-12% of EOC cases in North American populations [67]. Interestingly, the frequency of this subtype is much higher in Japan at 20-25% of ovarian carcinomas [88]. This increase in frequency does not appear to be primarily due to environmental factors in Japan as one study looking

at EOC patients living in the United States found that CCC was diagnosed over twice as frequently in Asians (Chinese, Japanese, Korean, Vietnamese, and Filipina) than Whites (11.1% of Asian patients versus 4.8% of White patients) [89]. One contributing factor to the higher prevalence of CCC in Asian women may be that this group of women has a higher prevalence of endometriosis, which is associated with the development of CCC [81].

The majority of CCC tumours present as early stage disease, likely due to their slow-growing tumour behaviour and characteristic growth pattern resulting in presentation as a large pelvic mass as opposed to a disseminated collection of small tumours [67, 90, 91]. However, despite its frequent early presentation, CCC is still considered to be one of the more lethal subtypes. Some studies have suggested that clear cell patients have poorer survival outcomes than stage-matched serous patients for any disease stage, while others have concluded that early stage clear cell patients have better outcomes than stage-matched serous patients, with the inverse being true for late stage patients [81, 91]. A recent meta-analyses of seven randomized trials found that advanced stage clear cell patients had significantly shorter progression-free survival (median 9.6 *vs* 16.1 months) and overall survival (median 21.3 *vs* 40.8 months) as compared to advanced stage serous patients [92]. One reason for the poor survival outcomes seen in CCC patients is likely that the response rates to traditional platinum-based therapy is particularly poor (11-56%) compared to the response rate of the serous subtypes (>70%) [90, 91, 93, 94]. These findings have led some clinicians to suggest that there is no additive survival benefit with the addition of chemotherapy to cytoreductive surgery in CCC patients [67]. It is thought that such poor chemotherapy treatment response rates in CCC are in part due to these tumours' low levels of proliferation as compared to serous tumours [90].

Clear cell tumours get their name from the clear appearance of the cells as a result of their abundant glycogen. Interestingly, the association between CCC and glycogen is also highlighted by their enrichment of glycogen metabolism genes as compared to HGSC [81]. However, both serous and endometrioid ovarian carcinoma cells can undergo changes to acquire a "clear cell" appearance, potentially leading to incorrect classification of these subtypes as CCC [95]. Pathologists therefore recommend consideration of other parameters in addition to the appearance of "clear cells" when deciding whether or not a tumour is of this subtype [95]. Though clear cell tumours tend to have a low mitotic rate, all CCCs are by definition high-grade and it is recommended that they automatically be graded as grade 3 [78, 96]. Grading using conventional grading systems could result in clear cell tumours being assigned a grade of 1 or 2, but such grades are not considered appropriate for this subtype [78]. In addition, it has been found that CCC patient prognosis is not correlated with the conventional Silverberg grading method [70].

Some subtypes of EOC are associated with endometriosis. CCC is included in this category and in fact has a stronger association with endometriosis than any other subtype. A recent large-scale international study found that women with self-reported endometriosis had an approximately three-fold greater risk of developing CCC than women without endometriosis [84]. In the case of CCC, the reason for this association is believed to be linked to the origin of this tumour type. CCCs are thought to develop originally from endometriosis (*i.e.* they are thought to be derived from ectopic uterine epithelium) [97]. In fact, clear cell tumours have actually been seen arising in endometriotic cysts [73]. In regards to an explanation for these tumours' association with the ovary, CCCs are thought to develop from endometrial tissue that has implanted on the ovary and therefore the involvement of the ovary in this subtype is secondary [69].

The endometriotic cyst is a hostile and unnatural microenvironment and it is not surprising that tumours arise under such conditions. There is repeated bleeding into the cyst during the menstrual cycle but no natural outlet for the blood. As a result, there is an accumulation of old blood within the cyst, resulting in a microenvironment with a high concentration of iron which can cause oxidative stress, resulting in cellular and DNA damage [98]. In addition, endometriosis is associated with a local inflammatory reaction and it has long been acknowledged that inflammation can promote tumourigenesis [34]. However, most women with endometriosis do not develop any of the endometriosis-associated forms of EOC, so further research is needed to be able to determine which women with endometriosis are at highest risk of developing EOC [84].

In contrast to HGSC tumours, *TP53* mutations (resulting in aberrant protein expression as assessed by IHC) are relatively infrequent in CCC tumours and therefore do not appear to be as important for the pathogenesis of CCC as they are for HGSC [77, 87, 99]. This subtype also has a lower frequency of *BRCA1/2* mutations than HGSC and is considered to be genomically stable [81]. One of the pathways that does seem to be important in CCC is the PI3K/v-akt murine thymoma viral oncogene homolog 1 (Akt) pathway. Up to 46% of cases have mutations in *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) (the gene that encodes PI3K) and the majority of these are activating mutations as demonstrated by phosphorylation of PI3K's target, Akt [100, 101]. Therefore, *PIK3CA* appears to function as an oncogene in CCC. The importance of the PI3K/Akt pathway for CCC is further highlighted by the fact that up to 37% of cases have been reported to have lost protein expression of phosphatase and tensin homolog (PTEN), which acts in opposition to PI3K [102, 103]. Interestingly, the frequency of mutations in the

PTEN gene in CCCs is actually quite low (5-8%), indicating that this subtype employs other mechanisms to silence PTEN protein expression [101, 104].

Another gene frequently mutated in CCC is *ARID1A* (AT-rich interactive domain 1A (SWI-like)). *ARID1A* encodes BRG1-Associated Factor 250a (BAF250a), a key component of the switch/sucrose-nonfermentable (SWI-SNF) chromatin remodelling complex which functions as a regulator of gene expression and chromatin dynamics. *ARID1A* has recently been reported to be mutated in 46-57% of clear cell tumours [97, 101]. The majority of the mutations cause truncations and the majority of mutated cases have loss of nuclear BAF250a expression [97]. In contrast to the mixture of somatic and germline *BRCA1/2* mutations seen in HGSC, all of the *ARID1A* mutations are somatic as deletion of *ARID1A* on one allele has been shown to be embryonic lethal in mice [97]. Interestingly, none of the HGSC cases examined had any *ARID1A* mutations, with mutations only occurring in endometriosis-associated subtypes [97]. However, the authors of these studies note that "the mechanism by which somatic mutations in *ARID1A* enable the progression of benign endometriosis to carcinoma is unclear" and further research is needed [97].

One additional protein that appears to be uniquely important in CCC is hepatocyte nuclear factor-1β (HNF-1β). Almost all clear cell tumours show protein expression of HNF-1β while this protein is rarely expressed in other subtypes [105, 106]. The precise function of this protein in CCC is not fully understood but it appears to be involved in the stress response, perhaps in response to the iron- and hypoxia-induced oxidative stresses characteristic of endometriotic cysts and CCC [98].

1.2.4 Endometrioid ovarian carcinoma

The prevalence of endometrioid ovarian carcinoma (EC) in North American populations is comparable to that of CCC, accounting for 8-11% of EOC cases [67]. The majority of cases present as early stage and low-grade disease, and there is relatively low mortality associated with this subtype compared to other subtypes [67, 73].

Like CCC, EC is also associated with endometriosis. A recent study found that women with self-reported endometriosis had approximately double the risk of developing EC as compared to women with endometriosis [84]. The authors of this study noted that the magnitude of increased risk might be misleadingly low as some cases of EC may have been misclassified as HGSC [84]. The association between endometriosis and EC, like CCC, can be explained by the fact that EC is also thought to develop from endometriosis, and therefore also involves the ovary secondarily [97].

The mutation profile of EC is also somewhat similar to CCC, potentially reflecting their shared cell type of origin. *ARID1A* mutations have been reported in 30% of cases of EC [97]. Loss of expression of the *ARID1A*-encoded protein, BAF250a, is also common, occurring in 21% of EC cases [97]. Other commonly mutated genes in EC include *CTNNB1* (catenin (cadherin-associated protein), beta 1) (which encodes for beta-catenin) with mutations occurring in 37-50% of EC cases, and *PTEN*, which has been found to be mutated in approximately 20% of cases [107-109].

1.2.5 Low-grade serous ovarian carcinoma

The low-grade serous subtype is much less prevalent than its high-grade counterpart, accounting for only 3-4% of cases of ovarian carcinomas in North American populations [67]. LGSC tumours tend to correspond to grade 1 serous ovarian carcinomas and have

several distinguishing features from HGSC [67, 80]. LGSCs usually have a lower mitotic index (reflective of the number of mitoses visible per high power field of view) than HGSCs [79]. Perhaps this lower level of proliferation contributes to the fact that LGSC does not respond well to traditional ovarian cancer chemotherapeutics and therefore, some oncologists do not administer adjuvant chemotherapy if the tumour is optimally debulked (no obvious residual disease) [78]. Unfortunately, like HGSC, LGSC also tends to present as late stage disease when optimal debulking becomes more difficult [67].

The cell type of origin for LGSC is still unclear with some researchers proposing that LGSC arises from fallopian tube epithelium like HGSC, while the majority still maintains that LGSC arises from the ovarian surface epithelium or cortical inclusion cysts [69, 78, 110]. This subtype appears to develop from serous ovarian tumours of low malignant potential (also known as serous borderline ovarian tumours) whereas this continuum is not proposed to involve high-grade serous tumours which are thought to arise from STICs in most cases [73]. Interestingly, it has just been reported that women with self-reported endometriosis have approximately double the risk of developing LGSC as compared to women without endometriosis [84]. This is the first time such a link has been identified and the biological explanation for this is not yet clear. LGSC is not thought to develop from the cells of endometriotic cysts, unlike the CCC and EC subtypes, and further studies examining this relationship are anticipated.

In contrast to HGSC, LGSC is not associated with *BRCA1/2* abnormalities or *TP53* mutations [73]. This subtype is not considered to be chromosomally unstable and tumours are usually diploid or near diploid [73]. The most common mutations found in LGSC are those in *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) or *BRAF* (v-raf murine sarcoma viral oncogene homolog B1). Activating mutations in either gene have been

reported in 68% of LGSCs, though these mutations appear to be mutually exclusive as mutations in both genes are not observed in the same tumour [111]. Such mutations result in constitutive activation of mitogen-activated protein kinase (MAPK)-mediated signalling and this appears to be an important pathway in LGSC [69].

1.2.6 Mucinous ovarian carcinoma

Mucinous ovarian carcinoma (MC) has a low prevalence rate among the other subtypes, accounting for only 3% of EOC cases in North American populations [67]. MC was previously thought to be more common but we now know that many tumours that were originally classified as MC were actually metastasized tumours from the gastrointestinal or biliary tracts [73]. The majority of MC tumours present as early stage disease as a large pelvic mass [67]. However, if they recur, there are no effective treatments as their response to current platinum/taxane therapies is poor [73].

Similar to HGSC, there is no association between MC and endometriosis [84]. The cell of origin of this tumour type is still not clear, but the current theory proposes that these tumours develop from transitional-type epithelium located at the tubal-peritoneal junction [69]. Unlike the serous, clear cell, or endometrioid subtypes of ovarian cancer, mucinous tumours do not display a Müllerian phenotype (resembling cells of the female reproductive tract) and instead more closely resemble gastrointestinal mucosa [69].

The most commonly mutated gene in MC is *KRAS*, with mutations reported in up to 85% of MC cases [112]. Human epidermal growth factor receptor 2 (HER2) has recently emerged as a candidate target protein in mucinous patients, with reported *HER2* gene amplification rates of 18.2-35.3% of mucinous carcinoma cases and overexpression of

HER2 protein in 15.2-29.4% of cases [113, 114]. There is now interest in targeting this subtype with anti-HER2 therapeutics [113, 114].

1.3 Autophagy in ovarian cancer

The role of autophagy in ovarian cancer in general, or its particular subtypes, has not been studied extensively. However, there have been some promising findings that indicate that modulating autophagy in this disease (or diseases) may be a beneficial therapeutic strategy.

As mentioned previously, the most commonly used chemotherapeutic drugs for treatment of ovarian cancer are carboplatin and paclitaxel. While there have been no published studies to date that have explicitly investigated the role of autophagy in response to carboplatin treatment in ovarian cancer (or any other cancer), there have been some studies that have investigated carboplatin's closely-related parent drug, cisplatin, and autophagy in ovarian cancer. Cisplatin and carboplatin are both platinum-based compounds that covalently bind to purine DNA bases, which leads to cellular apoptosis [115]. A recent study using both HGSC and EC cell lines found that cisplatin treatment induced autophagy in these cell lines and that addition of a chemical autophagy inhibitor or genetic inhibition of autophagy enhanced cisplatin-induced apoptosis [43]. An additional study assessed cisplatin-sensitive and cisplatin-resistant derivatives of an HGSC cell line and found that the cisplatin-sensitive cells showed signs of ER stress (the accumulation of unfolded or misfolded proteins in the ER lumen), whereas the cisplatin-resistant line appeared to overcome this stress by activating autophagy to facilitate the clearance of misfolded or unfolded proteins. This conclusion was supported by the finding that inhibiting autophagy in combination with

cisplatin treatment in the cisplatin-resistant cells resulted in an increase in ER stress and decreased viability as compared to treatment with cisplatin alone [116].

There are no studies to date that have looked at the role of autophagy in response to paclitaxel treatment in ovarian cancer, however, there have been some promising results in other cancer types. One study found that treatment with paclitaxel induced autophagy in lung carcinoma, glioma, prostatic carcinoma, and colorectal carcinoma cell lines. In addition, inhibiting autophagy in these cells concurrently with administration of paclitaxel decreased cell viability and/or proliferation as compared to treatment with paclitaxel alone [117]. It should be noted though that there is some concern that paclitaxel itself may inhibit autophagy as it is a microtubule-binding agent and autophagosome movement involves microtubules [30]. However, it is important to note that paclitaxel is a microtubule-stabilizing drug as opposed to a microtubule-destabilizing drug such as nocodazole or vinblastine [118]. While the role of microtubule-destabilizing drugs as autophagy inhibitors is fairly well accepted, the impact of microtubule-stabilizing drugs on autophagy is less clear. There is a recent report that claims that paclitaxel inhibits autophagy in a breast cancer cell line, however, others have found that paclitaxel may slow down the movement of autophagosomes somewhat, but the impact is much less severe than seen with treatment with a microtubule-destabilizing agent [30]. The fact that inhibiting autophagy concurrently with paclitaxel treatment resulted in enhanced paclitaxel efficacy in the previous study discussed also supports the notion that paclitaxel itself is not an autophagy inhibitor or else one would not expect to see an improvement in paclitaxel's efficacy with the addition of an inhibitor [117].

Despite the promising results that have been shown when combining autophagy inhibition with either paclitaxel or cisplatin treatment, it is still not clear exactly why either of

these drugs, or carboplatin, would induce autophagy. As cisplatin and carboplatin can damage DNA structure, it is possible that autophagy provides metabolites to fuel DNA repair [61]. Another reason that these chemotherapy drugs may induce autophagy relates to Bcl-2. Treatment of an HGSC cell line with carboplatin, cisplatin, or paclitaxel resulted in a decrease in Bcl-2 mRNA expression [119]. As Bcl-2 inhibits Beclin 1 from promoting induction of autophagy, it is possible that a decrease in Bcl-2 levels results in more active Beclin 1, leading to autophagy induction. One other resultant stress from treatment with these chemotherapeutics that may induce autophagy is ER stress. Treatment with either paclitaxel or cisplatin has been reported to induce ER stress and it is known that autophagy can help alleviate ER stress by degrading misfolded or unfolded proteins [116, 120].

In addition to the standard ovarian cancer chemotherapeutics, there has also been a promising report on the role of autophagy in response to bortezomib in ovarian cancer cells. One group of researchers found that treatment of transformed ovarian surface epithelial cells with bortezomib plus autophagy inhibition resulted in an increase in apoptosis compared to bortezomib treatment alone, and this increase was greater than that seen when the combination treatment was administered to immortalized (but not transformed) ovarian surface epithelial cells [64]. These findings are exciting because they not only show that bortezomib plus autophagy inhibition may be an effective treatment for ovarian cancer, but they also show that this treatment may have some specificity for ovarian cancer cells as compared to healthy cells.

One additional important piece of research regarding autophagy and ovarian cancer involves a study that investigated the contribution of autophagy to tumour dormancy which could play a role in tumour resistance to chemotherapy. Researchers found that when autophagy was induced *via* expression of the protein aplasia Ras homolog member I (ARHI)

in human HGSC-derived xenograft tumours in mice, that these tumours would remain dormant. Then, when ARHI expression was inhibited (and presumably autophagy as well), the tumours were able to rapidly regrow. However, if the mice were treated with CQ during ARHI expression, then when ARHI expression was inhibited, the tumours were severely compromised in their ability to regrow [63]. These findings support the conclusion that autophagy may contribute to tumour dormancy in ovarian cancer and that inhibiting autophagy in ovarian cancer patients may lead to a lower frequency of recurrences.

Though there is much research that remains to be done on the roles of autophagy in ovarian cancer, the results to date have been promising. The work encompassed in this thesis strives to add to our knowledge of this topic and encourage other researchers to continue investigating this important relationship.

Chapter 2: The autophagy protein LC3A correlates with hypoxia and is a prognostic marker of patient survival in clear cell ovarian cancer

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JES, KNT, and JJL designed the study. JES, KNT, HH, SE, NRW, SK, MA, SMG, PHW, CBG, DGH, and JJL were involved in acquisition of data and analysis/interpretation of data. JES and JJL wrote the manuscript. KNT, NRW, SK, SMG, PHW, and CBG edited the manuscript.

2.1 Abstract

Clear cell ovarian cancer subtypes exhibit metabolic features associated with resistance to hypoxia and glucose deprivation-induced cell death. This metabolic characteristic suggests that clear cell ovarian cancers activate survival mechanisms not typical of other epithelial ovarian cancers. Here we demonstrate that LC3A, a marker of autophagy, is related to hypoxia and poor prognosis in clear cell ovarian cancer. In 485 ovarian tumours, we found that LC3A was significantly associated with poor progression-free ($p = 0.0232$), disease-specific ($p = 0.0011$), and overall patient survival ($p = 0.0013$) in clear cell ovarian cancer patients, but not other subtypes examined. LC3A was an independent prognostic marker of reduced disease-specific [HR: 2.55 (95% CI 1.21-5.37); $p = 0.014$] and overall survival [HR: 1.95 (95% CI 1.00-3.77); $p = 0.049$] in patients with clear cell ovarian cancer. We also found a strong link between autophagy and hypoxia as LC3A staining revealed a significant positive association with the hypoxia-related proteins carbonic anhydrase-IX and HIF-1α. The functional link between hypoxia and autophagy was demonstrated using clear cell and high-grade serous cell lines that were subjected to hypoxia or hypoxia + glucose deprivation. Clear cell carcinoma lines displayed greater autophagy induction under hypoxia compared to the high-grade serous lines. Together, our findings indicate that hypoxia-induced autophagy may be crucial to the clinical pathology of clear cell ovarian cancer and is a potential explanation for histological subtype differences in patient response to chemotherapy.

2.2 Introduction

Ovarian cancer has been largely treated as a single disease, even though it is becoming apparent that ovarian cancer subtypes are distinct diseases that will require

discrete treatment strategies [77]. The major subtypes include high-grade and low-grade serous, clear cell, endometrioid, and mucinous [77]. The clear cell subtype presents a particular challenge as its response to conventional platinum-based therapy is particularly poor (11-56%) compared to the response rate of the more prevalent serous subtypes (>70%) [91, 93, 94]. Studies have shown that late stage (III/IV) clear cell patients have poorer progression-free survival (PFS) (median 9.6 vs 16.1 months) and overall survival (OS) (median 21.3 vs 40.8 months) than stage III/IV serous patients [92]. In contrast to other subtypes, clear cell tumours exhibit defined metabolic properties including upregulation of pathways involved in glycolysis, angiogenesis, and oxidative stress suggesting that these tumours have enhanced adaptations to respond to hypoxia and its downstream consequences [121-123]. One such adaptation that may be used by clear cell tumours is autophagy, a catabolic process which has been shown to promote ovarian cancer cell survival and dormancy *in vivo* [63].

In response to nutrient deprivation, hypoxia, chemotherapy, and growth factor withdrawal, cancer cells promote cell survival via autophagy [37-48]. During autophagy, cellular constituents are engulfed in a double-membraned vesicle called an autophagosome which fuses with a lysosome to degrade intracellular components such as damaged organelles and misfolded proteins. The current model proposes that autophagy promotes cellular and genomic integrity, acting as a barrier to tumour initiation, however, once a tumour is established, autophagy promotes adaptation to stress, resistance to apoptosis, and survival of the tumour [33]. Recently, several reports have validated and utilized an alternative method of assessing autophagy in formalin-fixed, paraffin-embedded (FFPE) tumour samples from a variety of tumour sites [124-128]. LC3 is a key component of the autophagy machinery that is incorporated into autophagosomal membranes. LC3A, one isoform of LC3, exhibits three

distinct staining patterns: diffuse cytoplasmic, juxtanuclear, and stone-like structures (SLS). LC3A SLS are believed to arise from hyper-activation of the autophagy pathway and are associated with poor patient prognosis in several cancer types [124-127]. Other studies have found LC3 upregulation in colorectal and gastrointestinal cancers, and in pancreatic cancer there was a correlation between high LC3 expression and poor patient outcome [129-131].

Here we report a study assessing LC3A in a 485-patient cohort encompassing all the major ovarian cancer subtypes to test the hypothesis that hyper-autophagy would correlate with poor patient survival. This included evaluation of LC3A SLS relationships with patient survival and markers of hypoxia such as HIF-1α and carbonic anhydrase-IX (CA-IX). The biological relevance of our findings was demonstrated by comparing subtype-specific cell lines and their induction of autophagy in response to hypoxia or combined hypoxia and glucose deprivation.

2.3 Methods

2.3.1 Study Population

The retrospective patient cohort used for the current study has been previously described [77]. Briefly, all patients were recorded in the Cheryl Brown Ovarian Cancer Outcomes Unit in the province of British Columbia from 1984-2000. All patients underwent surgery with no macroscopic residual disease and were given platinum-based chemotherapy. FFPE tumour specimens from these patients were used to construct a tissue-microarray (TMA) [77]. At the time of analysis, 15 cases were excluded from the original 500-case cohort in the current study as immunohistochemical analyses were not possible for technical reasons. The analyzable cohort of 485 patients is described in Table 2. Median follow-up time was 6.1 years (range 0.1 – 23.6 years). The follow-up times and number of events by

subtype for patients used in survival analyses are listed in Table 3. The entire follow-up period beginning with the date of surgery was taken into account for survival analyses. PFS was defined as survival without physical evidence of disease recurrence, disease-specific survival (DSS) was defined as survival without death due to ovarian cancer, and OS was defined as survival without death due to any cause. Data were censored for survival if, at the last point of contact, the patient had not reached the respective event. A univariate analysis of basic clincopathological parameters and DSS has been previously reported for this cohort [132]. All patients provided informed consent at the time of participation and the approval for this study was obtained from the University of British Columbia, British Columbia Cancer Agency Research Ethics Board (H02-61375).

Table 2. Patient and tumour characteristics

Clinico-pathological Parameters	Histological Subtype					
	High-grade serous	**Low-grade serous**	**Endo-metrioid**	**Clear cell**	**Mucinous**	**All Subtypes**
FIGO stage						
I	48 (24.7%)	1 (9.1%)	66 (55.5%)	67 (51.1%)	18 (60.0%)	200 (41.2%)
II	82 (42.3%)	6 (54.5%)	47 (39.5%)	56 (42.7%)	11 (36.7%)	202 (41.6%)
III	64 (33.0%)	4 (36.4%)	6 (5.0%)	8 (6.1%)	1 (3.3%)	83 (17.1%)
Grade						
1	0 (0%)	11 (100.0%)	78 (65.5%)		11 (36.7%)	100 (20.6%)
2	52 (26.8%)	0 (0%)	34 (28.6%)	N/A	17 (56.7%)	103 (21.2%)
3	141 (72.7%)	0 (0%)	7 (5.9%)		2 (6.7%)	281 (57.9%)
not graded	1 (0.5%)					1 (0.2%)
Age (years)						
median	61.22	63.49	54.12	55.33	54.99	57.14
range	37.59 – 85.96	33.54 – 81.63	29.45 – 88.07	28.10 – 89.05	25.36 – 76.65	25.36 – 89.05
Number of cases	194 (40.0%)	11 (2.3%)	119 (24.5%)	131 (27.0%)	30 (6.2%)	485 (100%)

Table 3. Follow-up and survival characteristics by ovarian cancer subtype

Variable	High-grade serous	Endometrioid	Clear cell
Median follow-up (range), years	5.4 (0.4 – 23.6)	7.9 (0.1 – 21.2)	6.5 (0.3 – 23.6)
Number of disease progressions	98	21	44
Number of ovarian cancer deaths	88	20	40
Total number of deaths	119	39	56

2.3.2 Immunohistochemistry

The TMA was stained with antibodies directed against the proteins LC3A, HIF-1α, CA-IX, cleaved caspase-3 (cl. Casp-3), and Ki67. The following antibodies and dilutions were used: α-LC3A (Abgent, San Diego, CA, USA; AP1805a; rabbit polyclonal; 1:50), α-HIF-1α (Santa Cruz, Santa Cruz, CA, USA; sc-10790; rabbit polyclonal; 1:10), α-CA-IX (gift from Dr. Stephen Chia; mouse monoclonal; clone M75; 1:10), α-cl. Casp-3 (Cell Signaling, Danvers, MA, USA; 9661; rabbit polyclonal; 1:100), α-Ki67 (Fisher Scientific, Nepean, ON, CA; RM-9106; rabbit monoclonal; clone SP6; 1:200). For all markers, the TMA was sectioned at 4 μm onto Superfrost plus slides (Fisher Scientific), incubated overnight at 37°C, and deparaffinized in xylene and graded alcohols. For all markers except Ki67, a Ventana Discovery XT autostainer was used for immunohistochemical staining (Ventana, Tucson, AZ, USA). All kits and reagents were from Ventana unless otherwise stated. For LC3A and cl. Casp-3 staining, Ventana's standard CC1 protocol was used for antigen retrieval. All blocking steps were included as part of the DABMap kit used for detection. Antibodies were manually added to the slides at the dilutions indicated above in Antibody Diluent and incubated for 60 minutes. A cross-adsorbed biotinylated goat α-rabbit immunoglobulin (Ig) G secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) was manually applied at a dilution of 1:500 for 32 minutes. Bound antibodies were detected using the

DABMap kit, counterstained with hematoxylin, and coverslipped manually with Cytoseal-60 (Richard Allan, Kalamazoo, MI, USA). Mouse ovarian follicles were used as a positive control for LC3A staining [133]. HIF-1α staining was performed using the ChromoMap kit. Ventana's standard CC2 protocol was used for antigen retrieval. Primary antibody was added for 2 hours without heat and UltraMap anti-rabbit HRP secondary antibody was applied for 16 minutes. CA-IX staining was performed using the DABMap kit. Ventana's mild CC1 protocol was used for antigen retrieval. Primary antibody was added for 32 minutes with heat and Universal Secondary antibody was applied for 32 minutes. Ki67 staining was performed on a Ventana BenchMark XT using the iVIEW DAB kit. Ventana's mild CC1 protocol was used for antigen retrieval. Primary antibody was added for 32 minutes with heat and the iVIEW biotinylated Ig secondary antibody was applied for 8 minutes.

2.3.3 Immunohistochemical scoring and analysis of markers

All clinical and pathological data as well as staining for other markers for each tumour sample were blinded to the pathologists during scoring. The LC3A staining patterns assessed included diffuse cytoplasmic, juxtanuclear, and SLS, all at 200x magnification as described previously [124]. The LC3A SLS pattern was scored independently by two pathologists (HH and SE) and their results significantly correlated (Spearman $r = 0.5637$, $p = <0.0001$). One scoring set for the LC3A SLS was used for the analyses reported in this manuscript. For the LC3A SLS staining pattern, the assigned score represents the total number of LC3A SLS per tumour core, and patients were grouped into categories of LC3A SLS-positive (LC3A SLS present in tumour) *vs* LC3A SLS-negative (no LC3A SLS present in tumour) for survival analyses. For the diffuse cytoplasmic staining pattern, patients were grouped according to the 67^{th} percentile into low *vs* high tumours. For the juxtanuclear

staining pattern, patients were grouped into categories of juxtanuclear-positive *vs* negative tumours. HIF-1α, CA-IX, and cl. Casp-3 staining were assessed on the basis of percent positive cells per tumour core and patients were separated into categories of high *vs* low expression based on the median for HIF-1α and positive *vs* negative for CA-IX and cl. Casp-3. Ki67 staining was assessed on the basis of percent positive epithelial (tumour) cells per tumour core and patients were separated into categories of high *vs* low expression based on the 75th percentile. For patients with duplicate tumour cores available for assessment, the score from the highest scoring core was used for subsequent analyses for all immunohistochemical markers assessed in this paper.

2.3.4 Cell lines and culture conditions

The cell lines used in this study were the clear cell lines ES-2, RMG-1, and TOV-21G and the high-grade serous lines OVCAR3, OVCAR4, and OV-90 (gifts from OvCaRe). For standard culture conditions, all cell lines except the OVCAR3 cells were maintained in RMPI 1640 Medium + 2.05 mM L-Glut, 10% heat inactivated fetal bovine serum (FBS), 100 U/ml Penicillin, 100 μg/ml Streptomycin, and 1 mM HEPES (all from Fisher Scientific). The aforementioned medium base and supplements were also used for the OVCAR3 cells, however, they were maintained in 20% FBS instead of 10%, and supplemented with an additional 8.4 μg/ml insulin, 7.6 μg/ml transferrin, and 10 ng/ml sodium selenite (additives all from Sigma, Oakville, ON, CA). All cells were maintained at 37 °C, 20% O_2, and 5% CO_2 in a water-jacketed Forma Scientific Incubator.

2.3.5 Autophagy induction assays

Each cell line was subjected in duplicate to three conditions: normoxia, hypoxia, or hypoxia + glucose-deprivation. Cells cultured in normoxia were plated in standard media and maintained under standard growth conditions. Cells cultured under hypoxia were plated in standard medium and then placed in a Hypoxic Glove Box (Coy Laboratory Products Inc., Grass Lake Charter Township, MI, USA) with final gas concentrations of 0.5% O_2, 5% CO_2, and 94.5% N_2 and maintained at 37°C. Similarly, cells were placed in hypoxia and glucose-free medium for the remaining condition using RPMI 1640 + L-Glut (-) D-Glucose (Life Technologies, Burlington, ON, CA) as the base medium. One set of cultures exposed to each of the three conditions for each cell line was treated with a final concentration of 10 μM HCQ (Acros Organics, Geel, BE) upon plating and the remaining set was left untreated. Cells cultured for three days were collected and lysed in total cell lysis buffer (2% SDS (Sigma), 0.1 M DTT (Fisher Scientific), 60mM Tris (pH 6.8) (Sigma), 10% glycerol (Fisher Scientific), 1 EDTA-free protease inhibitor cocktail tablet/10 ml (Roche, Laval, QC, CA) in dH_2O) at 99°C with shaking at 1400 rpm using a Thermomixer (Eppendorf, Mississauga, ON, CA) for 10 minutes. Proteins were resolved using 4-12% precast Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes (Pall Corporation, Ville St. Laurent, QC, CA). Membranes were probed with the primary antibodies α-LC3 (generated by Quality Control Biochemicals, Hopkinton, MA, USA according to ref [134] using the peptide sequence PSEKTFKQRRSFEQC; rabbit polyclonal; 1:2000) and α-β-Actin (Sigma; mouse monoclonal; clone AC-15; 1:40000); secondary antibodies were α-rabbit IgG (H&L) IRDye®800 conjugated (Rockland, Gilbertsville, PA, USA; 611-132-002; goat polyclonal; 1:10000) and Alexa Fluor 680 α-mouse IgG (Life Technologies; A10038; donkey polyclonal;

1:10000), respectively. Bands were quantified using the Odyssey program version 3.0 (LI-COR, Lincoln, NE, USA). LC3-II levels were normalized to β-actin.

2.3.6 Statistical analyses

Kaplan-Meier curves were plotted, log-rank tests performed for patient survival, and hazard ratios (HRs) and 95% confidence intervals (CIs) calculated using GraphPad Prism version 5.04 (GraphPad Software, La Jolla, USA). Prism was also used to conduct Mann-Whitney tests to compare LC3A SLS counts between patients grouped based on IHC marker status, stage, or age. Multivariate analyses were conducted using SPSS version 14.0 (IBM, Armonk, NY, USA). This study complied with reporting recommendations for tumour marker prognostic studies (REMARK) criteria [135].

2.4 Results

2.4.1 LC3A stone-like structures (SLS) are associated with poor prognosis in patients with clear cell ovarian carcinoma but not patients with other subtypes

It has been reported that the LC3A SLS staining may represent hyper-autophagy and that this is associated with poor patient prognosis in a variety of cancer types [124-127]. To determine whether prognosis in ovarian cancer is associated with LC3A SLS, LC3A staining was performed and analyzed on 485 ovarian tumour TMA samples. LC3A SLS staining was observed in the three major ovarian cancer subtypes represented in our cohort (high-grade serous, endometrioid, and clear cell) (Figure 3). To assess if any of the three reported LC3A staining patterns were a prognostic indicator for patients, we plotted Kaplan-Meier survival curves and conducted log-rank tests for PFS, DSS, and OS for each subtype and staining pattern [124]. The presence of LC3A SLS in clear cell ovarian tumours showed a strong

correlation with poor patient outcome as LC3A SLS were associated with an increased risk of relapse (PFS) [HR: 2.61 (95% CI 1.14-5.99); $p = 0.0232$], higher disease-specific death (DSS) [HR: 4.36 (95% CI 1.80-10.55); $p = 0.0011$], and reduced overall survival (OS) [HR: 3.52 (95% CI 1.64-7.56); $p = 0.0013$] (Figure 4). In contrast to clear cell ovarian tumours, we did not find any significant relationship between LC3A SLS and patient survival in high-grade serous or endometrioid cases (Figure 5). The small cohort size in mucinous and low-grade serous tumour categories (Table 2) precluded robust survival analyses. Despite the appearance of diffuse cytoplasmic and juxtanuclear LC3A staining in all subtypes, these staining patterns did not consistently correlate with patient survival in any subtype (data not shown).

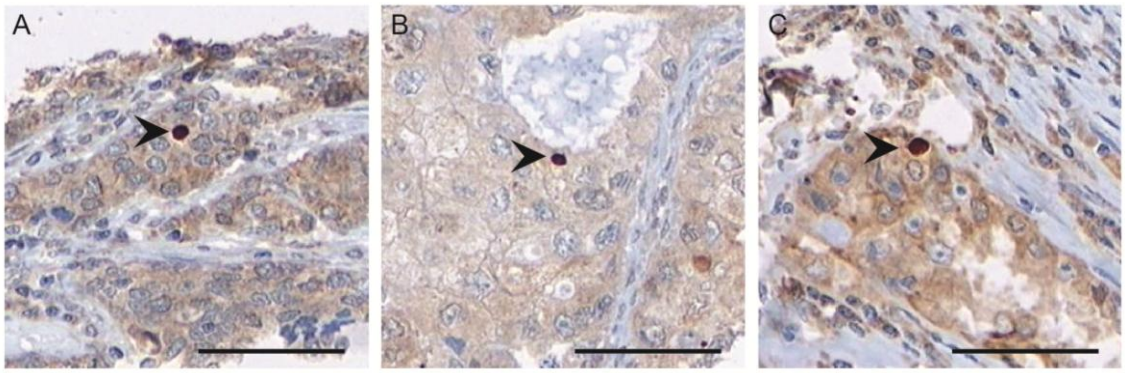

Figure 3. LC3A SLS are present in multiple subtypes of ovarian tumour specimens. LC3A SLS in (A) high-grade serous carcinoma, (B) clear cell carcinoma, and (C) endometrioid carcinoma. Arrowheads indicate LC3A SLS. LC3A SLS were defined as dense, spherical structures with an average diameter of 5 μM and typically enclosed within a cytoplasmic vacuole. Scale bar = 50 μm.

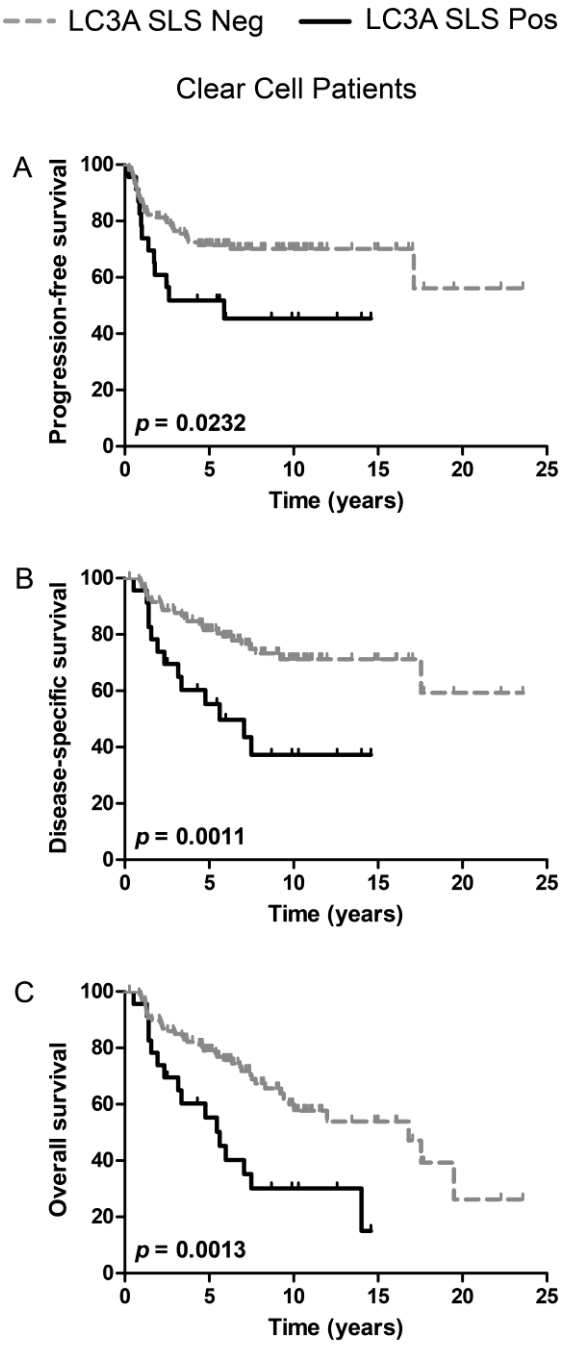

Figure 4. LC3A SLS are associated with poor patient prognosis in clear cell ovarian carcinoma.

Kaplan-Meier analyses of survival in ovarian clear cell carcinoma patients: (A) progression-free survival, (B) disease-specific survival, (C) overall survival. LC3A SLS Neg: $n = 108$, LC3A SLS Pos: $n = 23$. p values were calculated using the log-rank test.

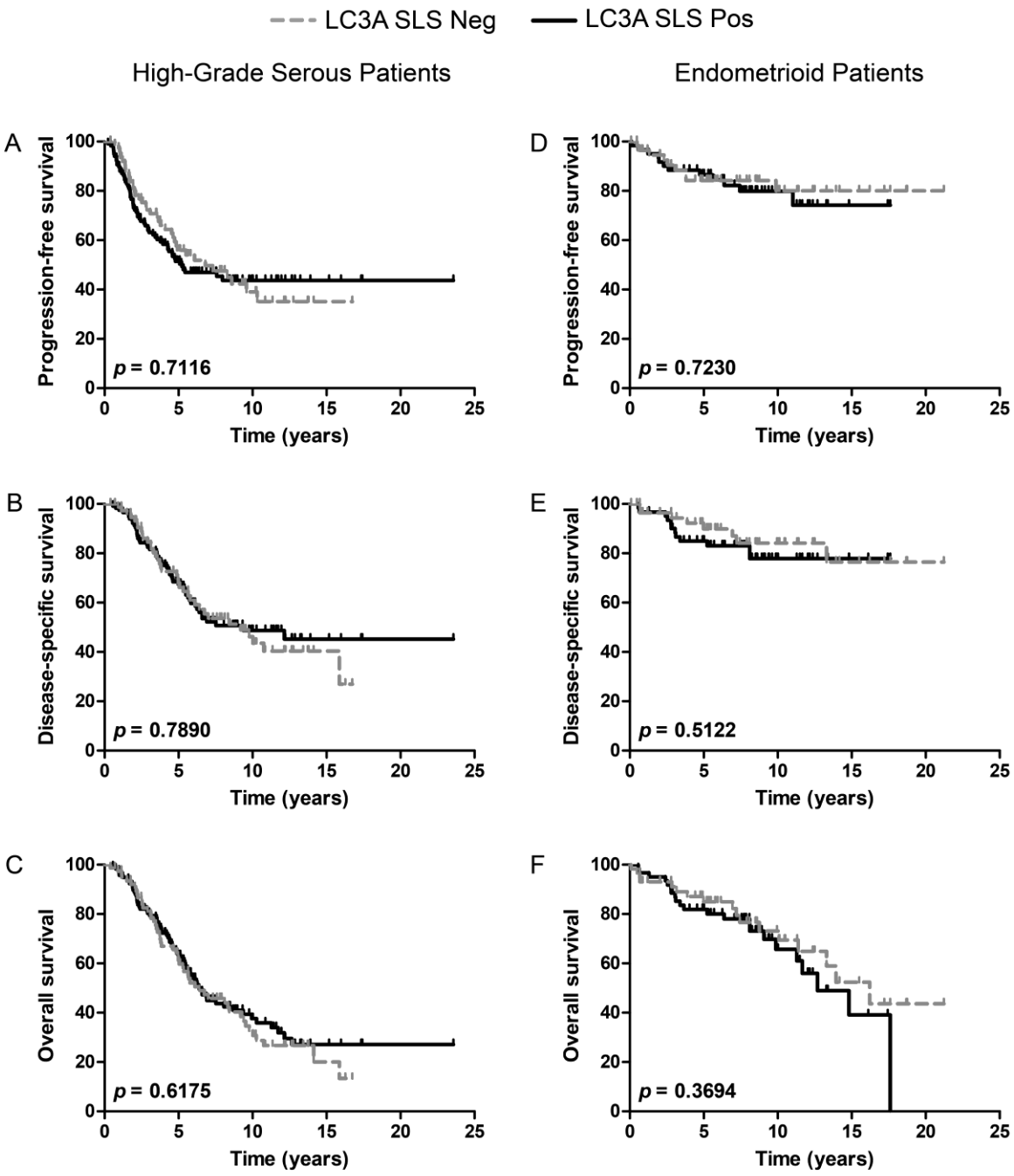

Figure 5. LC3A SLS are not associated with patient prognosis in high-grade serous or endometrioid carcinoma.

Kaplan-Meier analyses of survival in high-grade serous carcinoma patients: (A) progression-free survival, (B) disease-specific survival, (C) overall survival. LC3A SLS Neg: $n = 77$, LC3A SLS Pos: $n = 117$; Kaplan-Meier analysis of survival in endometrioid carcinoma patients: (D) progression-free survival, (E) disease-specific survival, (F) overall survival, LC3A SLS Neg: $n = 58$, LC3A SLS Pos: $n = 61$. p values were calculated using the log-rank test.

To examine the relationship between LC3A SLS and basic clinicopathological parameters in the clear cell patients, LC3A SLS scores for each tumour core were compared between patients with stage I, II, or III disease (Figure 6A). When all subtypes were assessed, there was no evident trend showing increasing LC3A SLS scores with more advanced stage disease and this was also the case when clear cell patients were excluded from the analysis. However, when the clear cell patients were examined alone, the stage III patients had significantly higher LC3A SLS scores than either the stage I or II patients (Figure 6A). Patient age exhibited no significant relationship with LC3A SLS regardless of subtype (Figure 6B).

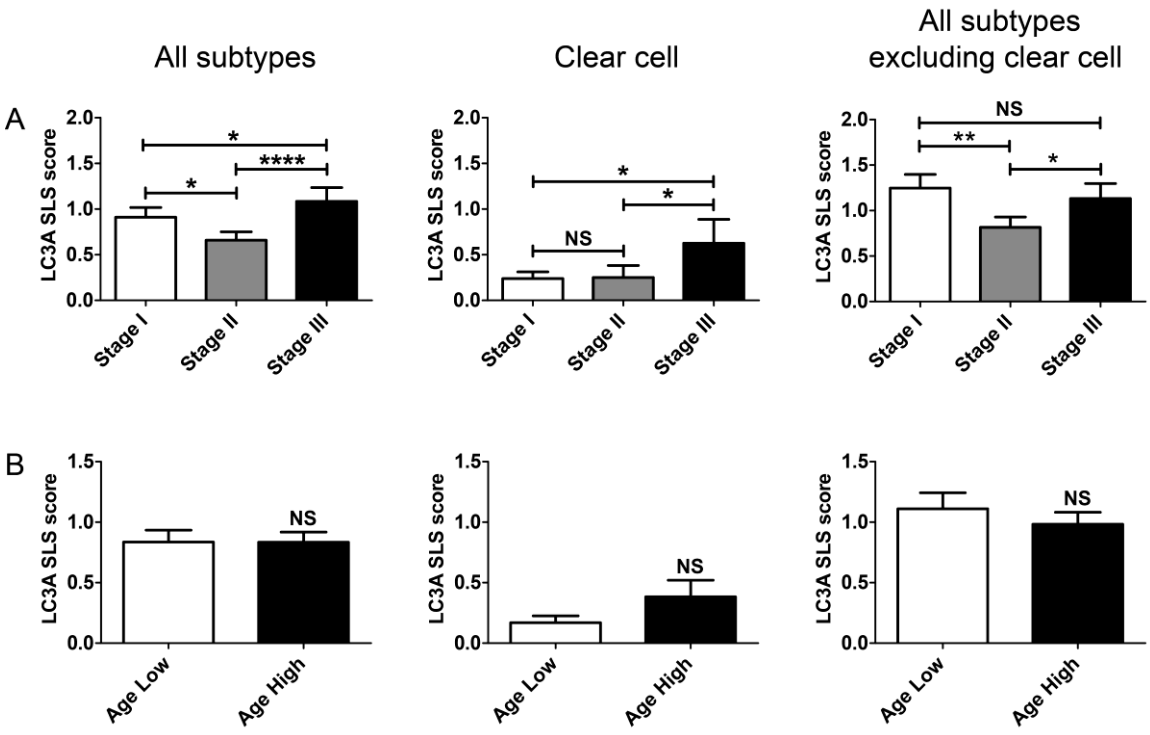

Figure 6. Relationship between LC3A SLS and basic clinicopathological parameters. Mean LC3A SLS score comparisons with (A) patient disease stage; (B) patient age (Low ≤ 57.14 years, High > 57.14 years). Error bars indicate standard error of the mean (SEM). *p* values were calculated using the Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, NS *p* = not significant.

The prognostic significance of LC3A SLS in the clear cell patients was further investigated by introducing LC3A SLS into a multivariable Cox proportional hazards regression model, together with patient age and disease stage. The model showed that LC3A SLS were an independent prognostic marker for DSS, and the only independent prognostic marker for OS, with calculated HRs of 2.55 (95% CI 1.21-5.37) and 1.95 (95% CI 1.00-3.77) respectively (Table 4).

Table 4. Multivariate analysis for survival of the patients with clear cell carcinoma

Variable	Progression-free survival		Disease-specific survival		Overall survival	
	HR (95% CI)	***p***	***HR (95% CI)***	***p***	***HR (95% CI)***	***p***
Stage						
I	0.11 (0.04-0.31)	<0.001	0.27 (0.10-0.73)	0.010	0.43 (0.16-1.11)	0.079
II	0.14 (0.05-0.40)	<0.001	0.35 (0.13-0.94)	0.037	0.48 (0.18-1.27)	0.141
III (ref)						
Age	0.97 (0.95-1.00)	0.041	0.99 (0.97-1.02)	0.462	1.02 (1.00-1.04)	0.134
LC3A SLS (pos *vs* neg)	1.51 (0.67-3.41)	0.324	2.55 (1.21-5.37)	0.014	1.95 (1.00-3.77)	0.049

2.4.2 LC3A SLS are associated with markers of hypoxia in patients with clear cell ovarian carcinoma and markers of proliferation and apoptosis in patients with any subtype

To investigate if LC3A SLS are associated with known markers of hypoxia, LC3A SLS scores between tumour samples were compared to high or low expression of HIF-1α and its transcriptional target, CA-IX. When all subtypes were examined together, LC3A SLS counts were significantly higher in tumours that displayed high levels of HIF-1α or CA-IX as compared to tumours that expressed low levels of the respective proteins (Figure 7). In clear cell cases alone, this significant association between LC3A SLS and CA-IX was also observed (Figure 7A) and the association between LC3A SLS and HIF-1α approached

significance ($p = 0.0681$) (Figure 7B). In contrast, if clear cell patients were excluded, the association in the remaining subtypes was no longer observed for both hypoxia markers supporting the concept that in clear cell patients, LC3A SLS may be an indicator of tumours undergoing hypoxia-induced autophagy.

We also examined the relationship between LC3A SLS and markers of apoptosis and proliferation by staining with antibodies specific for cl. Casp-3 and Ki67, respectively. In all subtypes, LC3A SLS counts were significantly higher in cl. Casp-3 positive tumours while a similar trend was found in the clear cell tumours ($p = 0.2171$) (Figure 8A). Highly

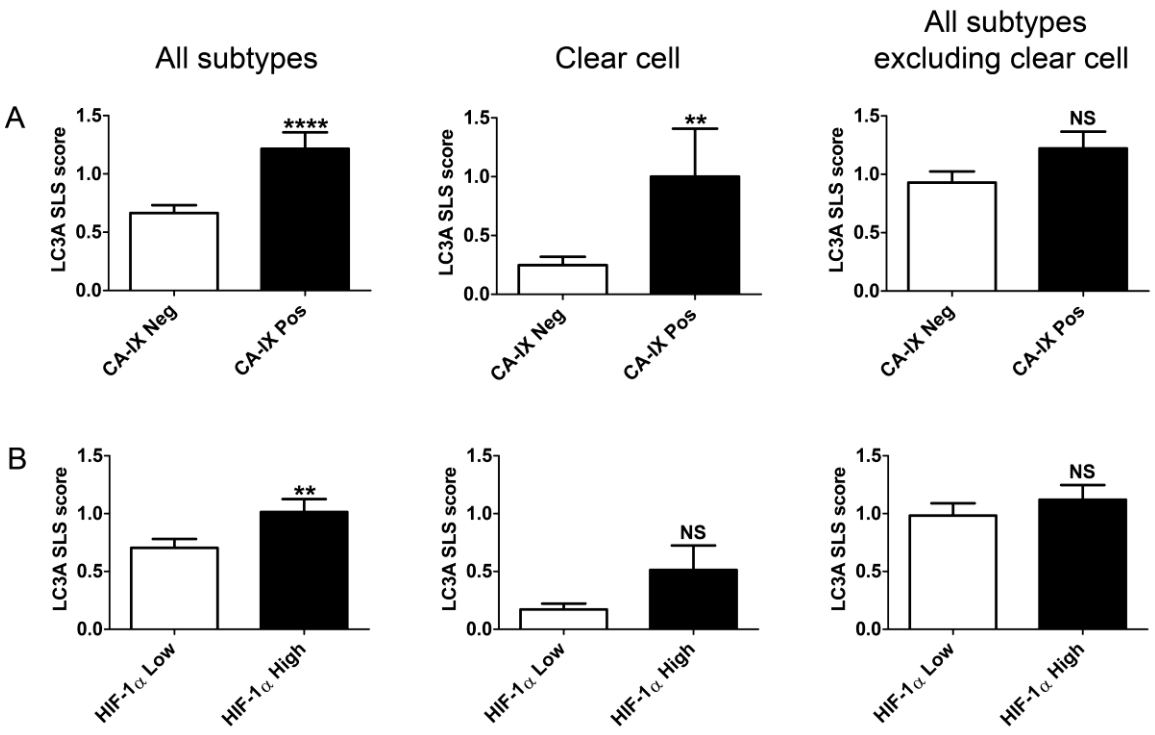

Figure 7. Relationships between markers of autophagy and hypoxia in clear cell and other ovarian cancer subtypes.

Mean LC3A SLS score comparisons with immunohistochemical markers (A) CA-IX and (B) HIF-1α. Error bars indicate SEM. *p* values were calculated using the Mann-Whitney test. $^{**}p < 0.01$, $^{****}p < 0.0001$, NS *p* = not significant.

proliferating tumours as evidenced by high Ki67 staining showed a positive association with LC3A SLS counts regardless of the subtype analyzed (Figure 8B).

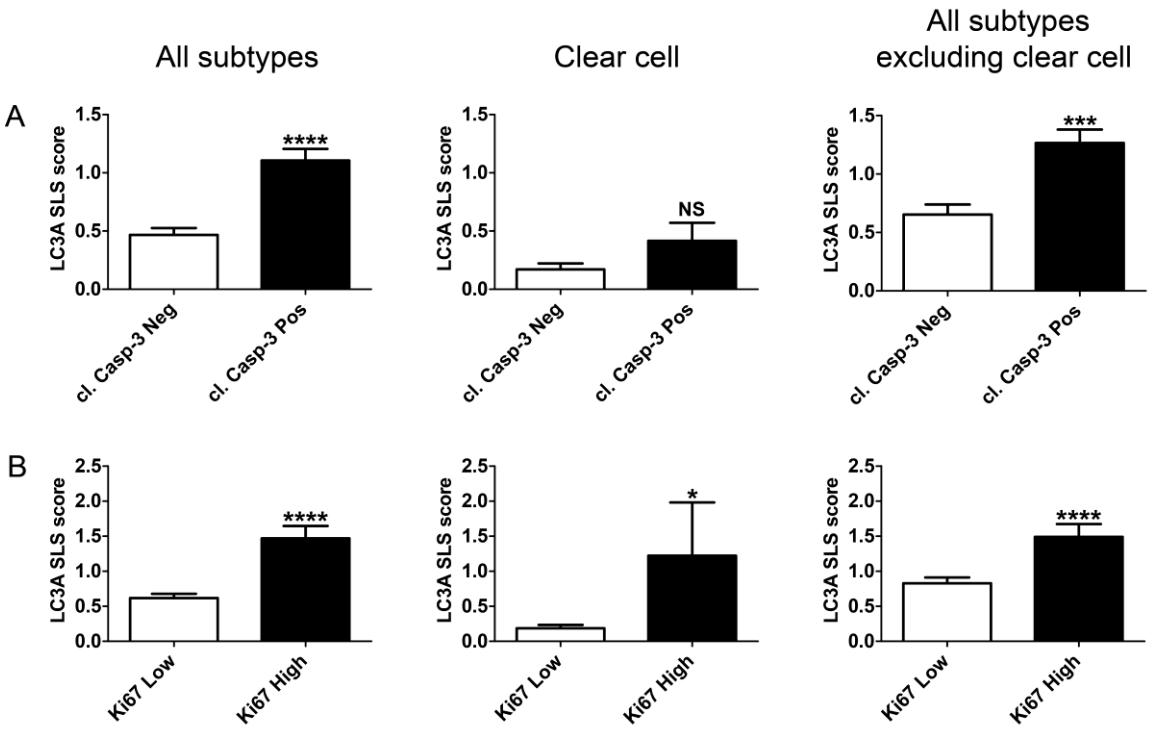

Figure 8. LC3A SLS correlate with markers of apoptosis and proliferation in all subtypes of ovarian carcinoma.
Mean LC3A SLS score comparisons with immunohistochemical markers (A) cl. Casp-3 and (B) Ki67. Error bars indicate SEM. *p* values were calculated using the Mann-Whitney test. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, NS p = not significant.

2.4.3 Induction of autophagy in response to hypoxia and glucose deprivation is dependent on ovarian tumour subtype

We next tested whether autophagy induction in response to hypoxia and glucose deprivation differed depending on the ovarian cancer subtype. Three high-grade serous

tumour cell lines (OVCAR3, OVCAR4 and OV-90) and three clear cell tumour cell lines (RMG-1, TOV-21G, and ES-2) were subjected to an autophagy induction assay [136]. Cells were cultured in duplicate under conditions of normoxia, hypoxia, or hypoxia and glucose-free medium for three days. One set of cultures was pre-treated with 10 μM HCQ to prevent autophagic degradation of LC3-II, the biochemical marker of activated autophagy. LC3-II in the hypoxic or hypoxic and glucose-free samples was compared to LC3-II in the normoxic samples. Using Western blot analysis, we observed that LC3-II was low or undetectable in all samples that were not treated with HCQ (Figure 9). However, in the HCQ-treated samples, two of the three clear cell lines displayed a substantial accumulation of LC3-II, indicating induction of autophagy, in the hypoxia or hypoxia and glucose-free samples (Figure 10A). In contrast all three of the high-grade serous cell lines displayed minimal to no accumulation of LC3-II in response to these metabolic conditions (Figure 10B). The levels of autophagy induction in response to hypoxia in the clear cell lines did not significantly increase with the

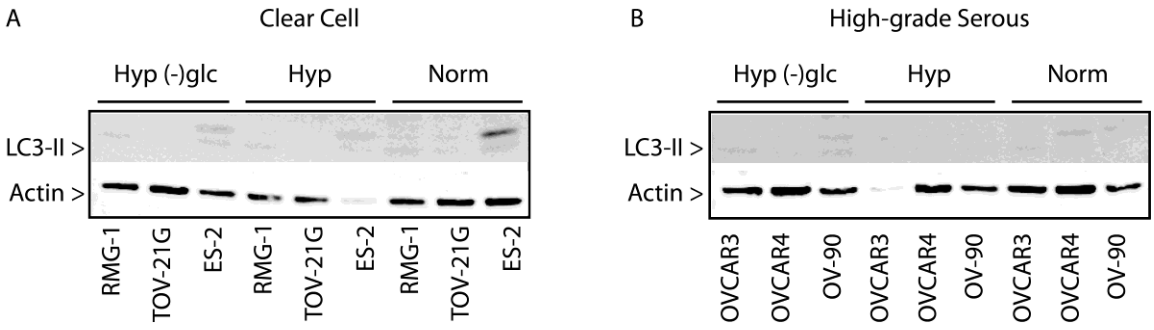

Figure 9. Clear cell carcinoma cells and high-grade serous carcinoma cells have minimal detectable LC3-II in the absence of HCQ under normoxia, hypoxia, or hypoxia + glucose-deprivation.

(A) Western blots of clear cell lysates; (B) Western blots of high-grade serous lysates. Norm = normoxia, Hyp = hypoxia (0.5% oxygen), Hyp (-)glc = hypoxia (0.5% oxygen) + glucose deprivation.

addition of glucose deprivation indicating that hypoxia alone is the crucial metabolic stressor required to activate autophagy in the clear cell subtype.

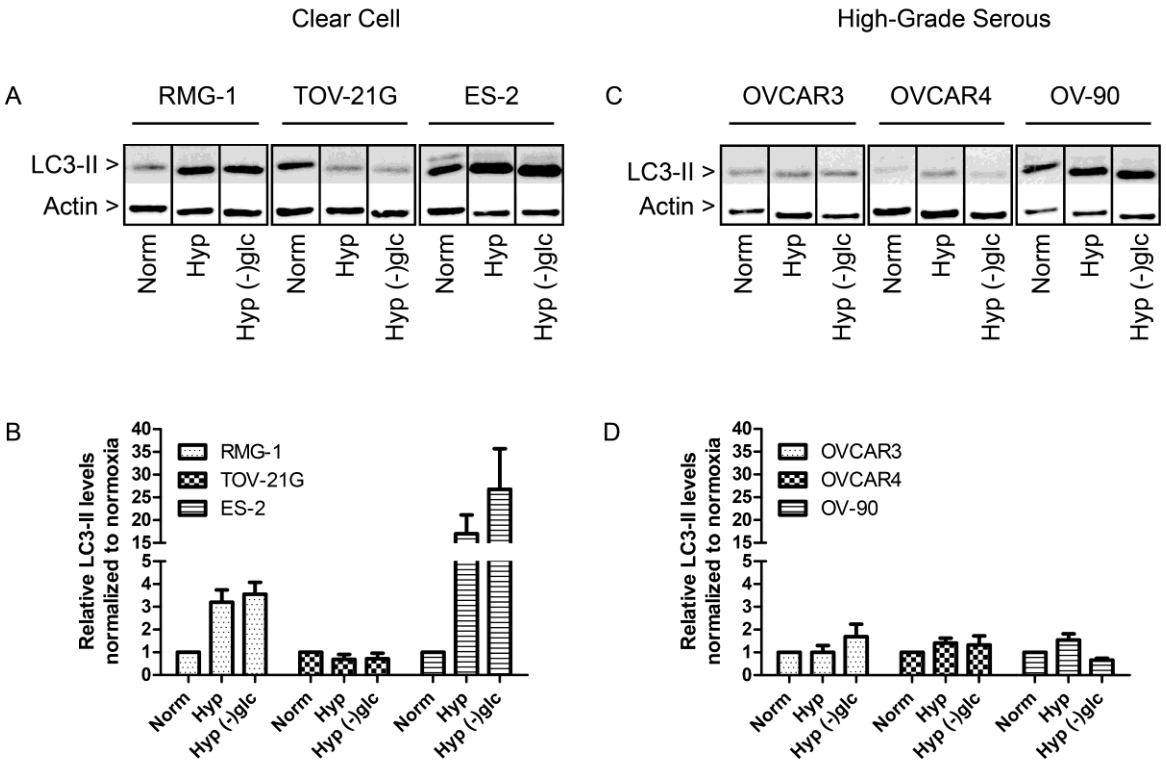

Figure 10. Clear cell carcinoma cells have higher autophagy induction in response to hypoxia and glucose deprivation than high-grade serous carcinoma cells.
(A) Western blots of clear cell lysates; (B) Western blot quantification of LC3-II levels relative to normoxia samples for clear cell lines (average of 3-4 independent experiments); (C) Western blots of high-grade serous lysates; (D) Western blot quantification of LC3-II levels relative to normoxia samples for high-grade serous cell lines (average of 3-4 independent experiments). LC3-II protein levels were normalized to β-actin. Error bars indicate SEM. Norm = normoxia, Hyp = hypoxia (0.5% oxygen), Hyp (-)glc = hypoxia (0.5% oxygen) + glucose deprivation. All samples were treated with 10 μM HCQ when subjected to the different culture conditions.

2.5 Discussion

Our results provide evidence for the autophagy marker LC3A as a prognostic indicator of poor survival in patients with clear cell ovarian carcinoma. Previous work suggests that LC3A SLS represent upregulation of autophagy, and in tumour types including breast, colon, lung, and endometrial, high numbers of LC3A SLS correlated with poor patient outcomes [124-127]. In the current study, we found the presence of LC3A SLS to be a strong predictor of poor survival in patients with clear cell ovarian cancer. This prognostic relationship was not observed in the other major subtypes in our cohort, including patients with high-grade serous or endometrioid ovarian cancers.

LC3A SLS in clear cell tumours are also associated with the markers of hypoxia, HIF-1α and CA-IX, implying a direct relationship between hypoxia and autophagy induction. Other groups have also reported positive associations between expression of autophagy-related and hypoxia-associated proteins in patient malignancies such as pancreatic cancer and melanoma [131, 137]. The observation of hypoxia-induced autophagy is supported by further *in vitro* mechanistic investigations using a variety of cell types [38-42]. A recent study found that clear cell cultures were better able to survive and proliferate under the conditions of hypoxia and glucose deprivation than high-grade serous cultures [121]. Indeed the upregulation of autophagy as an adaptation to hypoxia in clear cell tumours is supported by our observation that compared to high-grade serous tumour cell lines, clear cell tumour lines displayed a robust induction of autophagy when subjected to hypoxia or combined hypoxia and glucose deprivation. Based on these results, high levels of hypoxia-induced autophagy appear to be a unique feature of clear cell tumours. This property may reflect the intrinsic hypoxic nature of this histological subtype or an enhanced ability of clear cell tumours to respond to and survive in a hypoxic environment. Consistent with our

findings, there have been several studies that have reported increased expression of hypoxia-related genes or proteins in clear cell tumours compared to other subtypes of ovarian tumours or healthy ovarian tissue [106, 121-123]. Clear cell carcinoma is also associated with endometriosis which can cause iron-induced oxidative stress, resulting in the formation of reactive oxygen species (ROS) [84, 138]. ROS can also be produced by hypoxia and are a known inducer of autophagy [139, 140]. ROS produced via iron-induced oxidative stress or hypoxia may represent another stress under which clear cell tumours exploit autophagy to survive.

Similar to other studies investigating LC3A SLS in tumour specimens, we found an association between LC3A SLS and stage in clear cell tumours, indicating that high LC3A SLS counts reflect an aggressive neoplastic cell population with high ability for invasion and metastasis, and poor patient survival [127]. In addition, high Ki67 staining was associated with higher LC3A SLS counts, as reported in cutaneous squamous cell carcinoma which also supports the conclusion that LC3A SLS are present in more aggressive tumours [128]. Despite higher levels of proliferation, LC3A SLS correlated with tumours staining positively for the apoptosis marker, cl. Casp-3. The presence of apoptosis in these tumours may reflect a failed attempt to utilize hypoxia-induced autophagy. Although we found that clear cell tumours tended to have fewer LC3A SLS than tumours of other subtypes, this is not unexpected since each subtype of ovarian cancer is a unique disease [77]. Therefore, one would expect to see this variation in LC3A SLS numbers, for example between clear cell and high-grade serous tumours, just as it has been found that LC3A SLS numbers vary between different cancer types [124-127]. Despite several studies reporting an association of LC3A SLS and clinical outcomes, the functional link between LC3A SLS and hyper-autophagy has

yet to be confirmed. Therefore, interpretations arising from these clinical observations should be considered in light of this open question.

The lack of an association between autophagy and patient survival in other ovarian cancer subtypes may highlight the importance of hypoxia as an autophagy-inducing feature unique to clear cell carcinoma. This characteristic may compliment and provide additional criteria for classifying and implementing therapies for distinct subsets of ovarian carcinoma. Together, these data suggest that patients with clear cell tumours may benefit from targeted therapies that re-enforce pathways that promote addiction to autophagy combined with agents that compromise the ability of cells to utilize autophagy and promote tumour survival.

2.6 Acknowledgements

The authors thank Christine Chow and especially Katy Milne for their assistance with immunohistochemistry tissue preparation and staining. The authors also thank Vincent Poon and members of the CIHR Team in Investigating Autophagy Proteins as Molecular Targets for Cancer Treatments for their helpful comments and discussion. This work was supported by the Cheryl Brown Ovarian Cancer Outcomes Unit of the British Columbia Cancer Agency.

Chapter 3: Autophagy promotes carboplatin and paclitaxel resistance in a syngeneic mouse model of ovarian cancer

Adapted from: Jaeline E. Spowart[1,2,3], Dan Wu[4], Jenna Ries[1,2,3], Vincent I. Poon[1,2,3], Julian J. Lum[1,2,3] (Manuscript in preparation)

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JES, DW, and JJL designed the study. JES, DW, JNR, VIP, and JJL were involved in acquisition of data and analysis/interpretation of data. JES wrote the manuscript.

3.1 Abstract

Ovarian cancer is the most lethal of all gynaecological malignancies and has characteristically poor initial or sustained responses to conventional chemotherapies. One adaptation that ovarian tumour cells may employ to promote resistance to these treatments is autophagy. Autophagy is a survival process utilized by cells to degrade unnecessary or damaged cytoplasmic constituents. Recently, it has been reported that ovarian cancer cells use autophagy to resist the anti-apoptotic effects of the chemotherapy drug cisplatin. Here, we show that the cells of a syngeneic mouse model of ovarian cancer, known as ID8, induce autophagy in response to treatment with the most commonly used ovarian cancer chemotherapeutic drugs, carboplatin and paclitaxel. In addition, co-treatment of cells with the autophagy inhibitor HCQ and either chemotherapy drug results in an impairment of cellular proliferation recovery after withdrawal of treatment as compared to treatment with the chemotherapy drugs alone. Treatment of ID8 tumours grown *in vivo* with HCQ as a single agent significantly inhibited tumour growth compared to mice treated with PBS. Furthermore, co-treatment with carboplatin and paclitaxel plus HCQ resulted in a modest inhibition of tumour growth when compared to treatment with the chemotherapy drugs alone. Together, these results indicate that combining autophagy inhibitors with standard chemotherapy drugs may be a viable strategy to improve treatment responses in ovarian cancer patients.

3.2 Introduction

Autophagy is a survival process that can be used by cells in response to stresses such as hypoxia, nutrient deprivation, growth factor withdrawal, and chemotherapy [37-48]. During autophagy, a double-membraned vesicle known as an autophagosome sequesters and

engulfs cytoplasmic cargo and degrades the cargo upon subsequent fusion with a lysosome. The role of autophagy in chemotherapy response is of particular concern for cancer researchers and autophagy has now been shown to promote chemotherapy resistance in several different cancer cell types in response to a variety of chemotherapeutic agents [43-45, 47]. Such findings have prompted a growing interest in treating cancer patients with autophagy inhibiting drugs and a number of clinical trials are now underway, primarily employing HCQ as the agent to inhibit autophagy.[4] Unfortunately, many of the preclinical studies that have been conducted to date have been performed *in vitro* or in immunocompromised mouse models and there is a need for additional studies to be conducted in immunocompetent animal models.

Ovarian cancer is the leading cause of death amongst all gynaecological malignancies [66]. Though ovarian cancer patients have good prognoses if their disease is detected at early stage, the majority of cases are unfortunately detected in late stage as a result of largely asymptomatic early disease [67]. In addition, current treatments for ovarian cancer have poor initial efficacy or poor sustained efficacy, and patients often either do not respond to treatment or relapse [77, 82]. Ovarian cancer is a category of diseases that encompasses a variety of unique subtypes which include high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous [67]. Though each subtype is considered a distinct disease, the standard therapy for all subtypes is surgery with platinum- and taxane-based chemotherapy. The chemotherapy drugs paclitaxel and carboplatin are the standard of care for the treatment of advanced ovarian cancer [74]. Carboplatin exerts its effects on cells by covalently binding to purine DNA bases, which eventually leads to cellular apoptosis [115]. Paclitaxel is a microtubule-stabilizing agent which delays or blocks the metaphase-anaphase

[4] Source: www.clinicaltrials.gov

transition during mitosis, leading to mitotic arrest and eventual cell death [118]. Though carboplatin and paclitaxel are considered the most effective therapeutic agents to date for ovarian cancer, there is an acute need for improved treatment efficacy for these patients. Recent studies have found that autophagy is involved in ovarian cancer cell resistance to cisplatin (the parent drug of carboplatin), however, to our knowledge there are no studies that have examined carboplatin or paclitaxel in this context [43, 116].

The ID8 mouse model of ovarian cancer was originally developed to allow the study of events related to ovarian cancer in humans, using mice with intact immune systems. ID8 cells reliably form tumours in syngeneic mice and exhibit disease behaviour and characteristics similar to the high-grade serous subtype, the subtype that accounts for the majority of human ovarian tumours [67, 141]. Here we report an investigation into the role of autophagy in ovarian cancer treatment resistance to the drugs carboplatin and paclitaxel, both *in vitro* and *in vivo*, using the ID8 syngeneic mouse model of ovarian cancer.

3.3 Methods

3.3.1 Cell line and culture conditions

The generation of ID8 mouse ovarian cancer cells by Roby, *et al.* has previously been described [141]. Briefly, the authors harvested healthy ovarian surface epithelial cells from C57BL/6J mice and passaged the cells *in vitro* until a portion of the cells spontaneously transformed. The transformed cells were then cloned and tested for their ability to form intraperitoneal (i.p.) and subcutaneous (s.c.) tumours in syngeneic mice, upon injection into the respective sites. One of the clones that had tumourigenic capability was referred to as ID8.

The ID8 cells used in this report were a gift from Dr. Brad Nelson. ID8 cells were maintained in DMEM High Glucose Medium + 2.05 mM L-Glut, 4% heat inactivated FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 1 mM HEPES (all from Fisher Scientific), 4.2 μg/ml insulin, 3.8 μg/ml transferrin, and 5 ng/ml sodium selenite (latter three reagents all from Sigma). All cells were maintained at 37 °C, 20% O_2, and 5% CO_2 in a water-jacketed Forma Scientific Incubator.

3.3.2 Autophagy induction assays

Treatment with carboplatin (Novopharm, Toronto, ON, CA), paclitaxel (Biolyse Pharma, St. Catharines, ON, CA), and HCQ (Acros Organics) as indicated in Figure 11A was initiated 24 hours after ID8 cells were plated. Three days post-treatment, cells were collected and lysed in total cell lysis buffer (2% SDS (Sigma), 0.1 M DTT (Fisher Scientific), 60mM Tris (pH 6.8) (Sigma), 10% glycerol (Fisher Scientific), 1 EDTA-free protease inhibitor cocktail tablet/10 ml (Roche) in dH_2O) at 99°C with shaking at 1400 rpm using a Thermomixer (Eppendorf) for 10 minutes. Proteins were resolved using 4-12% precast Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes (Pall Corporation). Membranes were probed with the primary antibodies α-LC3 (generated by Quality Control Biochemicals according to ref [46] using the peptide sequence PSEKTFKQRRSFEQC; rabbit polyclonal; 1:2000) and α-β-Actin (Sigma; mouse monoclonal; clone AC-15; 1:40000); the secondary antibodies were α-rabbit IgG (H&L) IRDye®800 conjugated (Rockland; 611-132-002; goat polyclonal; 1:10000) and Alexa Fluor 680 α-mouse IgG (Life Technologies; A10038; donkey polyclonal; 1:10000), respectively. Bands were quantified using the Odyssey program version 3.0 (LI-COR). LC3-II levels were normalized to β-actin.

3.3.3 Generation of GFP-LC3 ID8 cells

Green fluorescent protein (GFP)-LC3 ID8 cells were generated by retrovirally transducing wild-type ID8 cells with the MIGR-IRES-hCD8-GFP-LC3 vector. In this system, GFP is tagged to the N-terminus of rat LC3 and remains associated with LC3 after C-terminal cleavage of pro-LC3 to form LC3-I. 48 hours post-transduction, cells were sorted using a BD Influx Cell Sorter (BD Biosciences, Mississauga, ON, CA) and GFP-positive cells were retained and used for subsequent relevant experiments.

3.3.4 GFP-LC3 cleavage assays

GFP-LC3 ID8 cells were cultured, treated, and cell lysates processed for Western blot analysis as described for the autophagy induction assay. Membranes were probed with the primary antibodies α-GFP (Abcam, Cambridge, MA, USA; rabbit polyclonal; ab6556; 1:5000) and α-β-Actin as described for the autophagy induction assay; secondary antibodies were α-rabbit IgG (H&L) IRDye®800 conjugated and Alexa Fluor 680 α-mouse IgG, respectively, as described for the autophagy induction assay. Bands were quantified using the Odyssey program version 3.0 (LI-COR). Within each lane, the ratio of the free GFP band to the LC3-GFP band was calculated.

3.3.5 Fluorescence microscopy

GFP-LC3 ID8 cells were plated into wells containing sterile microscope coverslips (Fisher Scientific) and left 24 hours to adhere to the coverslips. Cells were then treated with either 12.50 μg/ml carboplatin or 6.25 ng/ml paclitaxel or left untreated. Three days after treatment, media was removed from the wells and the cells were fixed with 3.65% formaldehyde in phosphate-buffered saline (PBS) (Sigma). The coverslips with adhered cells

were then mounted onto Superfrost®Plus microscope slides (Fisher Scientific) using Fluoroshield Mounting Medium (Abcam). Slides were observed using a BX53 system microscope (Olympus, Richmond Hill, ON, CA) and pictures were taken using a Nuance FX Multispectral Imaging System (Quorum Technologies Inc., Guelph, ON, CA).

3.3.6 Cell recovery assays – crystal violet absorbance

ID8 cells were plated at 500 cells/well in 3 ml of medium in 6-well plates. Cells were returned to the incubator and left to adhere for 2-3 hours. Once the cells had adhered to the plates, cells were treated in triplicate with carboplatin, paclitaxel, and HCQ as indicated in Figure 12A (left panel). Three days post-culturing cells, the media was removed from each well, the well was washed with 3 ml PBS, and 3 ml fresh media was added to the well. Four days post-media change, media was removed from all wells and cells were fixed for 10 minutes at room temperature with 1 ml/well 3.65% formaldehyde in PBS. Formaldehyde was then removed and each well was stained with 0.5 ml 0.1% crystal violet in dH_2O (Sigma) and plates were placed on an oscillator (Fisher Scientific) to distribute the stain amongst the cells for 15 minutes. Crystal violet was then removed and the wells were rinsed with an excess of tap water until no more dye was lifting from the wells. Plates were left to dry overnight. Crystal violet that had adhered to the cells was dissolved by adding 2 ml 10% acetic acid to each well and putting the plates on an oscillator for 25 minutes. Crystal violet solution in each well was mixed by pipetting up and down and then 100 µl from each well was transferred to a 96-well flat bottom ELISA plate (Fisher Scientific). The absorbances of the wells were read on a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm.

3.3.7 Cell recovery assays – cell counts

ID8 cells were plated at 3000 cells/plate in 18 ml media in 10 cm plates. Cells were returned to the incubator and left to adhere for 2-3 hours. Once the cells had adhered to the plates, cells were treated with carboplatin, paclitaxel, and HCQ as indicated in Figure 12B. Three days post-culturing cells, the media was removed from each plate, the plate was washed with 18 ml PBS, and 18 ml fresh media was added to the plate. Four days post-media change, all of the cells were collected from each plate and counted by running cells through a BD FACSCalibur (BD Biosciences) with SPHERO^{TM} AccuCount Fluorescent Particles (Spherotech, Lake Forest, IL, USA).

3.3.8 In vivo HCQ and chemotherapy treatment experiment

ID8 cells were cultured in 15 cm plates and grown to confluency on the day of tumour cell implantation. Cells were harvested, washed, and suspended in PBS at a concentration of eight million cells/200µl. Mice were injected s.c. in the flank with eight million cells each. All mice were C57BL/6J mice (Jackson Laboratory, Bar Harbour, ME, USA). After eight days, mice were divided into four tumour size-matched groups: chemotherapy, chemotherapy + HCQ, HCQ, and PBS. Mice in the HCQ and chemotherapy + HCQ groups received 60 mg/kg HCQ in PBS daily *via* i.p. injection for 16 days beginning eight days post-tumour cell implantation. Mice in the chemotherapy and chemotherapy + HCQ groups received 50 mg/kg carboplatin and 16.7 mg/kg paclitaxel, each injected i.p., twice per week for two weeks beginning 10 days post-tumour cell implantation. Mice in the PBS group were injected with PBS i.p. on the same days that chemotherapy treatments were given. Tumours were measured approximately three times per week with digital calipers (Fisher Scientific) beginning on day eight and continuing until three weeks post-treatment

completion. The area of each tumour was calculated according to the formula $a = l \times w$ where a is the area, l is the length of the tumour (largest measurement) and w is the width of the tumour. This experiment complied with the University of Victoria Animal Care Committee Research Ethics Board.

3.3.9 Statistics

Comparisons between treatment group means were made by conducting unpaired t tests using GraphPad Prism version 5.04 (GraphPad Software).

3.4 Results

3.4.1 ID8 cells induce autophagy in response to carboplatin or paclitaxel treatment

We first examined the effects of the chemotherapeutic drugs carboplatin and paclitaxel on autophagy induction in ID8 cells. ID8 cells were cultured in duplicate with varying doses of carboplatin or paclitaxel or no chemotherapy drug for three days. One set of cultures was also treated with 5μM HCQ to prevent autophagic degradation of LC3-II, the biochemical marker of activated autophagy. LC3-II in the chemotherapy-treated samples was compared to LC3-II in the samples that did were not treated with chemotherapy. Using Western blot analysis, we observed that LC3-II was low or undetectable in all samples that were not treated with HCQ (Figure 11A). However, in the HCQ-treated samples, the accumulation of LC3-II in the chemotherapy-treated samples was greater than in the sample that had not been treated with chemotherapy (Figure 11A), indicating induction of autophagy in the chemotherapy-treated samples.

To confirm that treatment with carboplatin or paclitaxel induces active, functional autophagy rather than a blockade of autophagic cargo degradation, we employed a GFP-LC3

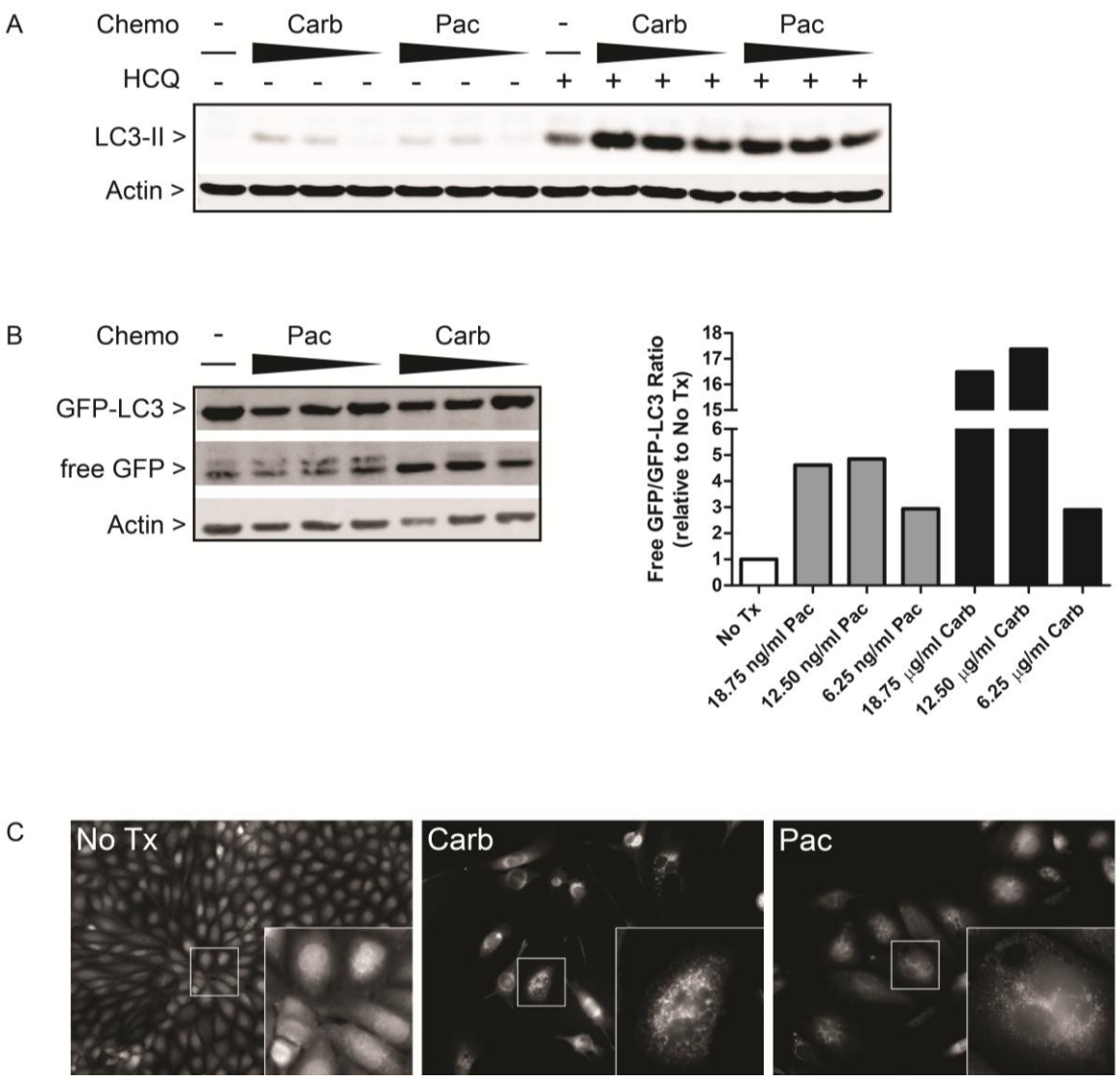

Figure 11. Treatment with carboplatin or paclitaxel induces autophagy in ID8 cells. (A) Western blots of ID8 cell lysates harvested from cultures treated with no chemotherapy drugs or treated with varying concentrations of carboplatin (carb) or paclitaxel (pac) +/- 5 μM HCQ for three days. Carb concentrations used (in decreasing order) were 18.75 μg/ml, 12.50 μg/ml, and 6.25 μg/ml. Pac concentrations used (in decreasing order) were 18.75 ng/ml, 12.50 ng/ml, and 6.25 ng/ml. (B) Left panel: Western blots of GFP-LC3 ID8 cell lysates harvested from cultures treated as in (A) except no cultures received HCQ treatment. Right panel: calculated ratios of free-GFP to GFP-LC3 for the blot to the left. Blots shown in (A) and (B) are representative of three independent experiments each. (C) Fluorescent microscopy images of GFP-LC3 cells either treated with no chemotherapy drugs, treated with 12.50 μg/ml carb, or treated with 6.25 ng/ml pac. The localization of GFP-LC3 can be seen as diffuse in the no treatment sample and punctate in the chemotherapy-treated samples.

cleavage assay. As autophagy progresses and the autophagic cargo begins to be degraded, the bond between GFP and LC3 is broken and free GFP is liberated. ID8 GFP-LC3 cells were cultured with varying doses of carboplatin or paclitaxel or no chemotherapy drug for three days. We compared the ratio of free GFP to GFP-LC3 in each sample and found that the ratio was higher in all chemotherapy-treated samples as compared to the sample that had not been treated with chemotherapy (Figure 11B), indicating that ID8 cells induce functionally active autophagy in response to treatment with carboplatin and paclitaxel. There was also a weak dose-dependent increase in autophagy induction with increasing drug concentration for each chemotherapy drug, though this trend was not observed in all experiments.

To visualize the formation of autophagosomes upon treatment with carboplatin or paclitaxel, ID8 GFP-LC3 cells were assessed by fluorescence microscopy. It was found that untreated cells exhibited a diffuse GFP-LC3 staining pattern, indicating that LC3 was not being incorporated into autophagosomes and, therefore, that autophagy was not being induced (Figure 11C, left panel). However, cells treated with either carboplatin or paclitaxel exhibited a punctate GFP-LC3 localization pattern indicating the presence of autophagosomes and, therefore, the induction of autophagy in response to these treatments (Figure 11C, centre and right panels).

3.4.2 Inhibition of autophagy compromises cellular proliferation after treatment with carboplatin or paclitaxel

To assess the requirement of autophagy for cell recovery after treatment with carboplatin or paclitaxel, ID8 cells were treated with varying doses of the chemotherapy drugs +/- 5 μM HCQ for three days. The drugs were then removed and the cells were left to recover for four days. After the recovery period, cells were fixed and then stained with

crystal violet. The crystal violet that had adhered to the cells was dissolved with acetic acid and the absorbances of the resulting solutions were read to obtain an estimate of the relative cell number between samples that had been treated +/- HCQ. It was found that the samples treated with HCQ in addition to the chemotherapy drugs had consistently lower absorbances than those treated with the chemotherapy drugs alone, even after adjusting for the toxicity of the HCQ alone (Figure 12A, left panel). We previously found that there was not a significant difference in cell number or viability between groups treated +/- HCQ after three days of treatment, before the four day recovery period (data not shown). Therefore, these results indicate that autophagy inhibition in combination with carboplatin or paclitaxel causes a synergistic inhibition of proliferation post-chemotherapy treatment.

These results were further validated by performing a similar recovery assay; however, the cells were counted at the end of the recovery period instead of being stained with crystal violet. Cell counts in the chemotherapy + HCQ treatment groups were consistently lower than those in the chemotherapy alone groups, even after adjusting for the toxicity of HCQ alone (Figure 12B), further validating the synergy of this treatment combination.

3.4.3 Autophagy inhibition in vivo suppresses tumour growth

To examine the effect of concurrent autophagy inhibition with carboplatin and paclitaxel treatment *in vivo*, mice were implanted with ID8 cells and allowed to form established tumours. Once tumours were established, mice were separated into one of four treatment groups: carboplatin + paclitaxel (chemo), carboplatin + paclitaxel + HCQ (chemo + HCQ), HCQ, and PBS (Figure 13). At the time of treatment initiation, the tumours were still somewhat inflamed and therefore a decrease in tumour size after treatment initiation is seen for all treatment groups, including the mice treated with PBS.

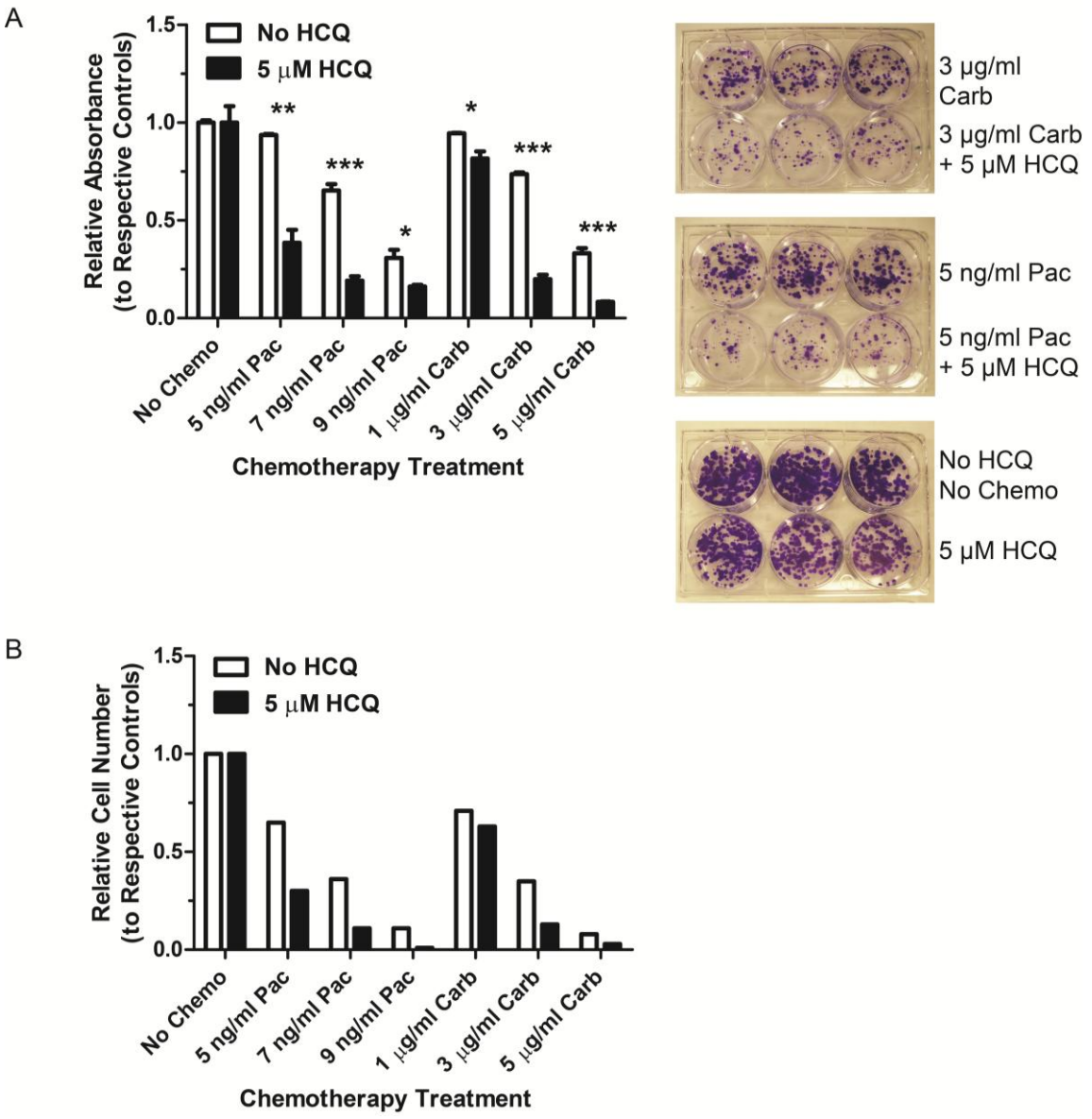

Figure 12. Inhibition of autophagy compromises cellular proliferation after treatment with carboplatin or paclitaxel.

Cellular proliferation recovery after chemotherapy treatment as assessed by (A) Left panel: crystal violet absorbance and (B) total cell counts. The experiments shown are each representative of three independent experiments. Data for samples that did not receive HCQ are shown relative to control samples that received no chemotherapy drugs and no HCQ. Data for samples that did receive HCQ are shown relative to samples that were treated with HCQ but not chemotherapy drugs. (A) Right panel: representative photographs of crystal violet staining of cells treated with or without HCQ in addition to carboplatin, paclitaxel, or no chemotherapy (chemo) (from a separate experimental replicate as that shown in the left panel). Pac = paclitaxel, Carb = carboplatin. Error bars indicate SEM. *p* values for (A) were calculated using an unpaired t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Interestingly, the HCQ alone appeared to have anti-tumour activity as a single agent as the HCQ group had significantly smaller tumours than the PBS group (Figure 13A). When the HCQ alone group was compared to the chemo alone group, the majority of the

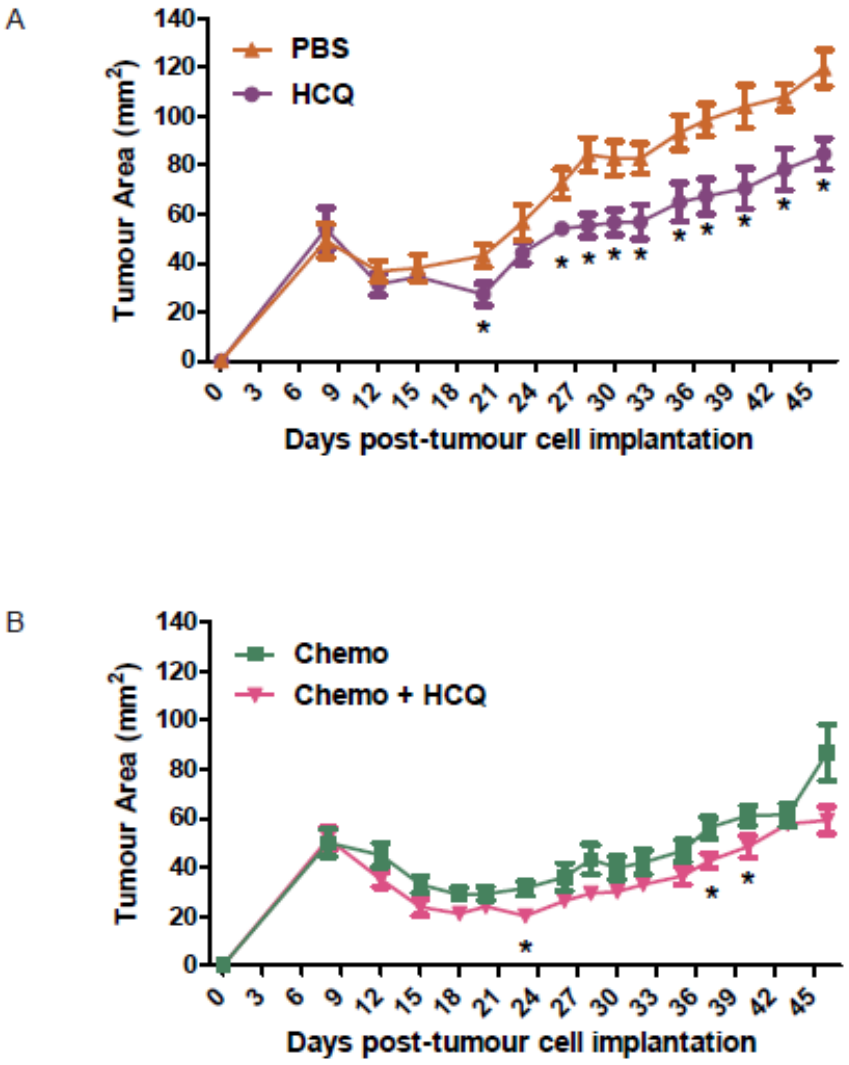

Figure 13. Treatment with HCQ suppresses tumour growth in ID8 tumour-bearing mice.

Mean (± SEM) tumour sizes over time for 9-10 mice per treatment group. Mice in (A) were treated with HCQ or PBS. Mice in (B) were treated with carboplatin +paclitaxel (chemo) or carboplatin + paclitaxel + HCQ (chemo + HCQ). HCQ treatments began on day 8 and chemotherapy treatments began on day 10. Treatments were completed on day 23. *p* values were calculated using an unpaired t test. $*p < 0.05$.

time points at which tumour measurements were taken did not show significant differences in tumour sizes between the two treatment groups (data not shown). In addition, the chemo + HCQ treatment group appeared to have modestly smaller tumours than the chemo alone group (Figure 13B). These findings indicate that autophagy plays a vital role in the *in vivo* growth of these tumours, both in response to chemotherapeutic stress, and potentially other stresses such as hypoxia and nutrient deprivation as demonstrated by the benefits observed when mice were treated with HCQ alone.

3.5 Discussion

Our results provide evidence for autophagy as a chemotherapy resistance mechanism exploited by ovarian carcinoma cells. Our findings that autophagy helps promote resistance to the commonly used ovarian cancer chemotherapeutic agents, carboplatin and paclitaxel, are consistent with other reports that ovarian cancer cells, including those of the high-grade serous subtype, employ autophagy to aid in their survival during treatment with carboplatin's parent drug, cisplatin [43, 116]. Interestingly, one of these reports noted that autophagy was utilized by ovarian carcinoma cells to aid in the degradation of misfolded or unfolded proteins, helping the cancer cells to avoid or overcome ER stress. While we have not specifically investigated whether this role for autophagy was also relevant in response to carboplatin or paclitaxel treatment in ovarian carcinoma cells, it seems very plausible that it would be. Carboplatin is closely related to cisplatin and the two drugs are thought to have similar mechanisms of action [115]. In addition, paclitaxel has been reported to induce ER stress in breast cancer cells and it is possible that it has a similar effect on ovarian carcinoma cells [120].

Interestingly, we found that HCQ as a single agent had a significant inhibitory effect on tumour growth *in vivo*. The exact reason(s) for this effect are not yet clear, but there are a few potential explanations. For example, ID8 tumours have been shown to have regions of hypoxia and autophagy is known to promote cell survival under hypoxia through a variety of mechanisms [42, 142]. It may be that autophagy is required by ID8 cells to survive and proliferate under hypoxic conditions within the tumours and that their survival and/or proliferation are compromised under hypoxia when autophagy is inhibited. In addition, just as the vasculature within solid tumours is often insufficient to supply adequate oxygen, it can also be insufficient to supply adequate nutrients. Autophagy can help cells to survive low nutrient levels by breaking down nonessential macromolecules to free up metabolites that can be used to fuel energetic processes [37, 48]. Autophagy therefore may also be required by ID8 cells to overcome low nutrient levels within the tumours.

As an extension of the above hypotheses, tumour cells with activating *RAS* mutations have been shown to be exquisitely sensitive to autophagy inhibition as they rely particularly heavily on autophagy to degrade damaged mitochondria that produce ROS and to maintain efficient cellular metabolism [143]. Though it has not been confirmed that ID8 cells have an activating *RAS* mutation, it has been reported that they show increased levels of Ras activation and therefore they may also be hypersensitive to autophagy inhibition [144]. We also acknowledge that it is possible that HCQ exerts its anti-tumour effects *via* an as yet undetermined mechanism that is not related to autophagy as has been reported by some researchers [145]. However, considering that autophagy can play a protective role in response to a variety of stresses commonly encountered by tumour cells, we maintain that it is likely that the anti-tumour effects of HCQ are at least in part mediated by its inhibition of

autophagy, though further experiments are required to support this hypothesis more definitively.

When HCQ was combined with chemotherapy treatment *in vivo*, there was a modest decrease in tumour growth as compared to chemotherapy treatment alone. We find these initial results to be promising and believe that they may be enhanced with some modifications of the treatment regimen. One way in which the tumour growth inhibitory effects of the treatment combination may be enhanced is by delivering the treatment for a longer period of time. CQ, HCQ's nearly identical parent drug, is known to take several weeks to reach steady state concentrations in humans and it may be that the mice were not treated for long enough to achieve sufficient autophagy inhibition to promote synergy with the chemotherapy drugs [146]. Increased inhibition of tumour growth may also be seen with the addition of a different class of drug to the treatment scheme. For example, it has been reported that concomitant inhibition of both autophagy and Akt was required to enhance cisplatin-induced apoptosis in skin carcinoma cells [45]. In this model, treatment with either cisplatin or an Akt inhibitor resulted in induction of autophagy. As ID8 cells have been reported to have enhanced Akt activation, adding an Akt inhibitor along with the chemotherapy drugs may result in even further induction of autophagy as compared to that seen with the chemotherapy drugs alone [144]. The increased dependency that the cells may have on autophagy in this scenario may mean that the addition of an Akt inhibitor to the chemotherapy + HCQ combination would prove effective for the treatment of ID8 tumours.

The enhanced efficacy of carboplatin or paclitaxel observed with the addition of an autophagy inhibitor in ovarian carcinoma cells lends support to the initiation of clinical trials in human ovarian cancer patients investigating this treatment combination. It should be

noted that the ID8 tumours cells have not been explicitly classified as any particular subtype of ovarian carcinoma. However, based on their cell type of origin, disseminated growth pattern when injected into the peritoneal cavity, and near-tetraploid chromosome number reflecting high genomic instability, these cells appear to resemble the high-grade serous subtype of human ovarian carcinoma [141]. Patients with high-grade serous tumours tend to have good initial response rates to carboplatin and paclitaxel, but have a high frequency of recurrence [82]. Therefore, high-grade serous tumours may be a good target for this treatment combination and it may be possible to achieve a lower frequency of recurrence in this patient population for whom better outcomes are desperately needed.

3.6 Acknowledgements

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Chapter 4: Concluding remarks

4.1 Chapter summaries and discussion

The objective of this thesis was to shed further light on the role of autophagy in ovarian carcinoma. In particular, I aimed to investigate evidence for the employment of autophagy by ovarian tumour cells to aid in their survival, and the contexts in which they utilize autophagy. I anticipate that such utilization ultimately negatively affects patient survival.

In Chapter 2, we investigated a retrospective cohort of nearly 500 ovarian carcinoma patients with tumours of various subtypes. In an effort to analyze autophagy levels in the tumours, we looked for a recently described structure known as an LC3A SLS which we believe to be representative of hyper-autophagy. We found that the presence of these structures correlated with poor patient survival, but only in the clear cell subtype. Interestingly, we also found that these structures correlated with markers of hypoxia, again uniquely in the clear cell subtype. These findings led us to investigate the autophagic behaviour of CCC cell lines under hypoxia and we found that CCC cell lines generally had a more robust induction of autophagy than HGSC cell lines under hypoxia. We therefore believe that the CCC subtype may have enhanced employment of and dependency upon autophagy to survive under hypoxic conditions.

One of the primary questions that remains from Chapter 2 is "what exactly is an LC3A SLS and how is it reflective of autophagy?" The group of researchers who first reported the existence of LC3A SLS, Sivridis, *et al.*, postulated that LC3A SLS are indicative of excessive levels of autophagic activity [124]. When investigating LC3A SLS in breast carcinoma specimens, they found that high numbers of these structures correlated with

tumour grade, which is in part reflective of proliferation. Sivridis, *et al.* concluded that LC3A SLS occur in tissues with high tumour cell turnover and are associated with an increased degradative process. In regards to the morphology and nature of LC3A SLS, Sivridis, *et al.* propose that they may represent structures known as "membranous whorls" which have been described by other groups. Membranous whorls result from massive degradation of membranous cellular components, leading to accumulation of membranes that form these whorls. Cells that exhibit membranous whorls may eventually die due to the excessive degradation of cytoplasmic components [147]. As noted in the introduction of this thesis, there are likely scenarios in which high levels of autophagy are induced for an extended period of time without respite, eventually leading to cellular destruction. LC3A SLS may be reflective of this particular type of autophagic activity and an LC3A SLS may in fact be the same thing as a membranous whorl.

Alternatively, even though LC3A SLS may be reflective of a hyper-autophagic response, is such autophagy even functional anymore or has the excessive burden of autophagic cargo exceeded the capacity of the autophagic machinery? It is very possible that the latter scenario is actually the case. However, a tumour that contains cells that have resorted to such an excessive autophagic response likely contains many cells that are performing very high levels of functional autophagy, just below the threshold of dysfunctional autophagy potentially represented by the LC3A SLS. This could explain why even though the frequency of LC3A SLS is quite low in the tumour types that have been examined, both by us and by Sivridis, *et al.*, there is still a correlation with patient survival for many cancer types because LC3A SLS are acting as an indicator of an entire tumour that is engaging in high levels of autophagy. Further experiments will be needed to confirm the exact nature of the LC3A SLS, but the strong links found between these structures and

cancer patient survival, as well as markers of hypoxia, indicates that they are biologically important.

Our findings that there may be a unique dependency of clear cell tumours upon autophagy for survival under hypoxia leads to the question of why this subtype would require autophagy in this context more so than other subtypes. One possible explanation may be in regards to CCC metabolism. Clear cell tumours show increased expression of HIF-1α, glucose transporter 1(Glut 1), and genes involved in glycolysis, indicating that CCC may be a particularly glycolytic subtype [121, 122, 148]. Glycolysis is a much less efficient form of metabolism as compared to oxidative phosphorylation in terms of ATP production [149]. It may be that clear cell tumours depend upon autophagy under hypoxia to provide metabolites to sustain their need for increased glycolytic metabolism.

As mentioned previously, a large proportion of CCC cases have activating mutations in *PIK3CA* [100, 101]. On one hand, it would appear that such mutations would result in suppression of autophagy in these tumours as PI3K activates mTOR which is traditionally thought of as an autophagy suppressor. However, activation of mTOR can also result in increased translation of *HIF-1a*, a known inducer of autophagy [38, 39, 150]. In fact, in CCC, the PI3K-Akt-mTOR-HIF pathway has been proposed as a therapeutic target and it is possible that activation of this pathway actually promotes autophagy induction in clear cell tumours as a result of the pro-autophagy effects of HIF-1α [81]. In addition, CCC is strongly associated with endometriosis and endometriotic cysts are characterized by high levels of iron-induced oxidative stress [84, 138]. Though this stress is not directly induced by hypoxia, it can lead to the development of ROS, a known inducer of autophagy [139, 140]. Together, there are various molecular characteristic of CCC that indicate that autophagy may be an important pro-survival mechanism of this subtype, particularly in response to hypoxia.

In Chapter 3, we utilized a syngeneic mouse model of ovarian carcinoma known as ID8 to investigate the role of autophagy in response to treatment with the commonly used ovarian cancer chemotherapeutic drugs, carboplatin and paclitaxel. We found that the ID8 cells did indeed induce autophagy in response to either carboplatin or paclitaxel treatment. In addition, autophagy appeared to promote resistance to these chemotherapeutic drugs as co-treatment with the autophagy inhibitor HCQ suppressed the proliferative recovery of the cells after withdrawal of the chemotherapeutics. To determine if autophagy inhibition could also enhance the efficacy of carboplatin and paclitaxel *in vivo*, we treated ID8 tumour-bearing mice with a combination of chemotherapy plus HCQ, chemotherapy alone, HCQ alone, or PBS. Surprisingly, HCQ as a single agent actually showed a significant inhibition of tumour growth as compared to the PBS-treated mice. This finding indicates that ID8 tumours may rely on autophagy for adaptation to stresses other than chemotherapy treatment. When comparing the mice treated with chemotherapy plus HCQ to those treated with chemotherapy alone, there was a modestly increased inhibition of tumour growth in the combination treatment group. This indicates that autophagy may indeed help ovarian tumour cells to resist chemotherapy treatment *in vivo* but further modifications of the treatment procedure may yield better results.

One of the key questions that remain from Chapter 3 is "why does treatment with carboplatin or paclitaxel induce autophagy in ovarian cancer cells and how does autophagy promote treatment resistance in this context?" As speculated in the discussion in Chapter 3, both a DNA damage response and/or an ER stress response could potentially be involved. Further experiments to compare the degree of DNA damage and protective surges in ATP levels between cells treated with the chemotherapy drugs +/- autophagy inhibition may support the role of autophagy as a mechanism to promote resistance to DNA damage. In

addition, analysis of the ER stress response, such as upregulation of PERK signalling and increased levels of ubiquitinated proteins, between cells treated with the chemotherapy drugs +/- autophagy inhibition may support the role of autophagy as a mechanism to degrade misfolded proteins and alleviate ER stress in response to treatment with carboplatin and/or paclitaxel. If autophagy does in fact play such a role, perhaps the addition of a proteasome inhibitor to this treatment combination to further potentiate ER stress may yield even better anti-tumour efficacy.

4.2 Integrating concepts from Chapters 2 and 3

While the findings of this thesis discussed in the previous section are exciting and point to relevant roles for autophagy in ovarian cancer, the relationship between the results of the two chapters warrants discussion. One key question that comes from comparing the two sets of results is "if autophagy does indeed contribute to treatment resistance in subtypes other than CCC as indicated by the results of the ID8 experiments, then why was an association between LC3A SLS and patient survival not found for these other subtypes?" It may be that the apparent discrepancy is linked to the nature of the different autophagic stimuli. To consider this, we must be mindful of the fact that the tumours assessed in Chapter 2 were removed from patients before the patients had received any chemotherapy treatment. Therefore, none of these tumours had yet encountered the stress of chemotherapy treatment. However, many, if not all, of these tumours already had regions of hypoxia and the cells of these tumours would therefore already be coping with this stress. As we found that CCC cells are more likely to induce autophagy under hypoxia than HGSC cells, it is logical that an indication of an existing autophagic response to hypoxia in clear cell tumours would correlate with better tumour cell survival and therefore poorer patient

survival. It also follows that there would not be an association between LC3A SLS and markers of hypoxia in high-grade serous tumours as there is in clear cell tumours.

In contrast, if autophagy is important for resistance to chemotherapy treatment in HGSC cells, and potentially cells of other subtypes as well, tumours with high pre-existing levels of autophagy before chemotherapy treatment may not actually be indicative of tumours that are able to use autophagy to promote chemotherapy treatment resistance. Tumours that can exploit autophagy in this manner may actually be those who would show a large induction of autophagy upon treatment with chemotherapy, but may actually have low levels of autophagy before treatment. If tumour cells already have high levels of autophagy, for example to cope with high nutrient demands, before even encountering chemotherapy, they may not be able to increase the levels any further to compensate for this additional stress. Perhaps the high-grade serous tumours with high numbers of LC3A SLS represent such a category of tumours, and this is why we did not see poorer patient survival associated with LC3A SLS in this subtype. In future clinical trials assessing autophagy inhibitors as an addition to cancer treatments, comparing the autophagy levels in patient tumours before and after the addition of the cancer treatment may help clinicians to distinguish the patients who would most likely benefit from the addition of autophagy inhibitors to their treatment regimen.

Alternatively, it is possible that LC3A SLS can be caused by factors other than autophagy in some tumour types and are therefore not indicative of hyper-autophagy in all tumour types. A study that compares the readout of LC3A SLS to electron microscopy (EM) analysis for the presence of autophagic structures, which is currently considered the gold standard in the field for assessment of autophagy levels in patient tissues, would help to address this question [47]. A correlation between high numbers of LC3A SLS and high

numbers of autophagosomes revealed by EM, would support the use of LC3A SLS as a readout of autophagic activity. In addition, performing such a study with a variety of tumour types may identify some tumour types in which a strong correlation exists, but others in which there is no such correlation. This type of rigorous study would likely be necessary for the translation of the assessment of LC3A SLS into clinical practice. If LC3A SLS are in fact not indicative of hyper-autophagy in subtypes other than CCC, this could be another reason why we do not see a relationship between LC3A SLS and patient survival in these other subtypes, even though tumours of these subtypes likely use autophagy to promote their survival.

4.3 Future Directions

The results of the research detailed in this thesis, as well as current knowledge regarding the molecular characteristics and disease behaviours of the different subtypes of ovarian carcinoma, indicate many potential anti-cancer treatments that may show enhanced efficacy if combined with autophagy inhibitors in the correct subtype(s).

The use of angiogenesis inhibitors has recently been demonstrated to be effective for the treatment of ovarian cancer, likely because inhibition of vasculature formation results in decreased delivery of oxygen and nutrients to tumour cells. As hypoxic clear cell tumours may be uniquely dependent upon autophagy, it may be that a strategic treatment combination for CCC patients would be the coupling of an angiogenesis inhibitor with an autophagy inhibitor [151, 152]. Pushing clear cell tumours into an even more hypoxic state may enhance their dependency upon autophagy and therefore treatment with an autophagy inhibitor in this context may yield maximal anti-tumour activity. Preclinical data indicates that this combination is effective for the treatment of glioblastoma tumours which utilize

autophagy to overcome hypoxia induced by an angiogenesis inhibitor, and perhaps clear cell tumours would also respond favourably to this treatment combination [54].

As high-grade serous tumours tend to have high initial response rates to carboplatin and paclitaxel as compared to other subtypes, this subtype likely represents the best candidate for treatment with carboplatin and paclitaxel in combination with autophagy inhibition. HGSC shows a strong pre-existing sensitivity to carboplatin and paclitaxel which can ideally be capitalized upon to yield good patient outcomes. In contrast, other subtypes such as CCC and LGSC which do not show strong initial response rates to carboplatin and paclitaxel upon which to capitalize, are likely to benefit most from treatment with different agents that are better targeted to the molecular characteristics of these subtypes [78, 90, 91, 93, 94].

The high frequency of *KRAS* mutations in mucinous and low-grade serous tumours indicates that these subtypes may be particularly sensitive to autophagy inhibition. Recent research has investigated non-ovarian tumour cells with activating *KRAS* or *HRAS* (v-Ha-ras Harvey rat sarcoma viral oncogene homolog) mutations, and found that these cells are particularly dependent on autophagy, showing impaired proliferation and decreased survival when autophagy is inhibited as compared to cells which do not harbour *KRAS* or *HRAS* mutations [143, 153]. Interestingly, pronounced effects of autophagy inhibition on these cells are seen in the absence of additional treatment with anti-cancer agents. Such findings warrant investigation of autophagy inhibition in mucinous and low-grade serous tumours harbouring *KRAS* mutations.

Another attractive treatment strategy for mucinous tumours may be the anti-HER2 monoclonal antibody trastuzumab (Herceptin®) combined with autophagy inhibition. It should be noted that trastuzumab has not yet been tested on a large scale as an agent for

treatment of MC. However, the high frequency of HER2 protein overexpression in MC, and a promising response from one MC patient treated with trastuzumab in a pilot study, has garnered interest in treating this subtype with trastuzumab [113, 114]. Autophagy has been shown to promote resistance to trastuzumab in breast cancer cells and it could potentially play a similar role in MC [154].

There are many exciting treatment options for the different subtypes of ovarian carcinoma, in addition to the ones listed above, that would potentially yield improved patient survival benefits upon combination with autophagy inhibitors. However, additional development of a few key tools would notably assist researchers in their attempts to further elucidate the roles of autophagy in the different subtypes of EOC in response to various stresses, and then translate these findings into practices that will yield clinical benefit. One such tool is subtype-specific mouse models of ovarian cancer. Many of the ovarian cancer animal models developed to date do not accurately reflect the subtypes commonly found in human patients [110]. Subtype-specific models will aid researchers in strategically determining which subtypes are likely to benefit from certain treatment combinations. Another desired tool for autophagy research is the development of a reliable method of assessing autophagy levels in patient tumours. Currently, EM is considered the gold standard for accomplishing this but the technique is cost- and labour-intensive and does not allow for assessment of autophagy in the commonly available FFPE format of archival tissue samples [47]. It is our hope that LC3A SLS may represent a method of assessing autophagy in FFPE samples, but further experiments are needed to definitively confirm the relationship between autophagy levels and LC3A SLS. One additional tool that will aid in the translation of ovarian cancer autophagy research to improved patient outcomes is an autophagy inhibitor that is safe for use in humans but has more potent inhibitory activity than the current

commonly used inhibitor, HCQ. Investigations to develop such a drug are currently underway by a variety of research groups and it is hoped that a safe and more potent autophagy inhibitor will soon be available.

In summary, the findings presented in this thesis have provided further evidence for the importance of autophagy in response to hypoxia and chemotherapy treatment in ovarian carcinoma. It is my hope that research continues in this field and that we can ultimately apply the results outlined in this thesis, along with additional results from current and future research, to improve the survival of ovarian cancer patients.

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Appendix A: Abbreviations

3-MA	3-methyladenine
Akt	v-akt murine thymoma viral oncogene homolog 1
AMBRA1	activating molecule in Beclin 1-regulated autophagy 1
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ARHI	aplasia Ras homolog member I
ARID1A	AT-rich interactive domain 1A (SWI-like)
ATF4	activating transcription factor 4
Atg	autophagy-related
ATP	adenosine triphosphate
BAF250a	BRG1-Associated Factor 250a (protein encoded by the *ARID1A* gene)
Bcl-2	B-cell CLL/lymphoma 2
Bcl-X_L	Bcl-2-like 1 protein
BNIP3	Bcl-2/adenovirus E1B 19-kDa interacting protein 3
BNIP3L	BNIP3-like protein (also known as Nix)
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
CA-IX	carbonic anhydrase-IX
CCC	clear cell ovarian carcinoma
CI	confidence interval

cl. Casp-3	cleaved caspase-3
CQ	chloroquine
CTNNB1	catenin (cadherin-associated protein), beta 1
	(gene that encodes for beta-catenin)
DNA	deoxyribonucleic acid
DSS	disease-specific survival
EC	endometrioid ovarian carcinoma
EM	electron microscopy
EOC	epithelial ovarian cancer (also known as ovarian carcinoma)
ER	endoplasmic reticulum
FBS	fetal bovine serum
FFPE	formalin-fixed, paraffin-embedded
FIGO	International Federation of Gynecology and Obstetrics
FIP200	200 kDa focal adhesion kinase family-interacting protein
Glut 1	glucose transporter 1
GFP	green fluorescent protein
HCQ	hydroxychloroquine
HER2	human epidermal growth factor receptor 2
HGSC	high-grade serous ovarian carcinoma
HIF-1	hypoxia inducible factor-1
HNF-1β	hepatocyte nuclear factor-1β
HR	hazard ratio
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
Ig	immunoglobulin

IHC	immunohistochemistry
i.p.	intraperitoneal
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LC3	microtubule-associated protein 1 light chain 3
LC3A SLS	LC3A stone-like structure
LGSC	low-grade serous ovarian carcinomas
MAP1LC3	microtubule-associated protein 1 light chain 3
MAPK	mitogen-activated protein kinase
MC	mucinous ovarian carcinoma
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
Nbr1	neighbour of BRCA1 gene 1
NH_4Cl	ammonium chloride
NS	not significant
OS	overall survival
PBS	phosphate-buffered saline
PE	phosphatidylethanolamine
PERK	PKR-like ER kinase
PFS	progression-free survival
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol-3-phosphate
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide (gene that encodes for PI3K)

PTEN	phosphatase and tensin homolog
REMARK	reporting recommendations for tumour marker prognostic studies
RNA	ribonucleic acid
ROS	reactive oxygen species
s.c.	subcutaneous
SEM	standard error of the mean
STIC	serous tubal intraepithelial carcinoma
SWI-SNF	switch/sucrose-nonfermentable
TMA	tissue-microarray
TP53	tumour protein p53
ULK1/2	unc-51-like kinase 1/2
UPR	unfolded protein response
Vps	vacuolar protein sorting