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Epithelial Ovarian Cancer: searching for new modulators of drug resistance.

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Abstract

Epithelial Ovarian Cancer (EOC) is the most lethal gynaecological malignancy due to the advanced stage at diagnosis and the development of drug-resistant recurrences after a good initial response to the therapy based on platinum drugs. Alterations of different cellular pathways, such as DNA repair, apoptosis and p53, have been identified as linked to the onset of platinum resistance in ovarian cancer. We performed a high-throughput shRNA-based screening to identify genes whose suppression enhanced cell sensitivity to platinum drug in different EOC cell lines (synthetic lethality). Bioinformatics analysis and a subsequent validation screening identified 8 genes possibly involved in platinum response. Our aim was to dissect the role of one of these genes, SGK2 (serum and glucocorticoid-regulated kinase 2), in the response to platinum in EOC cells.

We observed that 1) SGK2 overexpression conferred an increased platinum resistance to EOC cells, that acquired also a higher *in vitro* and *in vivo* growth rate respect to the control, and 2) by means of the SGK2 dominant negative (SGK2T193A/S356A) construct we demonstrated that SGK2 kinase activity was necessary to protect from platinum-induced death. For this reason, we tested the SGK kinase inhibitor GSK650394, showing that it was able to increase platinum-induced death only in SGK2-expressing EOC cells. Upon GSK650394 treatment, SGK2-expressing cells showed an accumulation of cytoplasmic autophagic vacuoles and increased expression of p62 and LC3II/LC3I, two known autophagy markers, suggesting that it induced alterations in the autophagy pathway. Evaluating additional critical markers of autophagy and monitoring autophagic flux, we confirmed that SGK2 inhibition (by silencing or pharmacological inhibition with GSK650394) induced a block of autophagy, favouring the accumulation of autophagic vacuoles already fused with lysosomes, but inefficient in the degradation of cell debris.

Overall we identified a new role for SGK2 in the regulation of autophagy that likely explains its role in the control of platinum response in EOC cells. Considering that induction of autophagy is an escaping mechanism by which EOC cells could overcome platinum-induced death, the combination of SGK2 inhibition and platinum might represent a promising strategy to improve the treatment of EOC patients.

Introduction

1.1 Epithelial Ovarian Cancers (EOCs)

Epithelial Ovarian Cancers (90% of ovarian cancers) represent the fourth commonest cause of female cancer death and the first one for gynaecological malignancy in the Western world. It was estimated that every year 220000 women develop Epithelial Ovarian Cancer worldwide (Jayson et al, 2014). EOCs are primarily a disease of postmenopausal women, occurring most commonly in sixth and seventh decades of life. Although a clear etiologic factor responsible for the development of ovarian cancer has not been identified, the risk of the disease is inversely proportional to the number of lifetime ovulations. Thus, factors associated with suppression of ovulation, such as increasing numbers of full-term pregnancies, longer duration of lactation, and oral contraceptives are associated with a decrease in ovarian cancer incidence. Factors associated with greater lifetime ovulation and/or greater lifetime estrogen exposure such as nulliparity, early age of menarche or late age of menopause, and use of hormone replacement therapy increase risk. Furthermore, inflammatory conditions such as endometriosis appear to increase the risk of ovarian cancer development, whereas tubal ligation and hysterectomy reduce this risk (Jelovac and Armstrong, 2011).

Early detection is the key to the successful treatment of ovarian cancer. However, the 75% of EOCs cases are diagnosed at advanced stage when ovarian tumour cells shed into the peritoneal cavity and metastasis are widely disseminated within the abdomen, respectively stage III and IV of the International Federation of Gynecology and Obstetrics (FIGO) staging classification for ovarian cancer (Prat, 2014)Two main reasons could be identified for this usual late diagnosis: 1) The disease indeed typically presents few specific symptoms at early stages, when it is localized to the ovary (FIGO stage I) and when it is extended also to tubes and uterus (FIGO stage II). Ovarian cancer patients exhibit generally 3–4 months of abdominal pain or distension, which might be mistakenly attributed to irritable bowel syndrome. 2) Available screening strategies, such as ultrasound and the evaluation of the cancer antigen CA125 tumour marker, are not specific and reliable to recognize early stages of the disease (Jelovac and Armstrong, 2011). Furthermore, contrast-enhanced computed tomography (CT) imaging, the standard non-surgical method for staging and assessing response, hardly detects small peritoneal deposits (Vaughan et al, 2011).

Epithelial Ovarian Cancer is not a single disease but it comprises a heterogeneous group of tumours recently reclassified on the basis on distinctive morphologic and molecular genetic features into two groups, type I and type II (Jayson et al, 2014). Type I tumours are clinically indolent, they usually present at a low stage and include low-grade micropapillary serous carcinoma, mucinous, endometrioid, and clear cell carcinomas (Figure 1). They are genetically stable and are characterized by mutations in a number of different genes, including mutations in BRAF and KRAS for serous tumours, KRAS for mucinous tumours, and CTNN1B (β-catenin) and PTEN mutations for endometrioid tumours, whereas mutations in the TP53 tumor suppressor gene are generally rare. Type II tumours, comprising high-grade serous carcinoma, malignant mixed mesodermal tumours and undifferentiated carcinomas (Figure 1), are highly aggressive neoplasms that rapidly grow and spread within the abdominal cavity. Type II tumors are characterized by the presence of TP53 mutations in almost all cases and by a high genomic instability (Jayson et al, 2014). Genes encoding for DNA repair proteins involved in the Homologous Recombination (HR) pathway are also frequently altered. Indeed HR repair of DNA damage is defective in roughly 50% of high grade serous cancers (Kurman and Shih, 2010; Jayson et al, 2014). Among these genes, somatic and germline mutations of BRCA1 or BRCA2 are the most frequent and can be observed in about 30% of the cases.

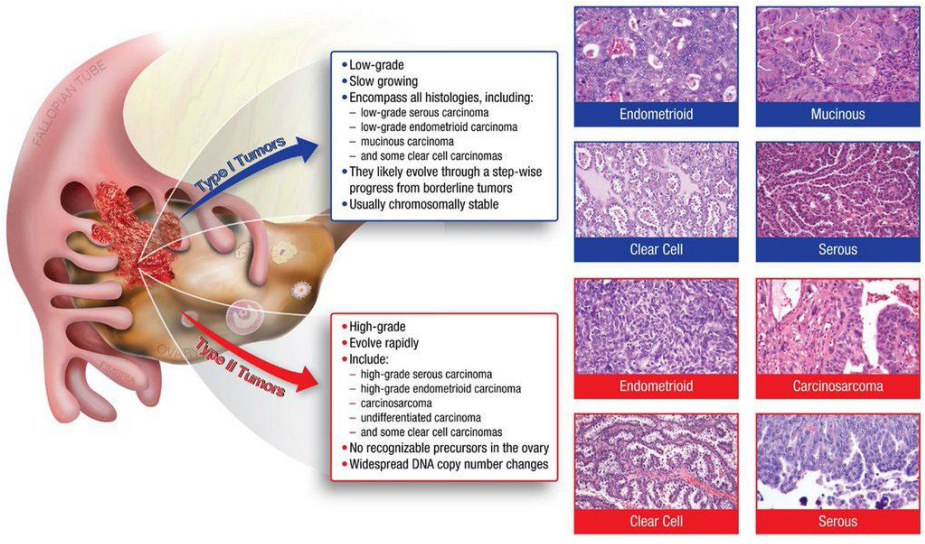

Figure 1: Classification and histopathology of Epithelial Ovarian Cancers. EOCs are classified according to distinctive morphologic and molecular genetic features into two groups: Type I tumours (in blue) include low-grade micropapillary serous carcinoma, mucinous, endometrioid, and clear cell carcinomas; Type II tumours (in red) comprise high-grade serous, endometrioid, clear cell carcinomas and malignant mixed mesodermal tumours (carcinosarcoma). The architectural complexity of tissue structures increases from Type I to Type II tumours (Kriplani and Patel, 2013).

In particular, women who have a deleterious mutation in the BRCA1 or BRCA2 gene present a 40–60% lifetime risk of developing ovarian cancer (Boyd, 2003). The mean age of onset of ovarian malignancy is significantly earlier in women with a BRCA mutations. Homologous recombination repair of DNA damage is defective in roughly 50% of high grade serous cancers (Kurman and Shih, 2010; Jayson et al, 2014).

The actual standard treatment of EOC consists of radical surgery and platinum-based chemotherapy. Depending of the disease extent, surgery can includes total hysterectomy (uterus and cervix removal), bilateral salpingo-oophorectomy (uterus plus both ovaries and fallopian tubes are removed), tumour debulking, and omentectomy. Surgery is performed to provide a histopathological diagnosis, to remove as much cancer tissue as possible, and to establish the FIGO stage of the disease. Regimens containing platinum have been the standard of care for almost 40 years worldwide. Actually, the standard first-line treatment after surgery is a combination therapy including a platinum compound and a taxane, usually carboplatin (that has reduced side effects respect to cisplatin) and paclitaxel, respectively. Platinum causes DNA damage inducing formation of DNA interstrand and intrastrand crosslinks, and subsequent single-strand and double-strand breaks. DNA damage triggers the arrest of cell cycle progression to repair, if the damage is limited, or to permanently eliminate the cells by inducing apoptosis when the damaged DNA is extended (Cooke and Brenton, 2011). Although the majority of ovarian cancer patients responds to initial chemotherapy, most of them ultimately develops disease recurrence with progression to chemotherapy resistance (Jayson et al, 2014).

1.1.1 Platinum resistance

Resistance to chemotherapy is a major unmet need in the treatment of Epithelial Ovarian Cancer and the main contributing factor in cancer-associated mortality. The initial response to platinum-based chemotherapy can be broadly classified into three groups: platinum-refractory, platinum-resistant and platinum-responsive. The platinum-refractory patients have an intrinsic (primary) drug resistance and they do not respond to platinum-based therapy showing progression during the course of the therapy. A genomics study of refractory disease shows amplification of the CCNE1 gene as a preadaptation, an early event, conferring primary platinum resistance (Cooke and Brenton, 2011). Platinum-resistant patients, instead, have an acquired (secondary) resistance selected by drug treatment, which is defined by less than six months of disease free period following the

last round of chemotherapy in patients that had an initial good response to the therapy. In particular, high-grade serous ovarian carcinomas, that account for most Epithelial Ovarian Cancer deaths, are highly heterogeneous with multiple, genetically distinct clones present prior to treatment. The presence of multiple tumour clones in a patient creates the opportunity for therapy-driven selection of an intrinsically resistant cancer subclone with selective advantages and it inevitably results in relapse (Castellarin et al, 2013) (Figure 2).

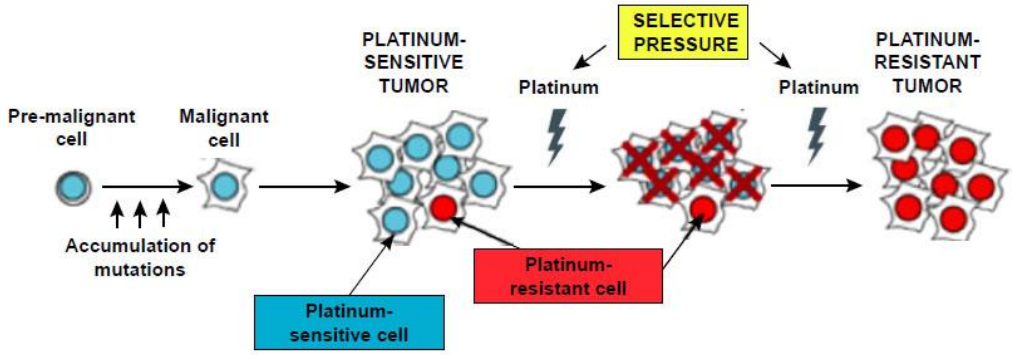

Figure 2: Model of platinum resistance development in Epithelial Ovarian Cancer. As indicated by the red cell, platinum-resistant clones are present in the tumour before treatment and they are selected once the chemotherapy has killed their platinum-sensitive counterpart. ("TANIGUCHI LAB -- Drug Sensitivity and Resistance in Cancer Chemotherapy", 2016)**.**

Thus, it is essential to improve understanding of clonal diversity in tumours prior to treatment, mechanisms of tumour adaptation and the acquisition of resistance following treatment to achieve more durable responses to therapy. Recurrent tumours further evolve by accumulating additional somatic or epigenetic mutations evolving over time under the selective pressure of platinum treatment (Vaughan et al, 2011). Occurrence of secondary mutations that compensate for BRCA1/2 deficiency (restoring the repair system of platinum-induced double-strand DNA breaks), alterations in drug influx and efflux and changes in binding affinity of the drug to intracellular target proteins are potential mechanisms of drug resistance (Agarwal and Kaye, 2003; Cooke and Brenton, 2011; Galluzzi et al, 2012). Mutations and amplification of PI3Kinase (found in 30–40% of ovarian tumours) stimulates cell survival and proliferation, and it represents possible causes of chemoresistance in clinical practice (Banerjee and Kaye, 2013). Moreover, general stress response pathways as autophagy, a survival mechanism that sustains cancer cells promoting recycling of cellular nutrients and energy production, were linked to

platinum resistance. For instance it has been demonstrated that ovarian cancer cells could progressively acquire chemoresistance by upregulating components of the autophagic pathway (Ren et al, 2010) (Figure 3).

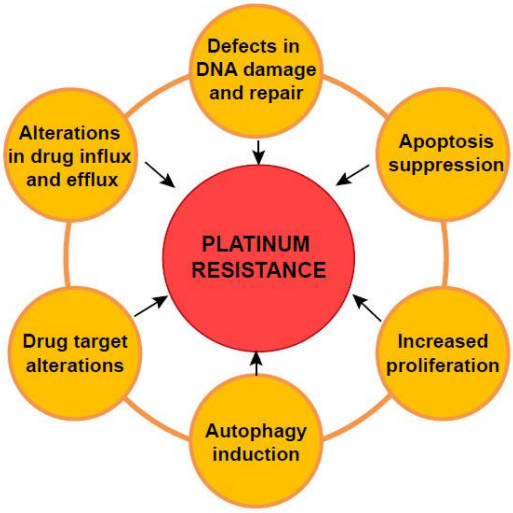

Figure 3: Summary of potential mechanisms involved in platinum resistance of Epithelial Ovarian Cancer. Alterations in drug influx and efflux, changes in binding affinity of the drug to intracellular target proteins, increased DNA damage repair, suppression of apoptosis, enhanced cell survival and proliferation, and exploitation of autophagy are some of the potential mechanisms involved in drug resistance of Epithelial Ovarian Cancer.

Therefore, the elucidation and the targeting of mechanisms, by which Epithelial Ovarian Cancer becomes resistant to chemotherapy, are crucial in the challenge to overcome platinum resistance. The major focus of actual clinical trials for the treatment of recurrent ovarian cancer regards the combination of current therapy with targeted biologic agents, such as antiangiogenic drugs (bevacizumab) or poly(ADP-ribose) polymerase (PARP) inhibitors (olaparib), offering potential for improved survival (Jelovac and Armstrong, 2011; Galluzzi et al, 2012). Indeed, tumours need new blood vessels to grow and to spread through vascular metastasis. Inhibition of angiogenesis by anti-VEGF (vascular endothelial growth factor) monoclonal antibodies like bevacizumab, that blocks activation of VEGF receptors through binding of their ligand, restrains tumour growth and can improve progression-free survival of patients with advanced ovarian cancer (Jayson et al, 2014). Also PARP inhibitors have shown significant clinical activity in ovarian cancer, particularly in tumours lacking DNA damage repair via homologous recombination because of mutated BRCA1 and BRCA2. Indeed, BRCA inactivity is a positive regulator for levels of poly(ADP-ribose) polymerase (D. Li et al, 2014), mediator of DNA single-strand break repair through

the base excision repair pathway. BRCA dysfunction renders cancer cells reliant on base excision repair and hence susceptible to PARP inhibition (Lord and Ashworth, 2012). Olaparib traps PARP at sites of DNA damage, blocking base-excision repair and resulting in the collapse of DNA replication forks and the accumulation of DNA double-strand breaks, causing cell death (Murai et al, 2012). Other studies investigate the possibility of targeting and modulating other receptors and pathways that are generally activated in ovarian cancer, including the RAS/RAF/MEK and PI3K/AKT/mTOR pathways, the ErbB and IGF family of receptors, mitotic check point, and the folate receptor with variable results (Banerjee and Kaye, 2013).

Based on these considerations and thanks to the development in high-throughput technologies, functional genomic studies can now be performed to identify new key mediators of platinum resistance of ovarian cancer. (Banerjee and Kaye, 2013).

1.2 Functional Genomic Screening

Identifying modulators of drug efficacy can be critical to recognize patients, that fail to respond to therapy, and may help to design more effective treatment protocols to overcome primary and acquired drug resistance in cancer. Functional genomics furnishes a powerful approach for interrogating gene function on large scale with the application of RNA interference (RNAi) technology (Lee et al, 2009). They require high-throughput tools such as liquid handling robot, a set of constructs to target specific pathways (small interfering RNAs, siRNA, or short hairpin RNAs, shRNA, libraries), data acquisition platforms able to recognize and quantify a specific phenotype (cell survival, apoptosis, migration, invasion, etc), complex computational analyses (Alvarez-Calderon et al, 2013). In loss-of-function high-throughput RNAi screenings, a series of genes are knocked down in cell lines with the use of siRNA or shRNA libraries. In the RNAi-based screens, the precise influence of individual genes on cell survival can be studied in the presence or absence of a low concentration of the given cancer drug, which causes only a small decrease of cell viability *per se*. In this way genes, whose suppression significantly enhances cell death induced by the drug, can be identified (Mullenders and Bernards, 2009). This represents the concept of synthetic lethality, in which the silencing of multiple genes or the combination between gene depletion and drug treatment results in cell death (Lord and Ashworth, 2013). Not every hit (gene) identified in an RNAi screen is a suitable candidate involved in modulation of drug response. It is important to verify that the

phenotype observed in the RNAi screen is caused by knockdown of the desired target gene excluding non-specific effects due to sequence identity between used siRNA/shRNA and unintended target transcripts (“off target” effects), by validating obtained results and in particular hits with druggable potential. Studies of synthetic lethal interactions not only provide important insights into the mechanism of drug action, but also identify potential combination strategies to limit treatment resistance and unveil functional candidates to serve as biomarkers for drug response, thus decreasing therapy-related toxicity and improving relapse-free survival (Alvarez-Calderon et al, 2013).

1.3 Serum and glucocorticoid-regulated kinases

The serum- and glucocorticoid-regulated kinases (SGKs) are a family of serine/threonine kinases consisting of three isoforms, SGK1, SGK2 and SGK3. SGK family belongs to the AGC (protein kinase A, G, and C families) kinase family comprising 60 members, some of which are intensely examined protein kinases such as AKT, S6K and RSK, while others are less studied enzymes such as SGKs (Arencibia et al, 2013).

SGKs were originally described as key enzymes in the regulation of several ion channels and pumps in the context of epithelial ion transport, but over time they were found involved in the regulation of cell growth, proliferation and survival. SGKs are also associated with several pathophysiological conditions, including hypertension (Lang et al, 2000; Schwab et al, 2008), fibrosis (Klingel et al, 2000; Fillon et al, 2002) and cardiovascular disease (Busjahn et al, 2002; Aoyama et al, 2005; Seebohm et al, 2008). In murine models, deregulation of SGK1 activity in cycling endometrium interferes with embryo implantation, leading to infertility, or predisposes to pregnancy complications (Salker et al, 2011; Monsivais et al, 2016; Lou et al, 2016). Recently, also their connections with cancer begin to be investigated (Sherk et al, 2008; Bruhn et al, 2010; Sommer et al, 2013; Bruhn et al, 2013; Gasser et al, 2014; Talarico et al, 2016).

1.3.1 SGK structure

SGK1, SGK2 and SGK3 are distinct but highly homologous proteins, sharing 80% amino acid sequence identity in their catalytic domain. They are products of three distinct genes localized on different chromosomes: SGK1 on chromosome 6q23, SGK2 on chromosome 20q12, and SGK3 on chromosome 8q12.2. SGK1 and SGK3 are ubiquitously expressed, while expression of SGK2 is most abundant in epithelial tissues. SGKs share similar domain structure and mechanism of activation. All SGK isoforms consists of three domains, an N-terminal variable domain, a catalytic domain and the C-terminal hydrophobic domain. They present two key regulatory sites, a serine in the C-terminal domain and a threonine in the activation loop of the catalytic domain, both of which require phosphorylation for complete activation (Figure 4).

Each SGK isoform is able to produce multiple splice variants. SGK1 has four distinct variants differing in the N-terminal region, two of which contain an endoplasmic reticulum localization motif (required for the association of SGK1 with the endoplasmic reticulum) and are more rapidly degraded by the 26S proteasome via polyubiquitin modification (Arteaga et al, 2007) (Figure 4).

Two variants of SGK2 and SGK3 have been described (Figure 4), but the functional consequence of the difference among them is as yet unknown (Bruhn et al, 2010). Furthermore, SGK3 differs from the other two SGK isoforms for the presence of an amino-terminal Phox homology (PX) domain, which interacts with phosphatidylinositol PtdIns(3)P targeting SGK3 to vesicle-like structures as early endosomes (Liu et al, 2000; Virbasius et al, 2001). SGK1 and SGK2 contain a truncated and non-functional PX domain and their localization is mainly cytoplasmic (Bruhn et al, 2010).

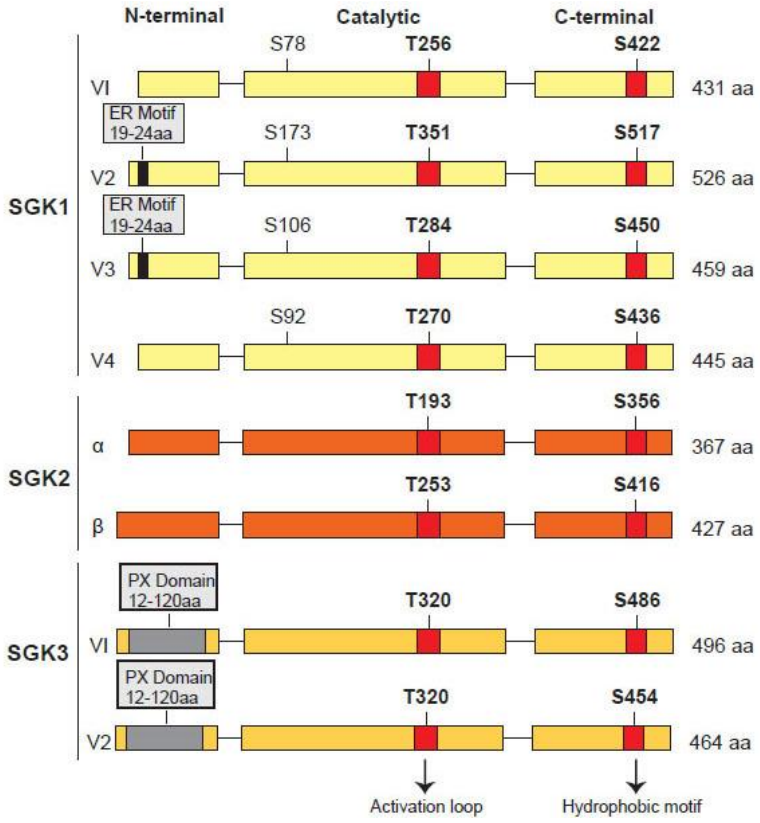

Figure 4: Schematic representation of SGK isoforms and their variants. SGKs share similar domain structure and mechanism of activation. The highlighted threonine in the activation loop of the catalytic domain and serine in the C-terminal domain require phosphorylation for complete activation of the molecule. SGKs differ in their N-terminal domain, that is specific for their cellular localization. (Modified from Bruhn et al, 2010).

1.3.2 SGK activation and regulation

SGK1 was initially discovered as an immediate early gene transcriptionally responsive to glucocorticoids and serum in mammary tumour epithelial cells (Webster et al, 1993). Subsequently, SGK1 gene transcription was shown to be upregulated by a multitude of other stimuli, including growth factors, such as transforming growth factor (TGF)-β1 (Waldegger et al, 1999), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (Mizuno and Nishida, 2001), and various cellular stressors such as ischaemic injury, heat shock and ultraviolet stress. SGK3 is an estrogen receptor transcriptional target regulated via two estrogen receptor-binding regions in its promoter (Wang et al, 2011), and SGK3 kinase activity is upregulated by glucocorticoid as dexamethasone (He et al, 2011). Transcription of SGK2 instead is not sensitive to those stimuli (Lang and Cohen, 2001) and despite its structural homology with the other two isoforms, it is not transcriptionally regulated (Kobayashi et al, 1999). SGK family is highly homologous to the

AKT kinase family. These three family members share similar domain structure and high sequence identity in the catalytic domain. SGK1, SGK2 and SGK3 are not constitutively active but they are regulated by post-translational modification, most frequently by phosphorylation and dephosphorylation mediated via the phosphatidylinositol 3-kinase (PI3Kinase) pathway and protein phosphatase 2A, respectively (Park et al, 1999). Activation of PI3Kinase by receptor tyrosine kinases (RTK) leads to 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylation and in turn phosphorylation and activation of both AKT and SGK. On the other hand, phosphatase and tensin homologue (PTEN) is a lipid phosphatase that antagonizes the activation of both SGK and AKT by catalysing the removal of the D3 phosphate from PtdIns(3,4,5)P3, and thus limiting, if not terminating, PI3Kinase signalling in cells (Cantley and Neel, 1999; Carracedo and Pandolfi, 2008).

SGK isoforms are regulated primarily by PDK1-mediated phosphorylation of a conserved threonine residue in the catalytic domain, and then phosphorylation of a serine residue in the hydrophobic motif (Figure 4). Both mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) are implicated in the phosphorylation of the serine residue in the C-terminal hydrophobic domain of SGK1, although the majority of evidence supports the role of mTORC2 (García-Martínez and Alessi, 2008) (Figure 5).

Endosomal localization is essential for SGK3 to co-localize with the kinase responsible for phosphorylating its C-terminal domain, and thus allowing PDK1 phosphorylation at threonine site for complete activation of SGK3. Mutation of the PX domain in SGK3 abolishes phospholipid binding and endosomal localization, and it is also associated with decreased SGK3 kinase activity (Xu et al, 2001).The kinase responsible for the phosphorylation of the C-terminal domain of SGK3 is unknown, but it could be mTORC2 (Figure 5).

Very few publications investigated SGK2 regulation and activation. *In vitro* studies have demonstrated that it is activated by PDK1 phosphorylation of the threonine residue in the catalytic domain (Kobayashi et al, 1999), but the kinase responsible for phosphorylation of the serine residue in the C-terminal domain is unknown; however, given the similarity in this domain between SGK2 and SGK1, it is possible that also SGK2 is phosphorylated by mTORC2 (Figure 5).

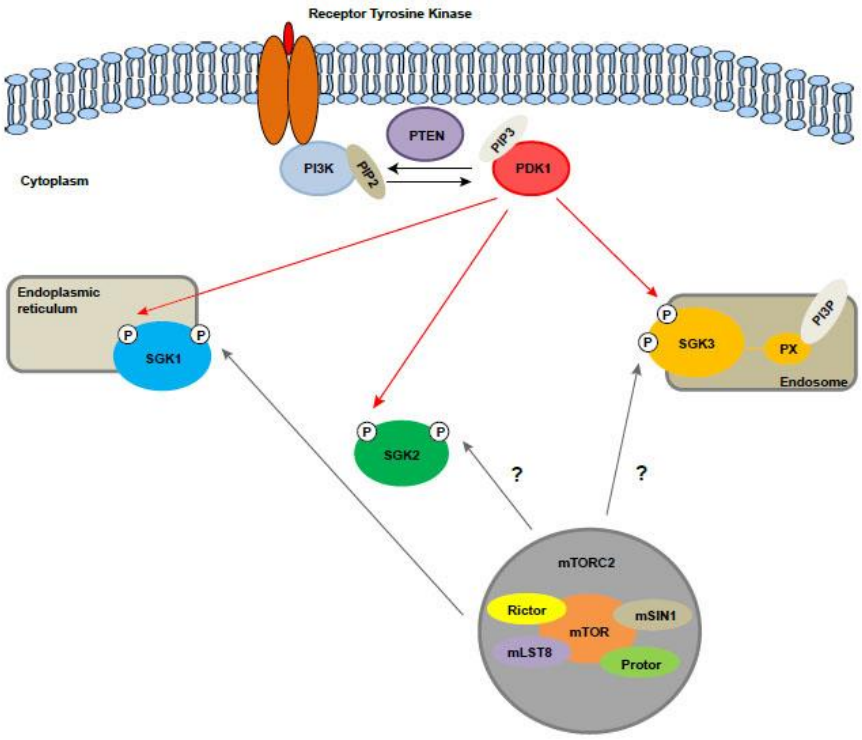

Figure 5: SGK activation and regulation. Activation of PI3Kinase by receptor tyrosine kinases leads to PDK1 phosphorylation, and in turn phosphorylation and activation of SGKs. SGK isoforms are regulated by PDK1-mediated phosphorylation of a threonine residue in the catalytic domain, and then by phosphorylation of a serine residue in the C-terminal domain. The kinase responsible for the phosphorylation of the C-terminal domain of SGK1 is mTORC2, and probably it may be the same also for SGK2 and SGK3. Rapid degradation of SGK1 occurs via the 26S proteasome following polyubiquitination at the endoplasmic reticulum. SGK3 is constitutively expressed, and through binding of PtdIns(3)P to its PX, SGK3 is able to localize to the early endosomes where it is fully activated through phosphorylation at two key residues. Very few information on SGK2 regulation and activation is available in literature. (Modified from Bruhn et al, 2010).

1.3.3 Role of SGKs in regulation of molecular and cellular functions

The SGK kinases are implicated in the regulation of a great variety of cellular factors, including ion channels, membrane transporters, cellular enzymes and transcription factors (Lang et al, 2006). The most studied function of SGKs regards their role in the regulation of renal sodium transport by stimulating the epithelial sodium channel (ENaC). In particular, SGK1 may control ENaC by phosphorylating the ubiquitin ligase Nedd4–2 (Asher et al, 2003), which otherwise ubiquitinates ENaC preparing the channel protein for clearance from the cell membrane and degradation (Staub et al, 1997). In this way SGK1 increases the abundance of ENaC channel protein within the cell membrane, favouring renal Na^+ reabsorption. The effect of SGK1 expression on ENaC is shared with SGK2 and SGK3, that have common consensus sequence, but SGK1 and SGK3 phosphorylate Nedd4–2 more efficiently than SGK2 (Palmada et al, 2004). With a similar mechanism, SGK1 and SGK3 (less evidences for SGK2) are able to stimulate also renal excretion of K^+

accomplished by ROMK1 ((Palmada et al, 2003), and to activate Ca^{2+} channel TRPV5 (Embark et al, 2004), Cl^- channel ClC-Ka (Embark et al, 2004), Na^+ channel SCN5A (Boehmer et al, 2003), K^+ channels KCNE1/KCNQ1 (Embark et al, 2003), K^+ channel KCNQ4 (Seebohm et al, 2008) and glutamate receptors GluR1/GluR6 (Seebohm et al, 2005) by increasing their abundance in cell surface. Stimulating cation channels, SGKs (in particular SGK1) contribute to the regulation of cell volume in a variety of cells (Huber et al, 2001; Wehner et al, 2001), and they participate in the control of blood pressure. Through the phosphorylation of Nedd4-2, serum- and glucocorticoid-regulated kinases control also several carriers and pumps, such as Na^+/H^+ exchanger NHE3 (Pao et al, 2010), glucose trasporter GLUT1 (Palmada et al, 2006), Na^+-glucose cotransporter SGLT1 (Dieter et al, 2004), amino acid transporters EAAT1–5 (Boehmer et al, 2006), and the Na^+-K^+- ATPases (Henke et al, 2003). Moreover, SGK2 is responsible for the regulation of the human organic anion transporter 4 (hOAT4), that plays critical roles in the body disposition of clinically important drugs, including anti-viral therapeutics, anti-cancer drugs, antibiotics, anti-hypertensives, and anti-inflammatories. Therefore SGKs are involved in maintenance of the ion homeostasis of cells, which is essential to sustain life processes, including proliferation, differentiation and apoptosis.

Serum- and glucocorticoid-regulated kinases are also involved in the regulation of some important cellular enzymes and transcription factors, favouring cell survival and proliferation. SGK1 and SGK3 were shown to phosphorylate and inhibit glycogen synthase kinase 3β (GSK3β), a kinase which phosphorylates the transcription factor β-catenin and participates in the regulation of cell proliferation (Sakoda et al, 2003). SGK1 and SGK3 could also regulate cell proliferation by phosphorylating and inhibiting tuberin (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40) (Aoyama et al, 2005; Bruhn et al, 2013). SGK1 interacts also with RAS/RAF/ERK signalling pathway at different levels. It phosphorylates and inactivates B-Raf kinase, and may thus interfere with B-Raf kinase-dependent cellular functions, critical for stimulation of cell proliferation (Zhang et al, 2001). SGK1 promotes ERK function through the phosphorylation of ERK2, which inhibits the TSC2/TSC1 (hamartin–tuberin) complex, promoting the activation of mTORC1 (Won et al, 2009).

SGK1 and SGK3 phosphorylate and inhibit the forkhead transcription factors such as FOXO3, leading to its exit from the nucleus and the consequent shutoff of its target genes (death receptor ligands, Bcl-2 family members, p27), contributing to cell survival (Brunet et al, 2001). The antiapoptotic effect of SGK1 regards also the activation of nuclear factor κB

(NF-κB). SGK1 indeed enhances the activity of NF-κB by association with and activation of IκB kinase beta (IKKβ). Active IKKβ phosphorylates IκB (the inhibitor of NF-kB) leading to release of NF-κB that enters into the nucleus to activate growth factor transcription (Zhang et al, 2005).

The unique localization of SGK3 at the early endosomes, where the class III PI3Kinase family catalytic subunit hVps34 (implicated in growth signalling to mTORC1) resides, raises the possibility that SGK3 may potentially modulate nutrient signalling via interaction with hVps34 (contributing to oncogenic cell growth during cell transformation and tumorigenesis, in an AKT independent manner) (Bruhn et al, 2013). SGK3 phosphorylates also Bad which binds to the chaperone 14–3-3 and it is prevented from traveling to the mitochondria where it triggers apoptosis (Lizcano et al, 2000).

The above evidences support the SGKs implication in the regulation of cell growth, proliferation and survival (all cellular processes dysregulated in cancer) and suggest that SGK proteins, originally described only as kinases responsible for regulating cellular ion channels and pumps, can also have a role in cancer onset and/or progression.

Potential roles of SGK2 in these pathways illustrated above are practically unknown in literature.

1.3.4 Serum- and glucocorticoid-regulated kinases in cancer

Activating mutations in SGKs are rare and the hyperactivation of these kinases is likely a result of increased expression and/or constitutive activation of upstream signalling. Indeed the mutational events behind constitutive PI3Kinase signalling, one of the most frequently dysregulated pathways in human cancer, or the loss of PTEN (Engelman et al, 2006) impact on SGK's involvement in malignancy, enhancing cell survival and proliferation of cancer cells. One example is represented by the case of dexamethasone, a glucocorticoid compound commonly used to relieve chemotherapy-related side effects with its anti-inflammatory properties. By binding and activating glucocorticoid receptor (upstream activator of important pathways as PI3Kinase signalling), dexamethasone is also able to suppress chemotherapy-mediated apoptosis in epithelial tumours (including breast and ovarian cancers) via the up-regulation of the pro-survival gene SGK1, which, in turn, inactivates important pro-apoptotic factors such as FOXO3 transcription factors and p27 (Melhem et al, 2009). Clinical trials to evaluate the therapeutic efficacy of AKT inhibitors for the treatment of breast cancer have reported that cancer cells resistant to the activity of

these compounds express high levels of SGK1, which share with AKT several substrates that regulate cell proliferation and survival (Sommer et al, 2013). Accordingly, SGK1 is highly expressed, in several tumour types including colon cancer (Lang et al, 2010), myeloma (Fagerli et al, 2011), prostate cancer (Szmulewitz et al, 2012) and non-small cell lung cancer (Abbruzzese et al, 2012) where it could increase cell survival and proliferation.
SGK3 is also frequently overexpressed in cancer. It functions as a PI3Kinase effector in the control of oncogenic signals promoting cell growth and migration (targeting the metastasis suppressor NDRG1 for degradation) of breast cancer (Gasser et al, 2014). Amplified (Liu et al, 2012) and overexpressed SGK3 is more common than AKT in hepatocellular carcinoma suggesting it may have a greater functional significance in the biology of this cancer (Liu et al, 2012), where it was found promoting growth and survival, inactivating GSKβ and Bcl-2 associated death promoter, respectively. Moreover, SGK3 was identified as a crucial effector of PI3Kinase/AKT-independent signalling in the pathogenesis of HCC; as shown by PI3Kinase mutant cancer cells with low AKT signalling that exhibit a selective dependency on SGK3 for viability (Vasudevan et al, 2009). In addition, it has been suggested that SGK3 is involved in cell survival signalling in estrogen receptor–positive breast cancer cells, potentially via Flightless-I (FLI-I) a downstream target of SGK3, which acts as a coactivator for the estrogen receptor, enhancing receptor activity, and promoting proliferation and survival (Wang et al, 2011). SGK3 is also an androgen receptor transcriptional target and promotes prostate cancer cell proliferation (Wang et al, 2014).
Very few evidences linking SGK2 to cancer are present in literature. SGK2 appeared among genes involved in oxidative stress indirectly related to heme-toxicity of consumption of red/processed meats, which is associated with higher risk of lung cancer (Lam et al, 2014). Deletion of the region on chromosome 20, where the imprinted genes SGK2 and epigenetic regulator L3MBTL1 are located, was found to dysregulate erythropoiesis and megakaryopoiesis, lineages commonly affected in chronic myeloid malignancies, increasing tumour incidence (Aziz et al, 2013).

1.4 Autophagy

Autophagy is an evolutionarily conserved and strictly regulated process, in which cytoplasmic material and organelles are sequestered in double membrane vesicles and degraded upon fusion with lysosomal compartments (Eskelinen, 2008). The degradation of proteins, lipids, glycogen and ferritin via autophagy promotes the recycling of cellular nutrients and enables energy production, allowing cell survival during stress situations (endoplasmic reticulum stress, hypoxia, oxidative stress, expression of aggregate-prone proteins, glucose deprivation, amino acid depletion, etc.) and in basal conditions. Depending on the delivery route of the cytoplasmic material to the lysosomal lumen, three different autophagic paths are known: macroautophagy (or simply autophagy), microautophagy, and chaperone-mediated autophagy. In macroautophagy, the target cytoplasm and/or organelle is first sequestered by a unique membrane structure (phagophore) into a double-membrane limited organelle (autophagosome), which then fuses with lysosomal (autolysosome) vesicles and delivers the engulfed cytoplasm for degradation by lysosomal enzymes, such as cathepsins and other acid hydrolases. (Mizushima and Komatsu, 2011). In microautophagy, the lysosomal membrane itself sequesters a portion of cytoplasm by a process that resembles pinching off of phagosomes or pinosomes from the plasma membrane pre-lysosomal sequestration stage (Li et al, 2012). In chaperone-mediated autophagy, proteins possessing a specific sequence signal are transported from the cytoplasm, through the lysosomal membrane, to the lysosomal lumen (Cuervo and Wong, 2014).

Macroautophagy is controlled by more than thirty highly conserved ATG genes (autophagy-related genes). The intricate process of autophagosome formation begins at the phagophore assembly site, where proteins of the UNC51-like kinase (ULK) complex (composed of ULK1 or ULK2 and ATG13, FAK family kinase interacting protein of 200 kDa (FIP200) and ATG101) assemble to initiate autophagosome formation. Next, in the nucleation stage, the activated ULK complex targets a class III PI3K complex, consisting of beclin 1 (Atg6 in yeast), vacuolar protein sorting 15 (VPS15), VPS34 and ATG14, to promote the local production of a pool of phosphatidylinositol 3-phosphate that is specific to autophagosomes (Lamb et al, 2013). In the expansion stage, the ATG12–ATG5–ATG16 complex facilitates the lipidation of the cytosolic form of microtubule-associated protein 1 light chain 3 (LC3I), that is converted into the LC3-phosphatidylethanolamine conjugate (LC3II) and recruited to the autophagosome membrane (Figure 6). The adaptor protein, sequestosome 1 (SQSTM1)/p62, binds both polyubiquitin on autophagy cargo via its

ubiquitin associated (UBA) domain and LC3 on autophagosome membrane via its LC3-interacting region (LIR) domain, which directs cargo to autophagosomes for degradation. The PB1 domain of p62 also interacts with itself, promoting self-aggregation. As p62 is an autophagy substrate, autophagy defects cause accumulation of p62, which perturbs signal transduction in multiple pathways (i.e. MAPK/ERK and PI3Kinase/mTOR signalling pathways) and increases cell stress. (White, 2012).

PI3Kinase/mTOR and AMP-activated protein kinase (AMPK) signalling pathways have emerged as the central conduit in the regulation of autophagy. mTOR can be activated by growth factors signal through the class I PI3Kinase/AKT pathway, and inhibited by AMPK and p53. Once activated, mTOR exerts a negative effect on autophagy by phosphorylating ULK1/2, which inhibits the downstream autophagy cascade (Figure 6). In contrast, AMPK can suppress mTORC1 signalling to stimulate autophagy through TSC1/2 phosphorylation. Several known tumour-suppressor genes (p53, PTEN, TSC1/TSC2) and tumour-associated genes (p21, AKT) stimulate or inhibit autophagy, respectively (Yu et al, 2010).

Tissues from mutant mice with defects in autophagy accumulate ubiquitylated protein aggregates, abnormal organelles, particularly mitochondria, as well as excess peroxisomes, endoplasmic reticulum, ribosomes and lipid droplets, normally eliminated through autophagy (Mizushima and Komatsu, 2011). The functional consequences of this failure of protein and organelle quality control are not entirely clear, but they are associated with the production of reactive oxygen species (ROS) and increased proteotoxicity. In mammals, defects in autophagy are implicated in either the pathogenesis or response to a wide variety of diseases, including neurodegenerative disease, chronic bacterial and viral infections, atherosclerosis, and cancer.

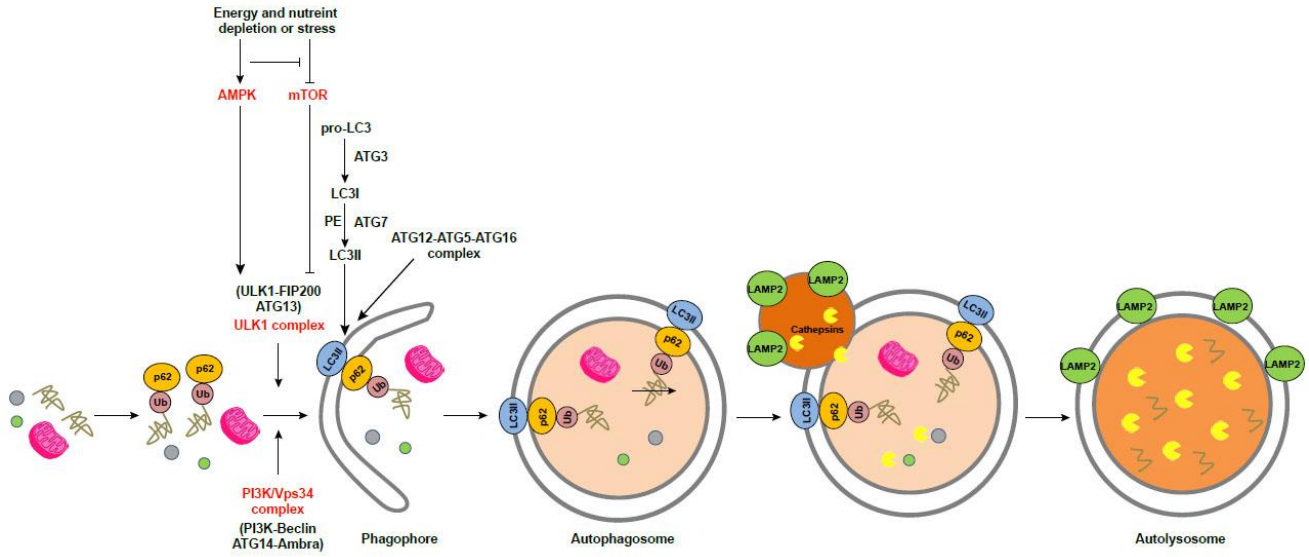

Figure 6: Schematic representation of autophagy signalling pathway. Initiation begins with the formation of the phagophore assembly site, mediated by ULKcomplex. Then, nucleation stage requires class III PI3K/VPS34 complex. Phagophore membrane elongation and autophagosome completion need two ubiquitin-like conjugation pathways. The first produces the ATG5–ATG12 conjugate, which forms a multimeric complex with ATG16L, whereas the second results in the conjugation of phosphatidylethanolamine to LC3I. LC3–PE (LC3II) is required for the expansion of autophagic membranes, their ability to recognize autophagic cargoes and the fusion of autophagosomes with lysosomes. The resulting autophagosome fuses with endocytic and lysosomal compartments, ultimately leading to formation of the autolysosome.

The role of autophagy in cancer is complex and may differ depending on tumour type or context. Autophagy can exert tumour-suppressive role through the elimination of oncogenic protein substrates, toxic unfolded proteins and damaged organelles. Alternatively, it can represent tumour promoting strategy, as in RAS-transformed cancer cells, where autophagy was found promoting their growth, survival, tumorigenesis, invasion, and metastasis (Lock et al, 2014). For instance, in pancreatic ductal adenocarcinoma, typically presenting KRAS amplification, autophagy was seen highly activated in the later stages transformation, and it was required for continued malignant growth *in vitro* and *in vivo* (Yang et al, 2011) So, cancer cells are more autophagy-dependent than normal cells and tissues, and when they are subjected to stressful conditions (i.e. hypoxia, chemotherapy, etc), autophagy constitutes an adaptive response rapidly upregulated to maintain metabolic homeostasis and ensure that cell growth is appropriate to changing environmental conditions, via increased catabolic lysis of proteins and organelles (Figure 7). For these reasons, induction of autophagy can represent an

attractive mechanism exploitable by tumour cells to develop resistance to chemotherapy, as for example, was observed in cisplatin-based chemotherapy that frequently results in acquired resistance of cancer cells. Cisplatin resistance, indeed, was shown to correlate with autophagy induction via activation of ERK survival pathway in a panel of ovarian cancer cells (Wang and Wu, 2014). Furthermore, autophagy was observed mediating cisplatin resistance in lung cancer, in particular, in hypoxic environment (Wu et al, 2015).
However, persistent or excessive autophagy is also shown to promote cell death following treatment with specific chemotherapeutic agents, either by enhancing the induction of apoptosis or mediating “autophagic cell death”(Sui et al, 2013) (Figure 7).
Autophagy modulation thus can represent an interesting potential therapeutic target in cancer in combination with chemotherapy or targeted agents.

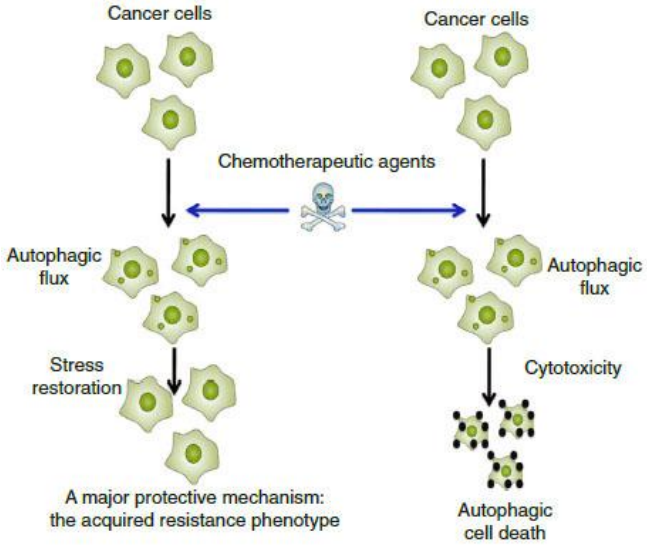

Figure 7: Dual role of autophagy for therapeutic purposes in cancer. On one hand (on the left), autophagy is activated as a protective mechanism to mediate the acquired resistance phenotype of some cancer cells during chemotherapy. On the other hand (on the right), autophagy may also function as a death executioner to induce autophagic cell death, a form of physiological cell death that is contradictory to apoptosis (Sui et al, 2013).

Aim of the study

Epithelial Ovarian Cancers are the most deadly disease among the gynecological malignancies, representing the fourth leading cause of cancer death in women in Western world. In the 75% of the cases EOCs are diagnosed at late stage, with tumour already spread throughout the abdominal cavity. The actual standard treatment of EOC consists of radical surgery and platinum-based chemotherapy. Although the majority of EOC patients responds to initial chemotherapy, most of them ultimately develops disease recurrence with progression to chemotherapy resistance, the main contributing factor in cancer-associated mortality. Therefore the identification of novel genes involved in chemoresistance is mandatory for the design of new therapeutic strategies.

Alterations of different cellular pathways such as DNA repair, apoptosis and p53, have been associated to platinum resistance mechanisms. Our aim is to identify new modulators inside these pathways, which could help to overcome drug-resistance in EOC. For this reason, we performed a high-throughput shRNA-based screening aimed to identify genes whose suppression enhanced cell sensitivity to platinum drug in different EOC cell lines (synthetic lethality). In particular, the study of one of the identified genes, SGK2, as a regulator of platinum response in EOC cells and its mechanism of action in platinum sensitivity are the focus of this PhD thesis.

Results

3.1 Identification of SGK2 as a mediator of platinum sensitivity in EOC cells.

Alterations in DNA repair, apoptosis and p53 pathways have been linked to the onset of platinum resistance in ovarian cancer (Konstantinopoulos et al, 2008). We used a loss-of-function screening targeting 680 genes belonging to these three pathways to evaluate whose suppression enhanced cell sensitivity to platinum drug in 2 different Epithelial Ovarian Cancer cell lines: MDAH 2774 and SKOV3 cells. To select genes potentially involved in the response to platinum, the used parameters were that two out of three shRNAs targeting each gene displayed a significantly enhanced and/or reduced survival after platinum treatment respect to control after Z-Score normalization and adjusted p-value for multiple testing (Benjamini and Hochberg, 1995). This first screening identified 50 genes potentially involved in the regulation of the response to platinum in Epithelial Ovarian Cancer. These data were then validated in a second screening performed using 5 shRNAs for each gene and 4 different cell lines: MDAH 2774, SKOV3, TOV112D and OV90. Using as pre-specified parameters that three out of five shRNAs targeting each gene displayed a significantly enhanced and/or reduced survival after platinum treatment respect to controls in at least 3 different cell lines, we identified 8 genes likely representing modulators of platinum drug resistance in EOC. Among these genes, the knocked-down of SGK2 significantly reduced cell viability in CBDCA-treated cells respect to controls (Fig.1A and Fig.1B). The validation of SGK2 as a regulator of platinum response in EOC cells and its mechanism of action are the focus of this PhD thesis.

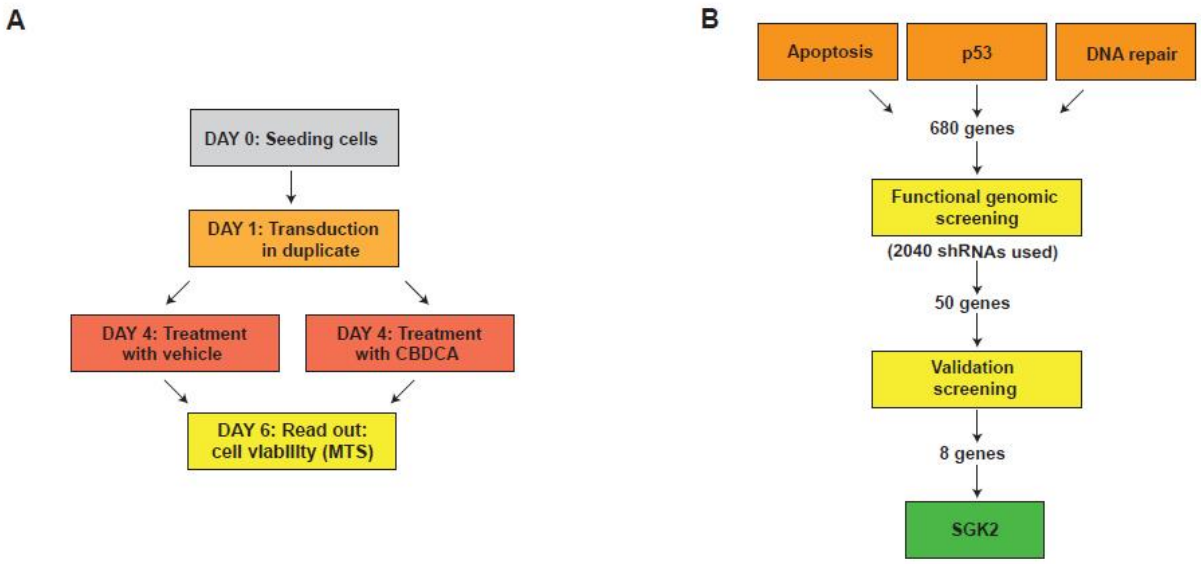

Figure 1: Identification of SGK2 as a mediator of platinum sensitivity in EOC cells. (A) Experimental design for the screening performed in presence or not of platinum drug treatment. The carboplatin (CBDCA) dose utilized was determined as the dose able to cause a 10-20% of cell mortality. (B) Schematic representation of the results of the loss-of-function high-throughput screening, conducted to unveil genes, whose silencing increase cell sensitivity to platinum drug (synthetic lethality), among which SGK2 was identified.

3.2 SGK2 silencing sensitizes EOC cells to platinum.

SGK2 is a serine/threonine kinase which belongs to SGK family, consisting of three distinct but highly homologous isoforms, SGK1, SGK2 and SGK3, that share 80% amino acid sequence identity in their catalytic domain (Bruhn et al. 2010). To validate and confirm screening results we transduced MDAH cells with three different SGK2 shRNAs (sh1, sh2, sh3) and, after 72 hours, we treated cells with platinum and transduced cells were analysed for SGK2 expression and cell viability. We observed that SGK2 shRNA 2 and shRNA 3 were able to silence specifically SGK2 expression, without altering the expression levels of the highly homologous genes SGK1 and SGK3, as shown by western blot analysis (Fig. 2, upper panels). SGK2 silencing increased MDAH cell death when associated to CBDCA treatment (synthetic lethality), compared to the platinum alone used at a concentration that caused about 20% of cell mortality (Fig. 2, lower graph). In untreated cells, SGK2 silencing (indicated by grey bars) had minimal effect on cell viability (Fig.2, lower graph). Given the high homology among the SGK family members, we investigated whether the silencing of SGK1 and SGK3 could alter cells sensitivity to CBDCA. Five SGK1 shRNAs and five SGK3 shRNAs, targeting different regions of the

respective genes, were used in MDAH cells. Two shRNAs were able to reduce SGK3 expression level (Fig.3A, upper panel), but SGK3 silencing was not associated to changes in cell viability upon platinum treatment, excluding SGK3 involvement in platinum-mediated cell death (Fig.3A lower graph). We also tried to silence SGK1, but we were not able to obtain changes in SGK1 expression neither with the use of shRNAs (Fig. 3B, upper panels and data not shown) nor with the use of SGK1 specific esiRNAs, as an alternative way of accomplish SGK1 silencing by RNA interference (Fig. 3B, lower panels). EsiRNAs are pools of siRNAs that target the same mRNA sequence and for this reason they are thought to improve the gene silencing respect to an unique target sequence. Indeed, in MDAH cells, SGK1 esiRNAs used at different concentrations, in a single transfection and after re-challenging the silencing with a second esiRNAs transfection (boost in Fig.3B), did not exert any effect on the expression of SGK1 protein (Fig.3B, lower panels) and mRNA (data not shown).

These data, along with the observation that the use of a SGK2 shRNA unable to silence SGK2 expression (SGK2 sh1 in Fig.2) did not modify the extent of platinum-induced cell death, strongly suggest that SGK2 is the SGK family member principally involved in MDAH cells sensitivity to platinum.

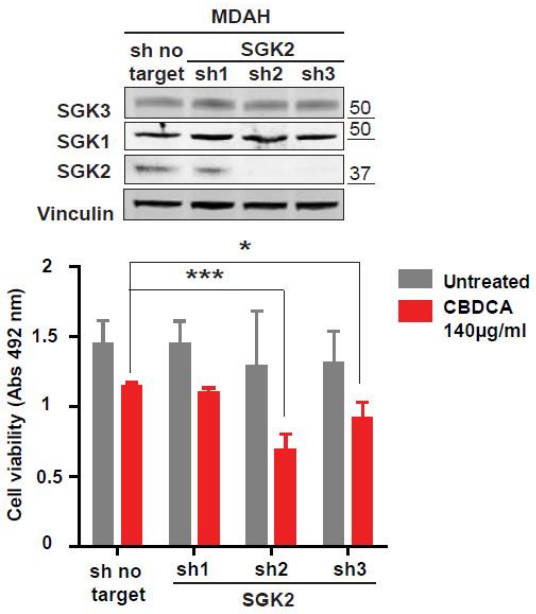

Figure 2: SGK2 has a role in platinum sensitivity in EOC cells. Graph reports the viability of MDAH cells transduced with shRNA no target and SGK2 shRNAs, and after 72h treated with CBDCA. Statistical significance was calculated using t-test. SGK2, SGK1 and SGK3 expression were analysed by western blot. Vinculin was used as loading control.

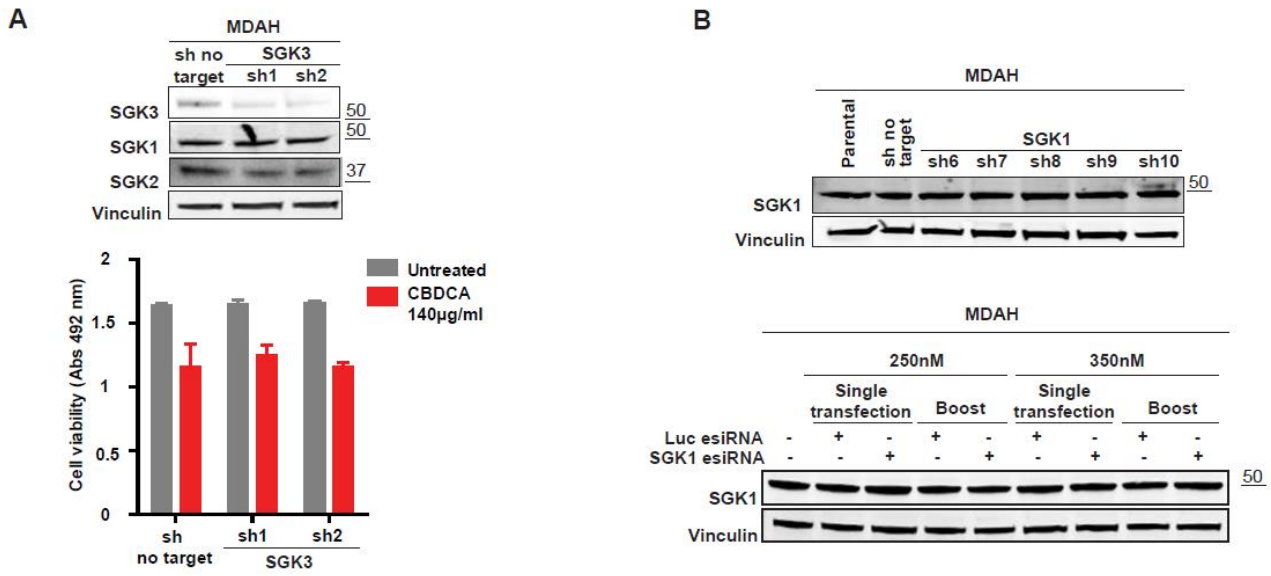

Figure 3: SGK3 is not involved in platinum sensitivity in EOC cells. (A) Graph reports the viability of MDAH cells transduced with shRNA no target and SGK3 shRNAs, and then treated with CBDCA. Statistical significance was calculated using t-test. The corresponding cell lysates were analysed by western blot. (B) Upward, western blot analysis of MDAH cells transduced with shRNA no target and SGK1 shRNAs. Below, cell lysates of MDAH cells transfected with different concentrations of luciferase esiRNAs (used as control) and SGK1 esiRNAs were analysed by western blot. Vinculin was used as loading control.

3.3 SGK2 overexpression confers an increased resistance to platinum drug.

Based on the above data, we tested the effect of SGK2 overexpression in cell response to platinum by stably overexpressing SGK2 wild type in OVCAR8 cells, that had very low (undetectable) levels of endogenous SGK2 protein (Fig. 4A, upper panels). Exogenous SGK2 expression in OVCAR8 resulted in an increased resistance to platinum treatment, reinforcing a possible role for SGK2 in platinum response in EOC cells (Fig.4A).

Since we did not resolve the role of SGK1 in platinum sensitivity of EOC cells using the knock-down approach, we checked if SGK1 overexpression could modify platinum sensitivity as observed for SGK2-overexpressing. To this aim we screened a panel of EOC cell lines available in our laboratory for the expression of SGK1 protein showing that all cell lines express moderated/high level of SGK1 expression (western blot in Fig.10). Since we did not find a SGK1-null cell line, we stably overexpressed SGK1 wild type in the same model used for SGK2 exogenous expression, namely the OVCAR8 cells. SGK1-overexpressing OVCAR8 cells showed the same sensitivity to platinum of the control cells (Fig.4B), suggesting a minor role for SGK1 in the response to platinum in this model.

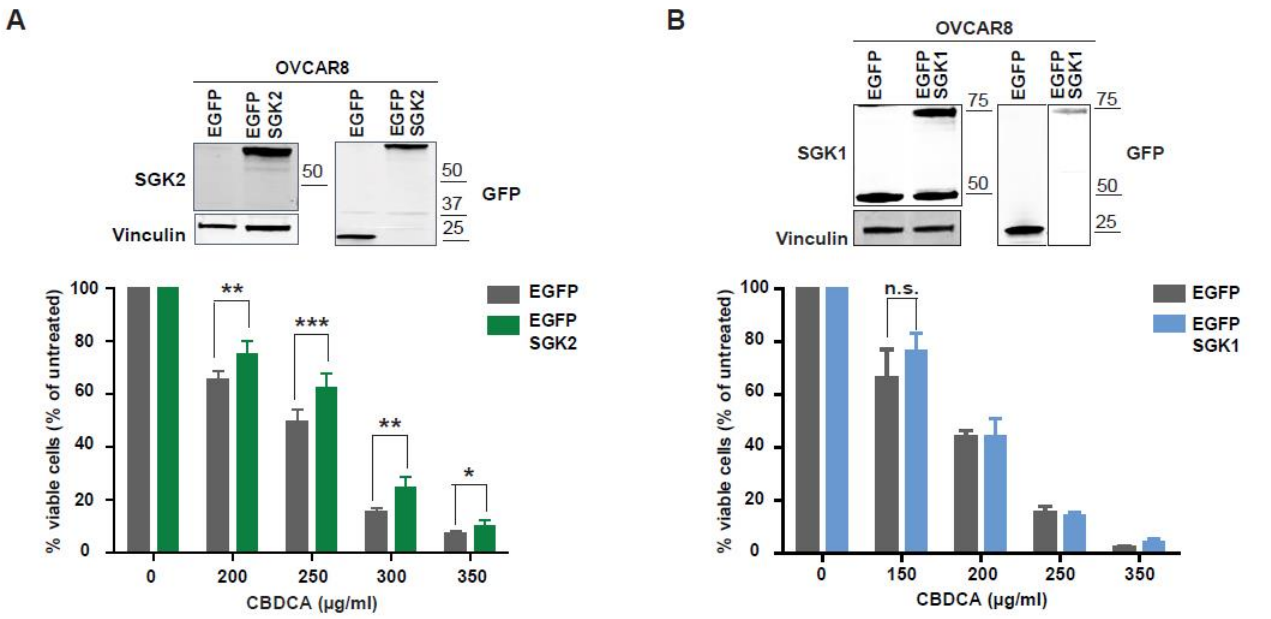

Figure 4: SGK2 overexpression increases resistance to CBDCA treatment in EOC cells. (A) Graph reports the viability of OVCAR8 cells stably overexpressing the empty vector and SGK2 wild type. Results are expressed as survival ratio between CBDCA treated and untreated cells. Statistical significance was calculated using t-test. SGK2 overexpression was confirmed by western blot. (B) Graph reports the viability of OVCAR8 cells stably overexpressing the empty vector and SGK1 wild type. Results are expressed as survival ratio between CBDCA treated and untreated cells. Statistical significance was calculated using t-test. SGK1 overexpression was shown by western blot. Vinculin was used as loading control.

3.4 SGK2-overexpressing cells present an increased *in vitro* and *in vivo* growth rate.

Based on the observation that SGK2-overexpressing OVCAR8 cells seemed to grow faster than the control cells in routine cell culture, we conducted a growth curve analysis to verify if SGK2 overexpression could provide an advantage in cell proliferation. Using this approach we verified that SGK2-overexpressing OVCAR8 cells had a significantly increased *in vitro* growth rate when compared to controls (Fig.5A). This data was confirmed by stably overexpressing SGK2 in a different EOC cell line, TOV21G (Fig.5B). On the contrary, SGK1 overexpression did not modify OVCAR8 growth rate respect to control cells (Fig.5C). Interestingly, *in vivo* experiments with subcutaneous injection of control and SGK2-overexpressing OVCAR8 cells in the nude mice (empty vector on the right flank, SGK2-overexpressing cells on the left flank) demonstrated that SGK2-overexpressing cells formed tumours with a lower latency time and with a faster growth (Fig.6A). Accordingly tumours explanted from mice one month after injection showed increased weight (Fig.6B) and size (Fig.6C) compared to controls. SGK2 overexpression

was maintained in explanted tumours (Fig.6D) and was associated with an increased cells proliferation as evaluated by Ki-67 staining of tumour sections (Fig.6E).

Overall these results suggest that SGK2 regulate *in vitro* and *in vivo* proliferation of EOC cells.

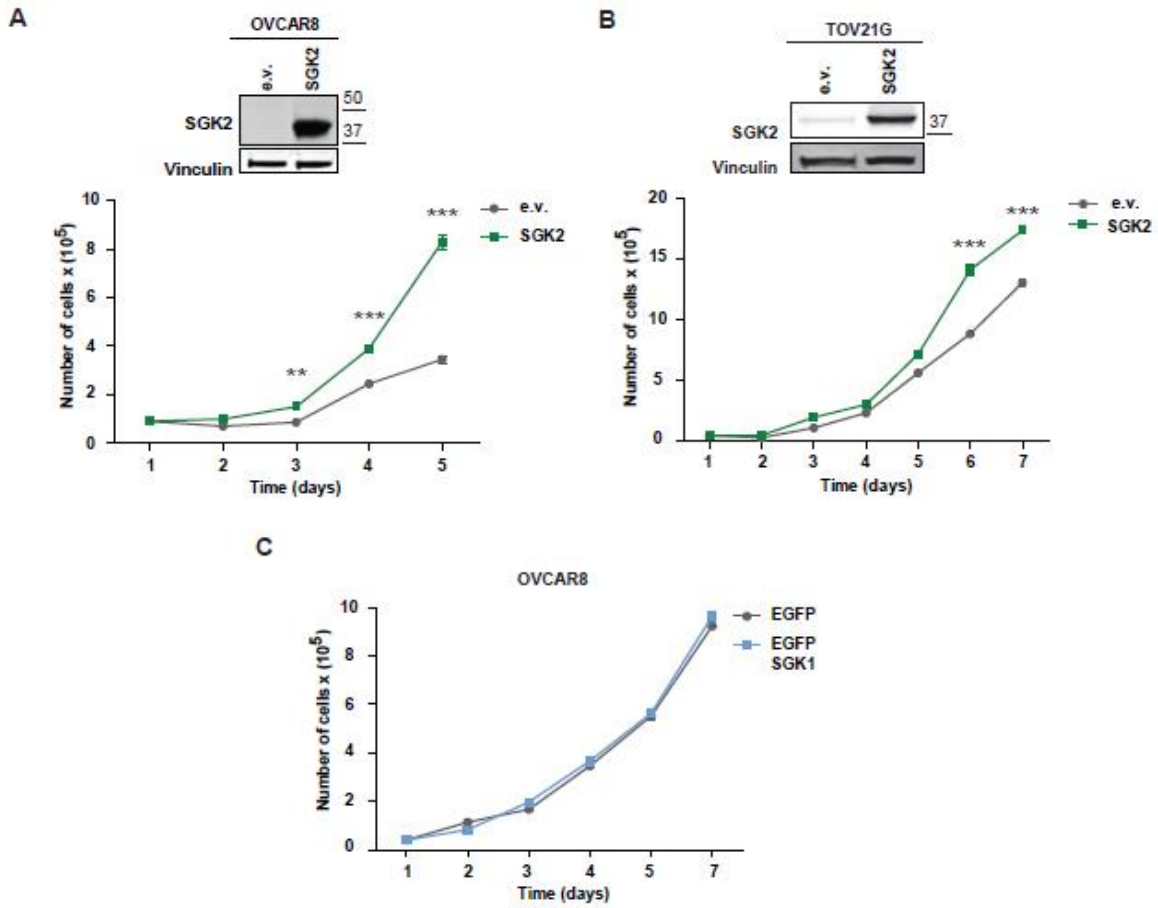

Figure 5: SGK2-overexpressing cells present an increased *in vitro* growth rate. (A) Growth curve of OVCAR8 cells stably expressing the empty vector (e.v.) and SGK2. Lysates were analysed by western blot. Vinculin was used as loading control. (B) Growth curve of TOV21G cells stably expressing the empty vector (e.v.) and SGK2. Lysates were analysed by western blot. Vinculin was used as loading control. (C) Growth curve of OVCAR8 cells stably expressing the empty vector and SGK1.

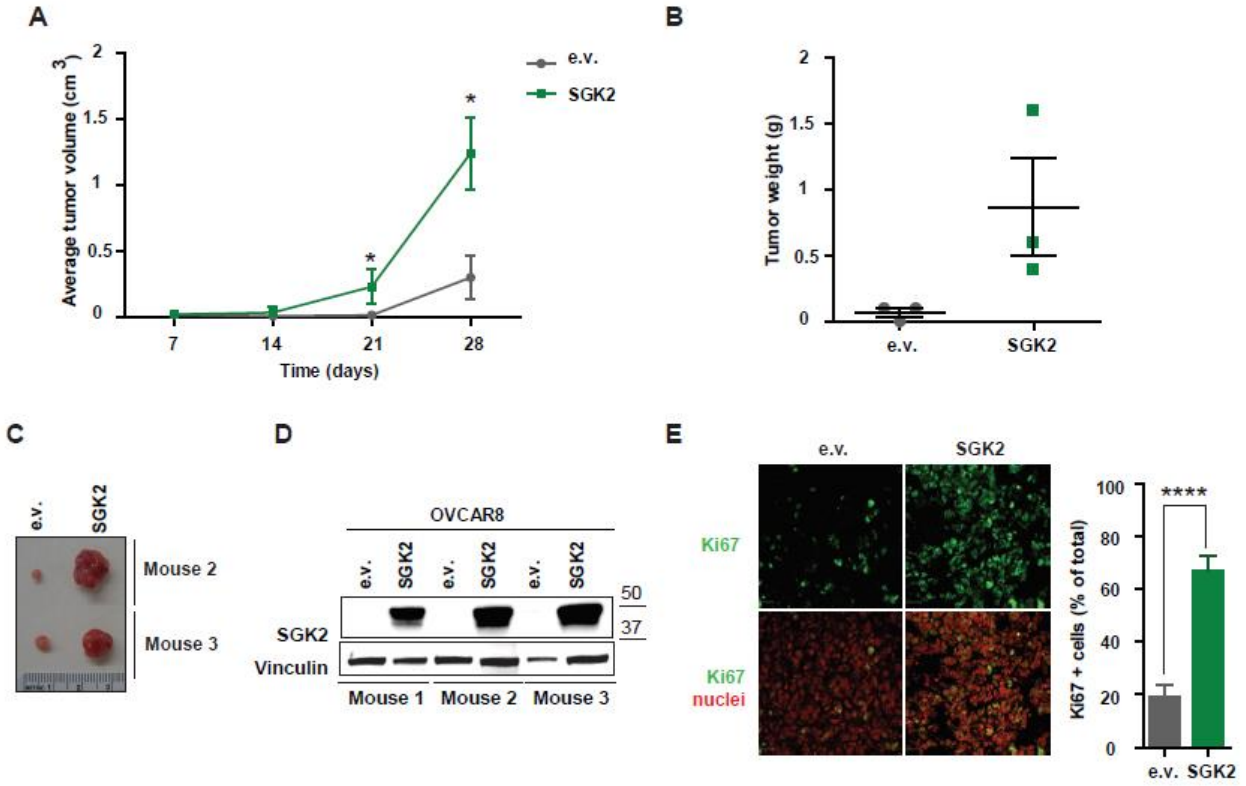

Figure 6: SGK2-overexpressing cells exhibit an increased *in vivo* growth rate. (A) Tumour growth in nude mice was monitored for a month. (B) At the end of the experiment, tumours were removed and weighted. (C) Photo shows representative explanted tumours. (D) Tumour lysates were prepared and analysed by western blot. Vinculin was used as loading control. (E) Tumour sections were stained with Ki67 staining. The graph reports the percentage of Ki67 positive cells.

3.5 SGK2 kinase activity is involved in EOC sensitivity to platinum treatment.

To analyse if the kinase activity of SGK2 was involved in the regulation of cell proliferation and/or platinum response in EOC cells, we generated a SGK2 dominant negative (DN) construct (SGK2$^{T193A/S356A}$) by mutating the two phosphorylation sites essential to activate the kinase (Fig.7A), following the approach used to generate a SGK1-DN protein (Brunet et al. 2001). SGK2-DN mutant was stably overexpressed in TOV21G cells, which were then treated with different doses of CBDCA. SGK2-DN mutant was able to reduce the survival of platinum-treated cells, mimicking the results obtained with SGK2 silencing (Fig.7B), indicating that SGK2 kinase activity was implicated in EOC sensitivity to platinum.

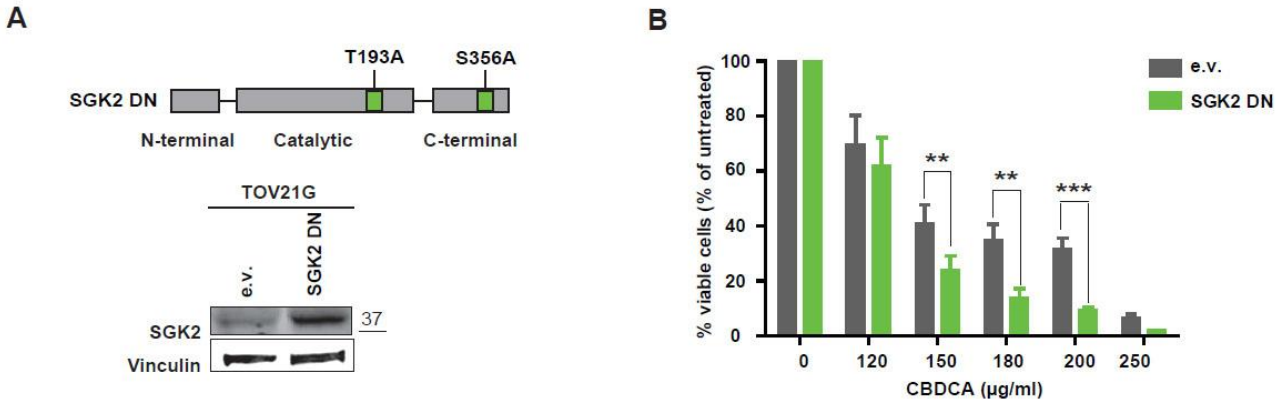

Figure 7: SGK2 kinase activity is involved in the regulation of EOC response to platinum treatment. (A) SGK2 dominant negative (DN) construct (SGK2$^{T193A/S356A}$). (B) Cell viability in TOV21G cells stably overexpressing the empty vector (e.v.) and SGK2 DN treated with different concentrations of CBDCA. Results are expressed as survival ratio between CBDCA treated and untreated cells. Significance was calculated using student t-test. TOV21G cell lysates were analysed by western blot. Vinculin was used as loading control.

3.6 The SGK1/SGK2 kinase inhibitor, GSK650394, reduces cell viability in combination with platinum.

The above data suggest that SGK2 kinase activity was necessary to increase platinum sensitivity in EOC cells. We thus tested a small competitive kinase inhibitor, GSK650394 (GSK), which was shown inhibiting the enzymatic activity of SGK1 and SGK2 with an in vitro IC50 (Inhibitory Concentration able to reduce the activity by the half) values of 62 and 103 nmol/L, respectively (Sherk et al, 2008). As a model we used MDAH cells in which we tested different GSK and platinum concentrations along with different schedules of treatment to identify the best combination treatment.

In the first case, MDAH cells were treated with increasing doses of GSK for 24 hours that was then removed before the administration of a fixed concentration of platinum (140µg/ml) for 16 hours to obtain a 20% of cell death (Fig.8A, upper schema). This schedule of treatment demonstrated that GSK did not alter MDAH cells viability used alone but increased platinum-induced cell death in a dose dependent manner (Fig. 8A lower graph). Since the lowest tested GSK concentration (35µM) was already able to significantly increase platinum-induced MDAH cell death, we used this GSK dose in a different schedule of treatment in which the SGK inhibitor was administered to the cells 24 hours before platinum and maintained during carboplatin treatment (140µg/ml for 16 hours) (Fig.8B, upper schema). We observed that when the SGK inhibitor was not removed during carboplatin treatment, it resulted more active in reducing cell viability when

compared with the other schedule (Fig.8B, lower graph). Therefore, we had chosen this schedule to test lower concentrations of GSK (5, 15, 25 and 35 μM) in association with different doses of platinum in MDAH cells. Using this approach, we confirmed that GSK used as single agent for up to 48 hours did not affect MDAH cells viability (Fig. 9). However, at any concentration used, GSK treatment significantly increased the platinum-induced death in a manner dependent on the amount of SGK inhibitor and platinum used (Fig. 9). Overall these data demonstrated that inhibition of SGK activity with GSK alone was not toxic for the cells, but it reduced cell viability when associated with platinum, mimicking the synthetic lethality observed by combining SGK2 silencing and CBDCA treatment.

3.7 GSK650394 is able to make SGK2-expressing cells more sensitive to platinum.

Taking into account these results obtained in MDAH cells, we tested the activity of GSK in a panel of EOC cell lines with different SGK2 expression. Interestingly, GSK was able to increase platinum-induced death in cells expressing detectable levels of SGK2 protein (i.e. MDAH, TOV21G, SKOV3), but not in EOC cells with undetectable SGK2 protein expression (i.e. COV318, TOV112D) (Fig.10). Moreover normal human ovarian epithelial cells HuNoEOC, used as a control, did not express SGK2 and were insensitive to GSK both when used as single agent and in combination with platinum (Fig 10).

GSK650394 inhibits the *in vitro* activity of both SGK1 and SKG2. Since in all tested cells SGK1 expression did not correlate with GSK activity (Fig 10), we more specifically addressed the role of SGK2, using TOV21G cells stably expressing the SGK2-DN protein (Fig. 7A). In these cells GSK had no effect on platinum sensitivity, while TOV21G cells expressing only the empty vector and thus maintaining active endogenous SGK2, responded as parental TOV21G cells to platinum associated to GSK (Fig 11A). These data indicated that SGK2 kinase activity was necessary to mediate the effect of GSK on platinum sensitivity in EOC cells.

Interestingly GSK also decreased the growth rate of SGK2-overexpressing OVCAR8 cells, as demonstrated by growth curve experiments in which GSK (20μM) treatment of 8 hours was sufficient to revert the increased growth of SGK2-overexpressing OVCAR8 cells (dark green line) to a growth rate (light green line) similar to that of the OVCAR8 cells with the empty vector (Fig.11B).

Together these data suggested that SGK2 is the principal mediator of GSK activity in EOC cells, while SGK1 and 3 seem to play a minor role in this model.

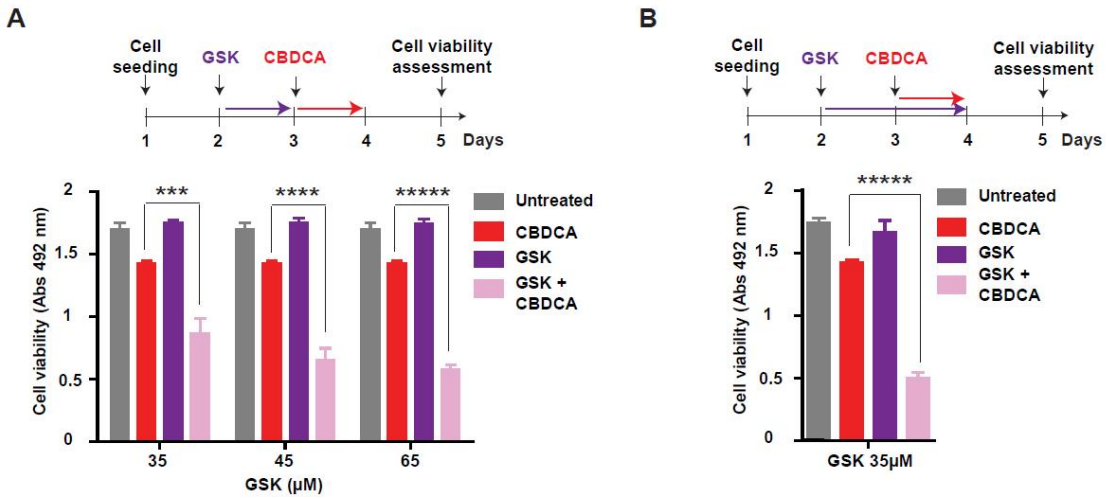

Figure 8: The SGK1/SGK2 kinase inhibitor, GSK650394, sensitizes MDAH cells to platinum.
(A) Graph reports cell viability of MDAH treated for 24 hours with different doses of GSK, and then with CBDCA 140μg/ml overnight. Significance was calculated using student t-test. (B) Graph reports cell viability of MDAH cells treated with GSK 35μM for 24 hours, and then with combination with CBDCA 140μg/ml for 16 hours. Significance was calculated using student t-test. Experimental timelines were shown.

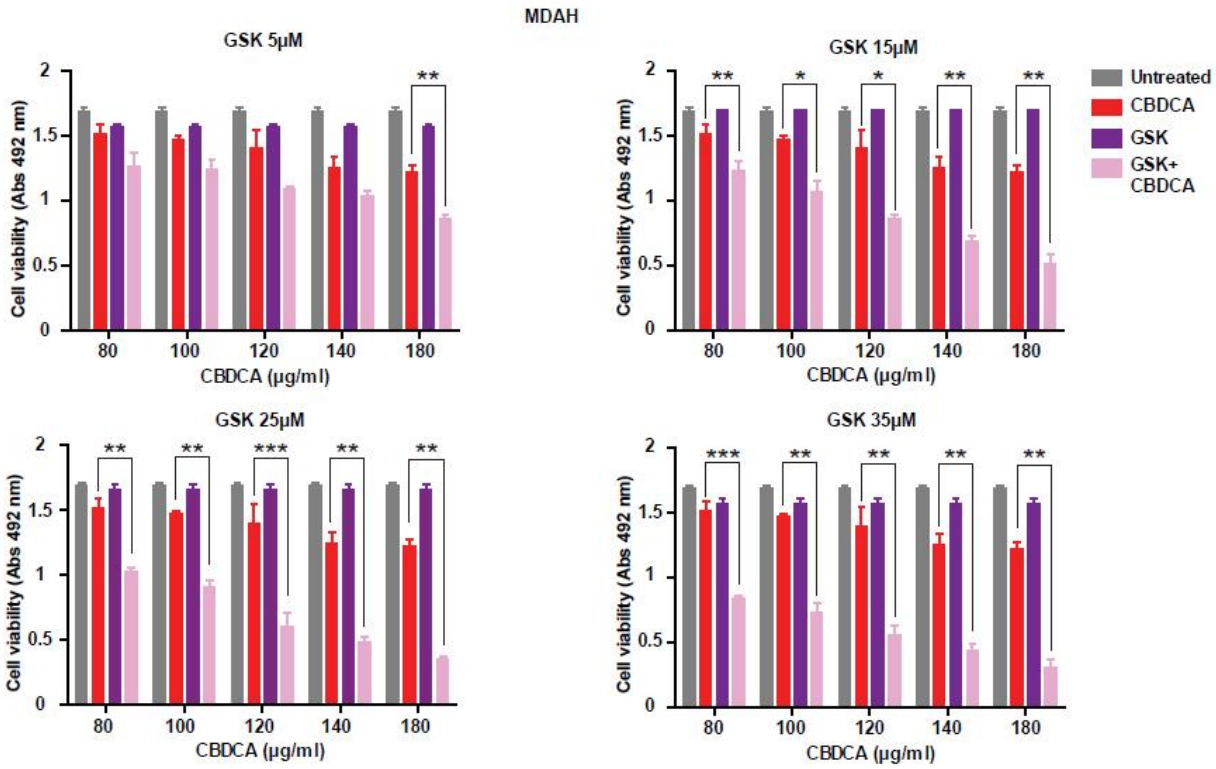

Figure 9: GSK650394 sensitizes MDAH cells to platinum-induced cell death. Graphs report cell viability of MDAH treated for 24 hours with GSK at doses 5, 15, 25, 35 μM, then combined with different concentrations of CBDCA overnight. Significance was calculated using student t-test.

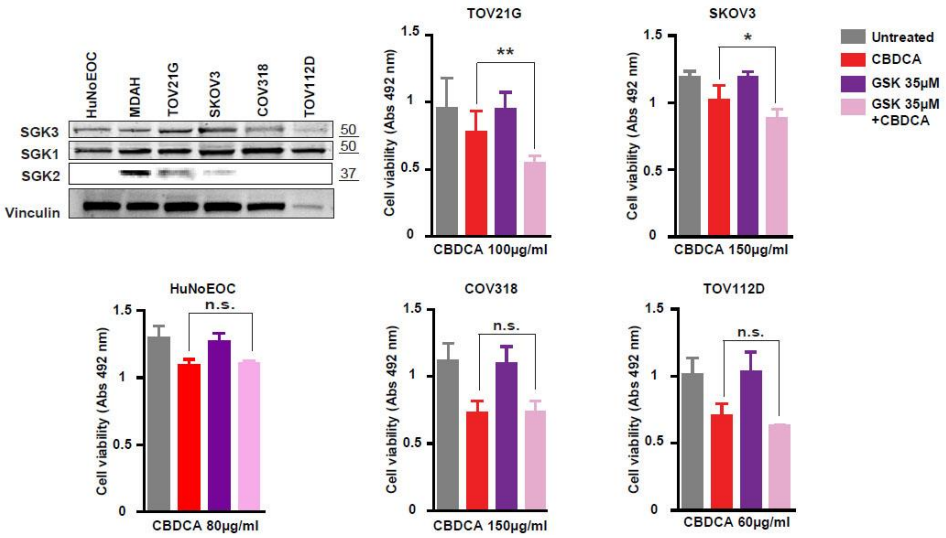

Figure 10: GSK650394 sensitizes SGK2-expressing cells to platinum. Cell viability of (upward) SGK2-expressing cell lines (TOV21G and SKOV3) and (below) EOC cell lines without SGK2 expression (HuNoEOC, COV318, TOV112D) treated with GSK, CBDCA and GSK+CBDCA. Significance was calculated using student t-test. SGK2 expression was analysed by western blot. Also SGK1 and SGK3 expression levels were reported. Vinculin was used as loading control.

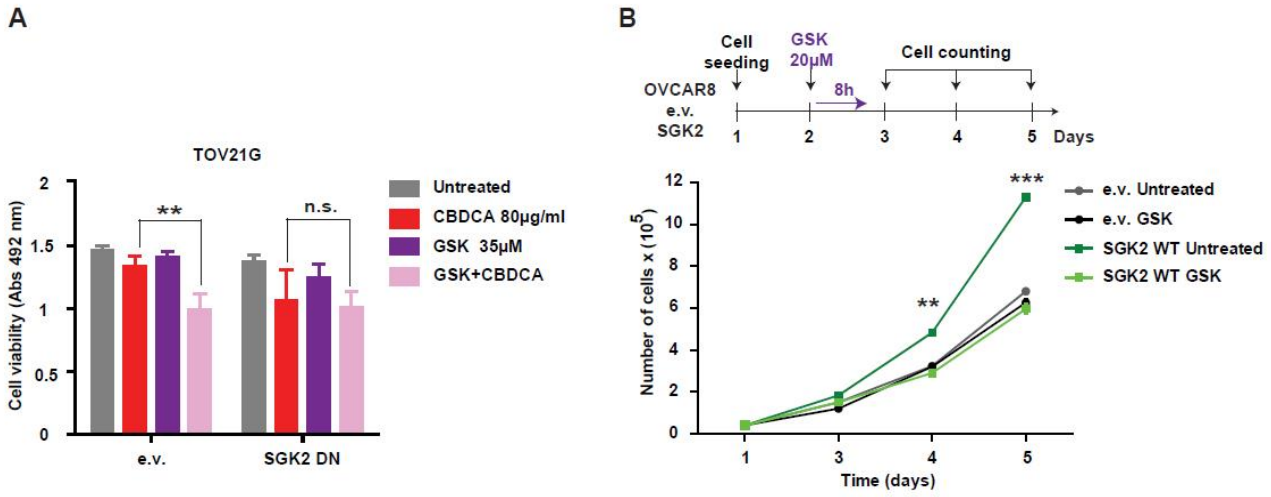

Figure 11: SGK2 mediates the activity of GSK650394 in EOC cells. (A) Cell viability of TOV21G cells stably overexpressing SGK2 DN and the empty vector control (e.v.), treated with GSK, CBDCA and GSK+CBDCA. Significance was calculated using student t-test. (B) Growth curve of OVCAR8 cells stably expressing the empty vector (e.v.) and SGK2, untreated (grey and dark green line, respectively) and treated with GSK (black and light green, respectively).

3.8 SGK2-expressing cells show formation of cytoplasmic vesicles upon GSK650394 treatment, possibly linked to autophagy.

We observed that only SGK2-expressing cells (such MDAH and TOV21G) showed the formation of cytoplasmic vesicles upon GSK and GSK+CBDCA treatment. Their presence was clearly visible at the optical microscope at 36h of treatment (Fig.12A), just before experiment was terminated and cell lysed for western blot analysis. It is known that the appearance of cytoplasmic vesicles could be a sign of altered autophagy (Klionsky et al, 2016). We thus evaluated the expression of the two important autophagy markers, LC3 and p62, by western blot in MDAH and TOV21G cells treated with CBDCA, GSK and GSK+CBDCA. In both cell lines LC3II/LC3I and p62 markedly increased in GSK-treated cells respect to untreated and CBDCA-treated cells (Fig.12B). The increase of LC3II/LC3I and p62 levels due to GSK treatment was dose-dependent, evident also at the lowest used concentrations (Fig.13A) and similar to the ones observable in cells exposed to bafilomycin A1 (Fig.13B), a known inhibitor of autophagy that prevents the fusion of autophagosome with lysosome (Yamamoto et al, 1998; Mizushima and Komatsu, 2011). This observation agreed with the fact that the increase of p62 protein expression was not accompanied by an increase in its mRNA expression, as evaluated by qRT-PCR experiments (Fig.13C), and was not linked to an increment of protein stability, as demonstrated by blocking *de novo* protein synthesis with cycloheximide (CHX) (Fig.13D). Overall these data suggested that p62 protein accumulation in GSK-treated cells is likely due to a block of its degradation via autophagy.

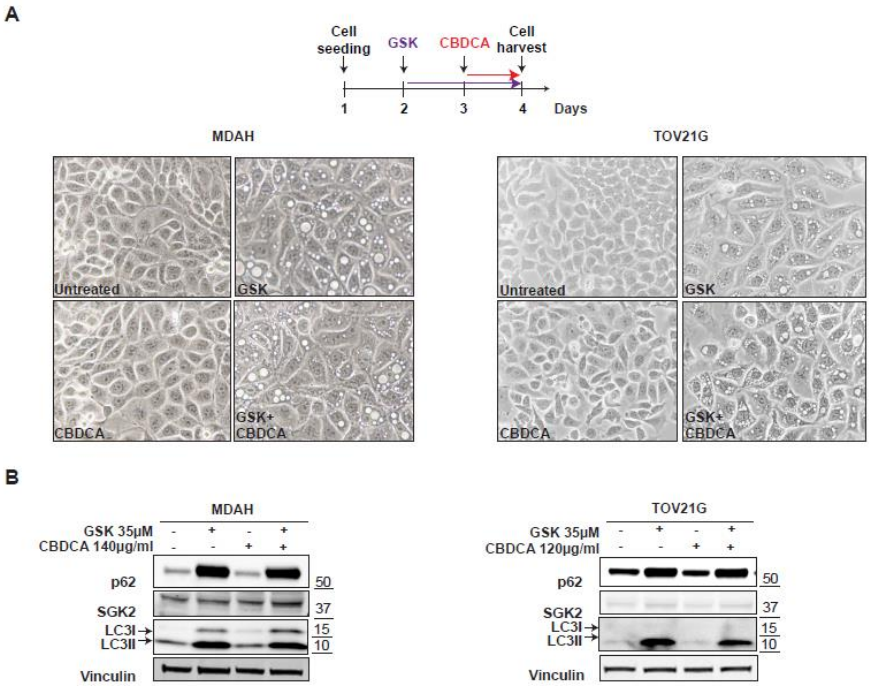

Figure 12: GSK650394 treatment triggers the formation of cytoplasmic vesicles in SGK2-expressing cells and it increases autophagy markers. (A) Pictures from optical microscope of MDAH e TOV21G cells treated with GSK 35µM (36h), CBDCA 140µg/ml (ON) and GSK+CBDCA. (B) Expression of SGK2 and autophagy markers LC3II/LC3I and p62 in MDAH and TOV21G cells, treated with GSK 35µM (36h), CBDCA 140µg/ml overnight and GSK+CBDCA. Vinculin was used as loading control. Experimental timeline was shown upward.

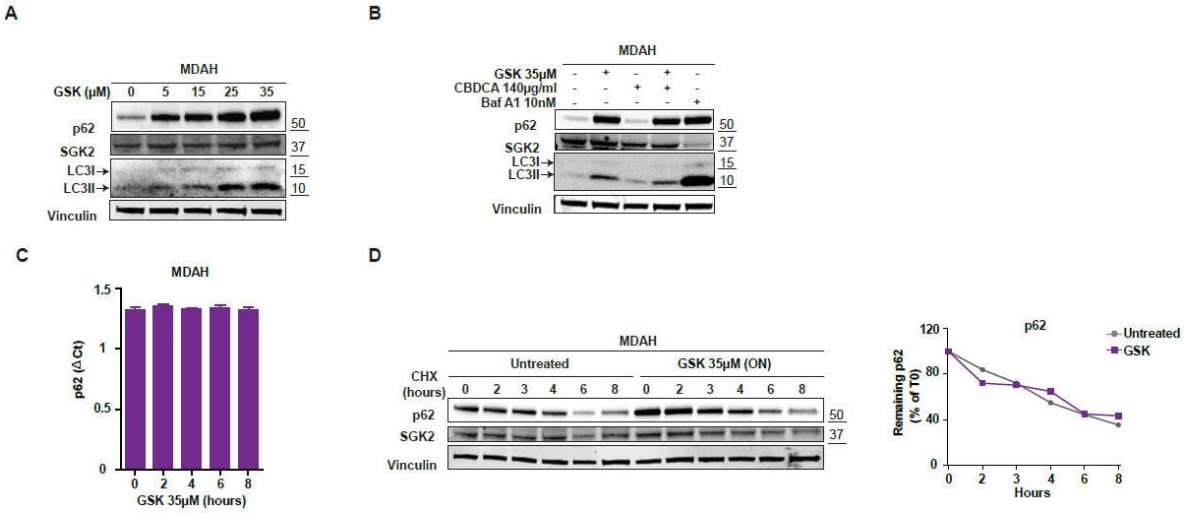

Figure 13: GSK650394 treatment increases autophagy markers, and in particular p62 protein increment could be indicative of a block of autophagy in GSK-treated cells. (A) SGK2, p62 and LC3II/LC3I expression in MDAH cells treated with different doses of GSK (5, 15, 25, 35µM). (B) Western blot analysis comparing MDAH cells treated with GSK(36h), CBDCA(ON) and GSK+CBDCA to MDAH cells treated with bafilomycin A1 (Baf A1) used at a concentration of 10nM for 36 hours. (C) Expression of p62 mRNA in MDAH treated with GSK 35µM for 2, 4, 6, 8 hours. Data are expressed in threshold cycle difference (ΔC_t). (D) Expression of p62 in MDAH untreated or treated with GSK 35µM overnight and then released in CHX containing medium for the indicated times. Vinculin was used as loading control. Graph on the right report the densitometric quantification of the blot for p62.

3.9 GSK treatment may increase cell sensitivity to platinum treatment via autophagy blockade.

The above data highlighted that SGK2 inhibition by GSK treatment may lead to ineffective autophagy accompanied by the accumulation of cytoplasmic vesicles (Fig.12A). To investigate the nature of these cytoplasmic vesicles, we used electron microscopic study to analyse MDAH and TOV21G cells treated with GSK for 36 hours. We observed that the cytoplasmic vesicles in GSK-treated cells were autophagic vacuoles. GSK-treated cells analysed showed a general markedly increased number of autophagic vacuoles with different characteristics that could be grouped in two main categories: 1) double-membrane autophagic vacuoles containing not yet degraded cytoplasmic material and organelles (present in 6% of MDAH cells (Fig.14b,14c) and in 8% of TOV21G (Fig.14f) examined cells; 2) big vacuoles, surrounded partially by membrane, enclosing material that seemed to be degenerated/degraded (Fig.14d,14g,14h). The first ones were clearly early or initial autophagic vacuoles named autophagosomes, containing morphologically intact cytosol and organelles; the last ones were late autophagic vacuoles containing degenerated or partially degraded cytoplasmic material, which could represent amphisomes/late autophagosomes if they fused with endosomes, or autolysosomes if fusion with lysosomes and degradation took place (Eskelinen, 2005). These cytoplasmic vacuoles were almost undetectable in untreated cells (respectively Fig.14a and 14e), overall confirming that GSK treatment blocks autophagy in EOC cells expressing the SGK2 protein.

Since with this approach was not possible to establish if the observed autophagic vacuoles in GSK-treated cells could represent autophagosomes already fused with lysosomes or not, we decided to analyse the expression and the localization of a known lysosomal marker (i.e. LAMP2) by immunofluorescence in control and GSK-treated TOV21G cells. While LAMP2 was evenly distributed in the cytoplasm of the untreated cells, it accumulated in the membranes of the autophagic vacuoles formed at 8 and 16 hours of GSK treatment (Fig.15), indicating that they have been already fused with lysosomes since LAMP2 is primarily localized in lysosomal membrane (Eskelinen et al, 2002).

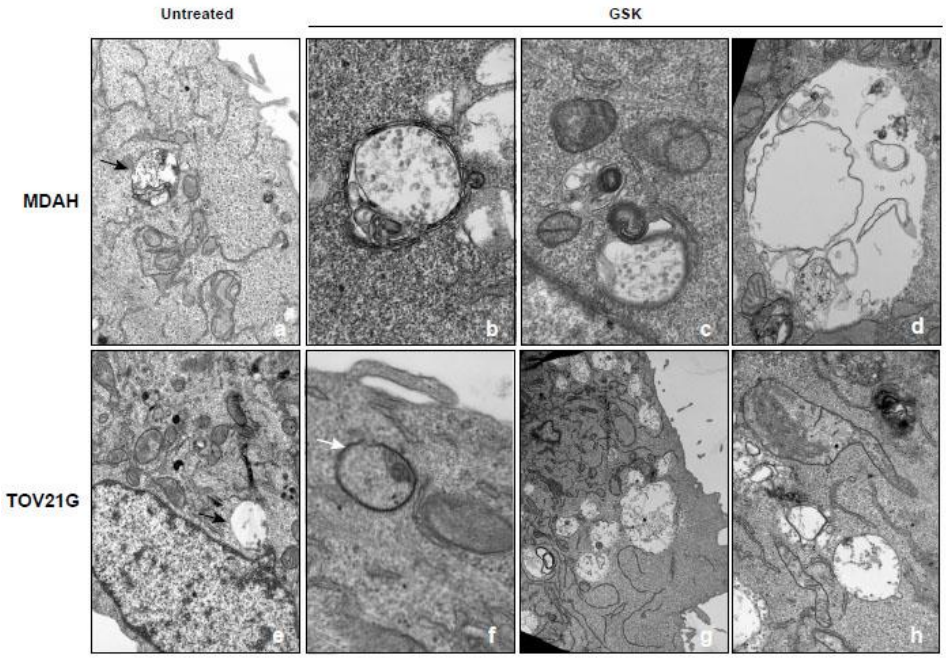

Figure 14: EOC cells present an increased number of autophagic vacuoles with different characteristics upon GSK treatment. Ultrastructure of autophagic vacuoles in MDAH and TOV21G cells untreated and treated for 36 hours with GSK 35 µM. (a) Untreated MDAH presented only few cytoplasmic vacuoles (black arrow). GSK-treated MDAH cells showed an increased number of (b-c) double-membrane autophagosomes and (d) big vacuoles (diameter 5-10µm) surrounded partially by membrane. (e) Untreated TOV21G cells displayed rare vacuoles (black arrow). GSK-treated TOV21G cells presented (f) double-membrane autophagosomes (white arrow) and (g-h) big vacuoles surrounded partially by membrane. (Magnification range: 20-75K)

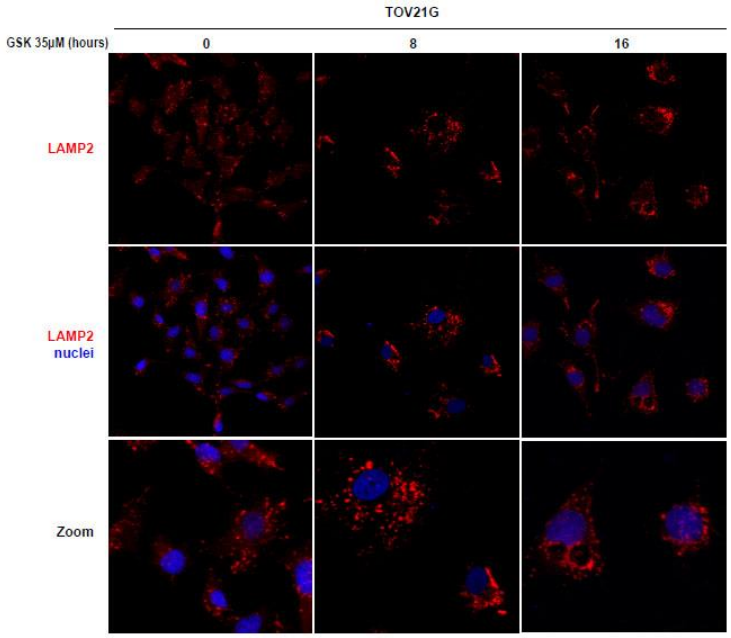

Figure 15: LAMP2 accumulates in the membrane of autophagic vacuoles in GSK-treated cells, suggesting that they have been already fused with lysosomes. Immunofluorescence analysis evaluating the expression and the localization of LAMP2 in TOV21G cells treated with GSK 35µM at 8 and 16 hours. Nuclei were stained with propidium iodide.

Based on this observation, we evaluated the expression of critical markers of autophagy in a GSK treatment time-course (4, 8, 16, 24, 36 hours) in MDAH and TOV21G cells to confirm our hypothesis. Data show that LAMP2 expression increased in both the cell lines with GSK time-course progression, suggesting that autophagic vacuoles were fused with lysosomes but degradation did not occur (Fig.16), in accordance with the observation that LAMP2 was not degraded, like in its normal turnover, and it accumulated in the membranes of the autophagic vacuoles formed upon GSK treatment, as previously observed in immunofluorescence analysis (Fig.15). Similarly Rab7, an accepted marker of late endosomes (which comprise lysosomes), accumulated in GSK-treated cells with a similar, although less evident, trend (Fig.15). Particularly interesting was the study of the lysosomal aspartic protease cathepsin D (catD) in western blot. CatD is a protein synthesized on the endoplasmic reticulum as a pre-pro-enzyme (pre-pro-catD, 52 kDa), processed with removal of the signal peptide to yield pro-catD (48 kDa) in Golgi apparatus and targeted to the lysosome, where it is activated by the acidic pH of lysosomal lumen (active catD, 34 kDa) produced by H+ v-ATPase proton pump (Richo and Conner, 1994). In our study we detected a decrease of the active catD expression accompanied by an accumulation of its inactive precursors during GSK treatment time-course progression (Fig.16). This evidence indicates that cathepsin D was not processed in its active form, possibly for the lack of the proper lysosomal pH, and therefore it was unable to degrade the autophagic material (Fig.16).

It was known that the blockade of any step downstream of autophagosome formation increases the number of autophagosomes while decreases the number of autolysosomes (Mizushima et al, 2010). To monitor the autophagic flux, we used the mRFP-GFP-LC3 construct that allows to detected the subcellular localization of LC3. This method is based on the concept of lysosomal quenching of GFP in GFP-labeled autophagic substrates such as LC3 (Mizushima et al, 2010). At the neutral pH of autophagosomes, green fluorescence of GFP and red fluorescent protein overlap resulting in yellow puncta, whereas the low pH inside the lysosome quenches the fluorescent signal of GFP, which makes undetectable the delivery of GFP-LC3 to lysosomes; in contrast, RFP exhibits more stable fluorescence in acidic compartments (Katayama et al, 2008), and mRFP-LC3 can readily be detected in autolysosomes as red puncta. This method depends on the acidification and degradation capacity of the lysosome. It is, therefore, sometimes possible that autolysosomes are observed as yellow, depending on the activity of lysosomal enzymes and the speed at which the acidic lysosomal pH quenches the GFP signal (Mizushima et al, 2010). Analyses

of confocal microscopy immunofluorescences of MDAH cells, transfected with mRFP-GFP-LC3 construct, showed an increased number of yellow puncta after 36 hours of GSK treatment, compared to the untreated control, in which most puncta were red (Fig.17), confirming that there was an accumulation of neutral-pH autophagic vacuoles where degradation did not occur.

In this way we established that GSK treatment might increase cell sensitivity to platinum treatment by altering the acidic pH of lysosomes, at which lysosomal enzymes cannot function properly, and degradation did not occur in evaluated autophagic vacuoles.

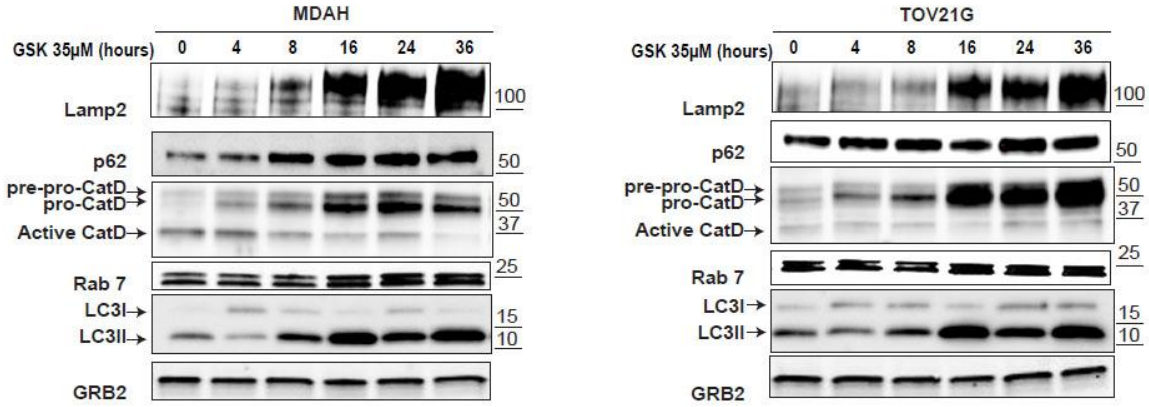

Figure 16: GSK treatment causes autophagy blockade preventing degradation of material after the fusion of autophagic vacuoles with lysosomes. Lysates from MDAH and TOV21G cells treated with GSK 35μM at 4,8,16,24,36 hours were analysed by western blot. GRB2 was used as loading control.

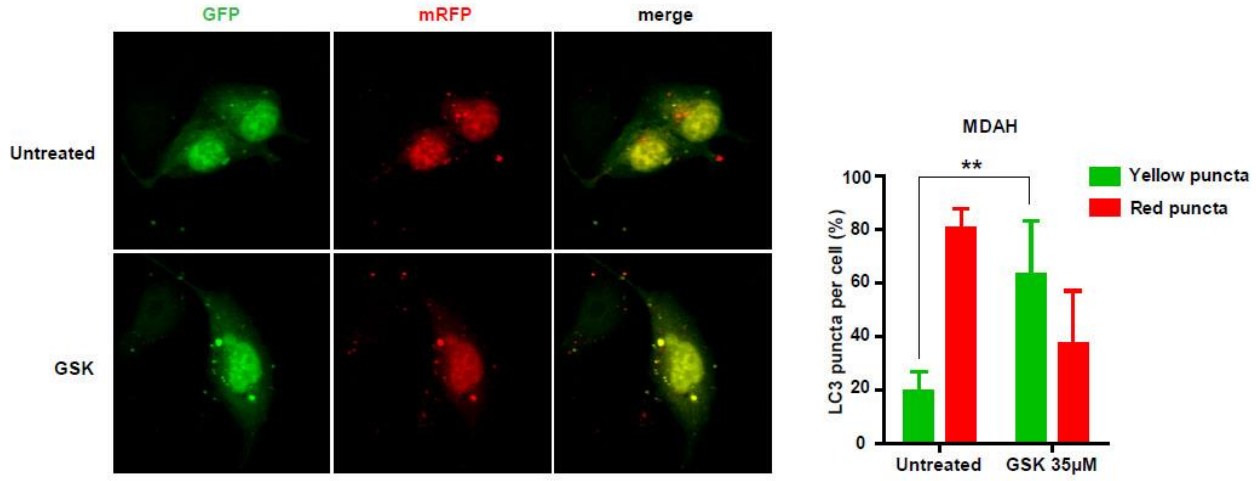

Figure 17: GSK treatment increases the number of yellow puncta in EOC cells transfected with mRFP-GFP-LC3. MDAH cells, transfected with mRFP-GFP-LC3, were untreated or treated with GSK 35μM for 36 hours. Note that most puncta are red in untreated MDAH cells, whereas most puncta in GSK-treated MDAH cells are yellow. Graph reports the percentage of LC3 puncta counted per cell (number of cells=10). Significance was calculated using student t-test.

3.10 SGK2 may have a role in EOC platinum sensibility via autophagy modulation.

To confirm that autophagy blockade observed upon GSK treatment was really due to SGK2 inhibition, we examined how the analysed autophagy markers behaved in presence of SGK2 overexpression or SGK2 silencing. We observed that, by treating stably SGK2-overexpressing OVCAR8 cells with GSK, CBDCA and the combination of GSK+CBDCA, p62 accumulation, due to GSK treatment, was completely abolished in presence of SGK2 overexpression. Interestingly, in SGK2-overexpressing OVCAR8 cells treated with GSK and GSK+CBDCA it is also possible to evidence a LC3II/LC3I inversion respect to the control (Fig.18). On the other hand, silencing SGK2 in MDAH and TOV21G cells, resulted in the increase of LAMP2 expression, in the decrease of active CatD, and in LC3II/LC3I and p62 increase (Fig.19) similar to what observed with GSK treatment (Fig. 16). Moreover, SGK2-silenced MDAH cells, transfected with mRFP-GFP-LC3 construct, showed an increased number of yellow puncta, compared to the control (Fig.20), suggesting that the accumulation of neutral-pH autophagic vacuoles seen with GSK treatment (Fig.17) was due to SGK2 inhibition.

Overall these data supported the possibility that SGK2 inhibition (through SGK2 silencing or pharmacological inhibition) increased cell sensitivity to platinum treatment, by blocking autophagy at the degradation step of material accumulated in autophagic vacuoles already fused with lysosomes.

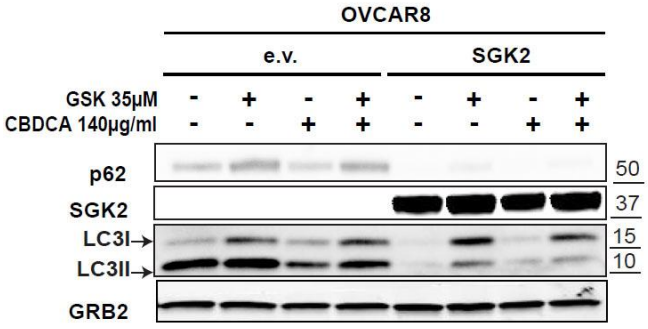

Figure 18: SGK2 overexpression completely abolishes p62 accumulation. Western blot analysis of lysates from OVCAR8 cells overexpressing empty vector (e.v.) and SGK2, treated with GSK, CBDCA and GSK+CBDCA. GRB2 was used as loading control.

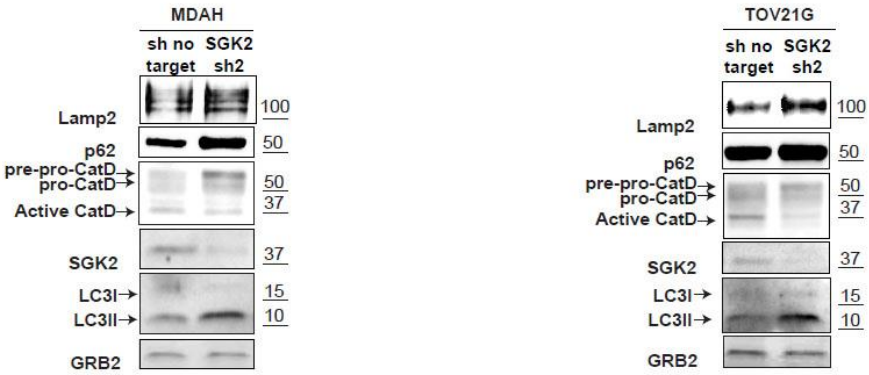

Figure 19: SGK2 silencing confirms autophagy blockade observed with GSK treatment. Lysates from SGK2-silenced MDAH and TOV21G cells were analysed by western blot. SGK2 silencing and Lamp2, p62, CatD, LC3I/LC3II expression are shown. GRB2 was used as loading control.

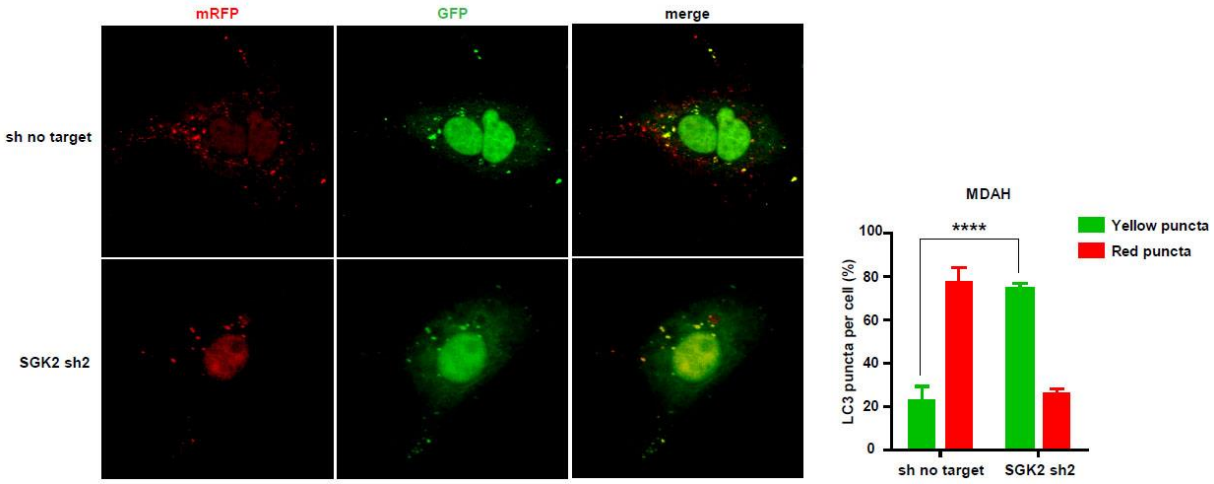

Figure 20: SGK2 silencing increases the number of yellow puncta in EOC cells transfected with mRFP-GFP-LC3. MDAH cells, transfected with mRFP-GFP-LC3, were transduced with sh no target or with SGK2 sh2. Note that most puncta are red in MDAH cells with sh no target, whereas most puncta in SGK2-silenced MDAH cells are yellow. Graph reports the percentage of LC3 puncta counted per cell (number of cells=10).
Significance was calculated using student t-test.

3.11 Platinum treatment was able to stimulate autophagy in EOC cells.

According to our data, SGK2 inhibition could increase cell sensitivity to platinum by blocking autophagy. This assumed that platinum treatment was able to induce autophagy in EOC cells. It is known that activation of autophagy is a hallmark in tumor cells treated with chemotherapy and it may constitute a mechanism exploitable to develop resistance to chemotherapeutic agents, but the role of autophagy in platinum-treated ovarian cancer remains to be clarified (Wang and Wu, 2014). To evaluate if platinum could be able to induce autophagy in our EOC model, we treated MDAH cells for 3, 6 and 16 hours with different doses of CBDCA from 140µg/ml (used in previous experiments) to 1000 ug/ml to detect changes in analysed autophagy markers. In this way, we observed an increase in LC3II/LC3I and active CatD, LAMP2 maintained its basal expression level, whereas p62 decrease at higher CBDCA concentrations at 6 and 16 hours of treatment, indicative of autophagy induction (Fig.21).

In this way, we confirmed that platinum treatment was able to stimulate autophagy in EOC cells.

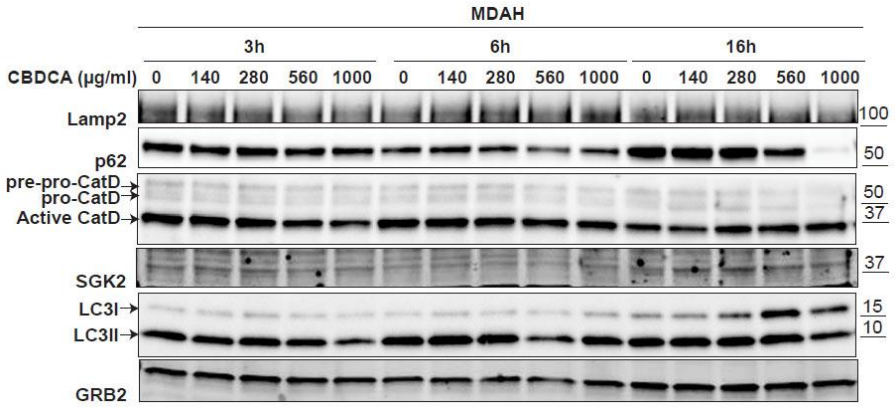

Figure 21: Platinum-treated EOC cells show autophagy stimulation. Lysates from MDAH cells treated with different doses of CBDCA (140, 280, 560, 1000 µg/ml) for 3, 6, 16 hours were analysed by western blot. Lamp2, p62, CatD, SGK2 and LC3I/LC3II expression are shown. GRB2 was used as loading control.

3.12 SGK2 binds to and colocalize with p62

All previous results demonstrated that SGK2 inhbition resulted in p62 accumulation. This accumulation could be a consequence of authophagy block or a direct effect of SGK2 on p62 function that eventually leads to ineffective autophagy. We thus tested if SGK2 directly interacted with p62 using co-immunoprecipitation experiments in untreated and GSK-

treated MDAH and TOV21 cells. Indeed, in both models endogenous SGK2 and p62 readily co-precipitated, and their interaction increased upon GSK treatment (Fig.22).
Immunofluorescence analysis confirmed that in MDAH and TOV21G cells p62 and SGK2 accumulated and co-localized around the cytoplasmic vacuoles (Fig.23).
These data suggested the possibility that SGK2 could have a role in EOC platinum sensibility by modulating autophagy via its interaction with p62.

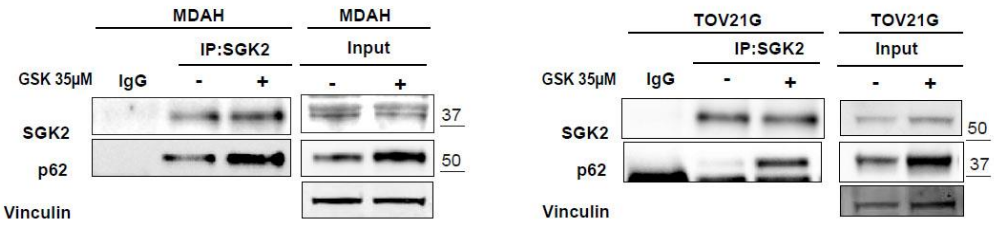

Figure 22: SGK2 interacts with p62. Co-immunoprecipitation (IP) analysis of endogenous SGK2 from MDAH and TOV21G cells untreated or treated with GSK. IPs were evaluated by western blot for the presence of p62. Expression of SGK2 and p62 proteins in the corresponding lysates (Input) is reported. Vinculin was used as loading control.

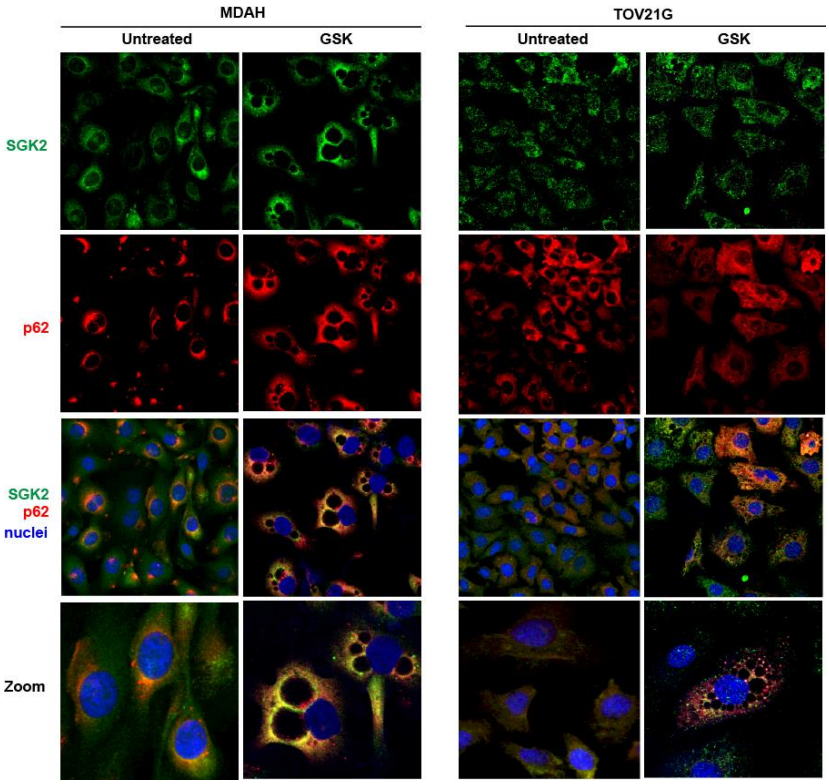

Figure 23: p62 accumulates and co-localizes around cytoplasmic vesicles formed upon GSK treatment. Immunofluorescence evaluating the expression and localization of SGK2 (green) and p62 (red) in MDAH (left panel) and TOV21G (right panel) cells, comparing untreated cells and cells treated with GSK 35μM for 36 hours. Nuclei were stained with propidium iodide.

Discussion

The overall survival of women with Epithelial Ovarian Cancer has not shown significant changes since platinum-taxol based-association treatment was introduced more than 30 years ago. The clinical history of most EOC patients continues to be characterized by late-stage presentation of the disease, initial good response to platinum-based treatment and subsequent development of recurrences with progression to chemotherapy resistance, making ovarian cancer currently largely incurable (Bowtell et al, 2015). Platinum-based therapy remains the standard of care and it has proved difficult to progress beyond it (Vaughan et al, 2011).

However, in the last years accumulating clinical evidences suggest that the combination between targeted therapies and standard chemotherapy could significantly improve the disease free survival and the quality of life of selected group of patients. For instance, the use of PARP inhibitors as maintenance therapy after platinum-based chemotherapy in patients, carrying germinal mutations in BRCA1/2 genes, showed excellent clinical results (Oza et al, 2015; Mirza et al, 2016). These findings open the way to consider combination therapies as a valid approach in EOC management to prevent and/or overcome the emergence of platinum resistance. The search for novel therapeutic targets is crucial to develop new treatment strategies. It has been already demonstrated that the employment of powerful techniques, such as synthetic lethal high-throughput RNAi screens, represents an interesting tool to identify new potential and highly selective therapeutic targets for combination strategies to overcome platinum drug resistance (Alvarez-Calderon et al, 2013). For this reason, we had used, in combination with chemotherapy, a loss-of function approach targeting 680 genes which belong to pathways already identified as linked to the onset of platinum resistance in ovarian cancer, namely DNA repair, apoptosis and p53 (Konstantinopoulos et al, 2008). This screening identified, among the other genes, SGK2, a member of the SGK family, consisting of three distinct but highly homologous genes, SGK1, SGK2 and SGK3. SGK2 is the most poorly studied member of the SGK family and very little information is available about its functional roles and its possible implications in cancer. However, there are accumulating evidences that link SGK1 and SGK3 to the regulation of cell growth, proliferation and survival, impacting also on cancer onset and/or progression (Sherk et al, 2008; Bruhn et al, 2010; Sommer et al, 2013; Bruhn et al, 2013; Gasser et al, 2014; Talarico et al, 2016), suggesting that these kinases could have a role

in cancer onset and/or progression. Indeed, SGKs were originally described as serine and threonine kinases responsible for regulating cellular ion channels and pumps (Palmada et al, 2003; Henke et al, 2003; Boehmer et al, 2003; Embark et al, 2004; Seebohm et al, 2008) and their role in cancer was therefore underestimated.

Here we report for the first time a possible role for SGK2 expression in Epithelial Ovarian Cancer response to platinum therapy through the regulation of autophagy, therefore opening a new possible therapeutic approach to overcome platinum resistance in EOC patients.

We showed that SGK2 silencing sensitized EOC cells to platinum treatment and, vice versa, that SGK2 overexpression conferred an increased resistance to platinum drug. Moreover, SGK2-overexpressing cells presented an increased *in vitro* and *in vivo* growth rate. Our data suggested that, in EOC cells, this SGK2 activity was not shared with the two other members of the family, SGK3 and SGK1, although, due to technical difficulties, we should better delineate the role of SGK1 platinum resistance in ovarian cancer. To address this point SGK1 gene knockout in EOC cells with the CRISPR technology is in progress and will likely resolve the remaining doubts on SGK1 role in platinum response.

We proved that SGK2 kinase activity was involved in EOC platinum sensitivity, exploring the effect of SGK2 dominant negative mutant in EOC cells treated with platinum. The efficacy of SGK2 kinase activity inhibition was confirmed also through the use of the SGK1/SGK2 kinase inhibitor, GSK650394, in association with platinum. In order to define the best way to administer this combined treatment, we tested different combination schedules between platinum and GSK650394. Data pointed out that the pre-treatment with GSK650394, which was maintained also during administration of platinum drug, markedly potentiated platinum efficacy. The response to this combination was observed only in EOC cells expressing detectable levels of SGK2 protein (i.e. MDAH, TOV21G, SKOV3), but not in EOC cells with undetectable SGK2 protein expression (i.e. COV318, TOV112D). Moreover, normal human ovarian epithelial cells (HuNoEOC), used as a control, did not express SGK2 and were insensitive to GSK650394 both when used as single agent and in combination with platinum. Even if GSK650394 is known as kinase inhibitor of both SGK1 and SKG2 (Sherk et al. 2008), its activity was found correlating only with SGK2 expression in the tested EOC cell lines.

SGK2-expressing cells, upon GSK650394 treatment, showed also a marked cytoplasmic accumulation of two morphologically distinct types of autophagic vacuoles: 1) typical double-membrane early autophagic vacuoles called autophagosomes, containing

morphologically intact cytosol and organelles, and 2) big late autophagic vacuoles with degenerated or partially degraded cytoplasmic material, where it was not possible to establish only from their morphologic evaluation if fusion with lysosome and degradation has already occurred. Alterations in autophagy pathways were suggested by the increased expression of p62 and LC3II/LC3I, two recognized markers of autophagic activity (Mizushima et al, 2010), in GSK650394-treated SGK2-expressing cells. Treatment with the autophagy inhibitor bafilomycin A1, known to prevent autophagosome-lysosome fusion (Yamamoto et al, 1998), highlighted accumulation of p62 and LC3II/LC3 similar to that observed with the treatment with the SGK inhibitor, leading us to consider the possibility SGK inhibition blocked autophagy process at the same step. Analysing the expression of additional critical markers of autophagy (LAMP2, Rab7 and cathepsin D), we confirmed that GSK650394 treatment induced autophagy blockade favouring the observed accumulation of autophagic vacuoles. In particular, the lysosomal membrane protein LAMP2 was not degraded via autophagy, like in its normal turnover (Eskelinen et al, 2002), and it accumulated in the membranes of the autophagic vacuoles formed upon GSK650394 treatment, indicating that they have probably already fused with lysosomes but degradation of autophagic material did not occur. This failure in degradation could be at least in part attributed to the lysosomal enzyme cathepsin D, which was found no longer processed in its active form, probably for the lack of the proper lysosomal acidic pH as the use of mRFP-GFP-LC3 construct highlighted, and accumulated as inactive precursors in GSK-treated cells. The pattern of expression of autophagy markers upon GSK650394 treatment was similar to the one observed with SGK2 silencing, suggesting that SGK2 inhibition (by GSK650394 or SGK2 shRNAs) could sensitize EOC cells to platinum drug via autophagy blockade. Accumulation of p62 and LC3II/LC3I, observed when SGK2 was inhibited, was indeed completely abolished by overexpressing SGK2; this suggests that an increase in p62 clearance, indicative of autophagy stimulation, occurred and it could represent a possible explanation for the increased resistance to platinum drug observed when SGK2 was overexpressed in EOC cells.

We showed that SGK2 interact directly with p62. The high number of p62 putative phosphorylation sites (Matsumoto et al, 2011) suggests that p62 could represent a possible target of SGK2 phosphorylation, through which SGK2 could modulate autophagy. We evaluated the phosphorylation level of p62 at S351, important in selective autophagy (Ichimura et al, 2013), with a proper antibody, but we did not observe any changes in EOC cells where SGK2 was inhibited. Further analysis is required to find evidences supporting

the hypothetical p62 phosphorylation by SGK2. Our results highlighted also another possible interesting link between SGK2 and autophagy. H^+ v-ATPase proton pump is responsible for the lysosomal appropriate acidic pH at which cathepsin D is processed in its active form and it is able to degrade autophagic material. We could hypothesize that cathepsin D may not be active when SGK2 was inhibited because the lysosomal H^+ v-ATPase could not function. It would be interesting to investigate if SGK2 could be implicated in the regulation of H+ v-ATPase, since the SGK family has been already known as regulator of several ATPases (Henke et al, 2003).

Autophagy has been recognised as one of the adaptive response rapidly upregulated by cancer cells to sustain cellular nutrient requests and energy production, to ensure cell survival also in stressful conditions like under the pressure of chemotherapy, although the implicated molecular mechanisms still remain not fully understood (Ren et al, 2010). Indeed, the role of autophagy in cancer is complex and may differ depending on tumour type or context. In our models, platinum treatment resulted in the stimulation of autophagy, confirmed by western blot with the characteristic LC3II/LC3I increase and p62 decrease in expression (Fig.21). We suggest that this autophagy induction is a possible survival mechanism exploited by tumour cells to overcome the stress induced by chemotherapy. Therefore blocking autophagy with SGK2 inhibition could result in the observed increase in platinum sensitivity of EOC. This possibility was in accordance with the fact that SGK2 inhibition either via silencing or via GSK treatment did not affect the viability of untreated EOC cells but strongly increased platinum induced cell death.

In the light of these findings, we will evaluate in future studies if the combination of SGK2 inhibition and platinum treatment could exert the same efficacy also *in vivo* experiments or in primary cell cultures from EOC patients. Overall we believe that targeting autophagy via SGK2 inhibition could represent a promising strategy to improve response to platinum in EOC patients, in the challenge to overcome platinum resistance.

Materials and methods

5.1 Cell lines

MDAH2774 (ATCC CRL-10303), OVCAR8 (NCI 60-0507712), SKOV3 (ATCC HTB-77), TOV112D (ATCC CRL-11731), TOV21G (ATCC CRL-11730), COV318 (ECACC 07071903) and OV90 (ATCC CRL-11732) cells were maintained in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 10% heat-inactivated FBS. Immortalized Human Ovarian Epithelial cells (HuNoEOC) (ABM T1074) were grown in Pigrow I medium supplemented with 10% heat inactivated FBS. 293FT cells (Invitrogen Inc.), used for lentivirus production, and 293T17 (ATCC CRL-11268), used for retroviral production, were grown in DMEM supplemented with 10% heat-inactivated FBS (Sigma-Aldrich Co.).

5.2 Reagents

Carboplatin (CBDCA) (TEVA Italia) was used for *in vitro* experiments. GSK650394 is a SGK1/SGK2 kinase inhibitor purchased from Tocris Bioscience (3572). Cycloheximide (CHX) was purchased from Sigma.

5.3 Loss-of-function screening

The used shRNA library was purchased from Sigma-Aldrich Co. Before to perform our screening analysis, we treated MDAH 2774 and SKOV3 cells with increasing doses of CBDCA for 16 hours to identify the appropriate dose to use in screening experiments, able to cause a 10-20% of cell mortality. The timeline of the functional genomic screening has been structured as follows: on day 1, 1000 MDAH cells/well and 700 SKOV3 cells/well were seeded in 96-well plates using a robotic liquid handling Hamilton's MICROLAB STARlet; on day 2, cells were transduced in duplicate with three shRNAs for each of the chosen 680 genes (2040 shRNAs used); 72 hours post transduction, in each round of the screening one 96-well plate was treated with CBDCA (MDAH cells were treated with CBDCA 140µg/ml and SKOV3 with CBDCA 250µg/ml) for 16 hours and the other represented the untreated control. Cell viability was evaluated 24 hours after the end of treatment using CellTiter 96 AQueous cell proliferation assay kit (Promega). The screening was performed twice on each cell line and the statistical analysis was conducted. Strictly Standarized Mean Difference (SSMD) was calculated on log2 (untreated/treated) for positive and negative controls: this value was always higher than 1.7 indicating appropriate

quality of the screenings performed (Zhang, 2007). We applied the Z-score normalization to account for plate-to-plate variation (Malo et al, 2006). shRNAs that presented synthetic lethality were ranked using a moderated t-test statistics (Smyth, 2004). To account for multiple testing problem, Benjamini and Hochberg's method (Benjamini and Hochberg, 1995) was applied to control the false discovery rate at a level of 5%. Three different sh target sequences were used for each gene, and in the final list of genes involved in synthetic lethality with platinum drug we only included those ones for which at least two shRNAs showed the desired effect. The results of the first screening were then validated in a second screening performed using five shRNAs for each gene in four different EOC cell lines: MDAH, SKOV3, TOV112D and OV90 cell lines. We took into account only the genes for which at least three out of five shRNAs displayed a significantly enhanced and/or reduced survival after platinum treatment respect to controls in at least three different cell lines.

5.4 Lentiviral production

Lentiviral particles were produced in 293FT cells, which were transfected, using calcium phosphate method, with the lentiviral based shRNA constructs and lentiviral system vectors pLP1, pLP2, and pVSV-G. The lentiviral particles were collected from the culture medium of these cells after 48h and 72h to transduce MDAH cells.

5.5 Cell viability assay

MDAH cells were seeded in 96-well culture plates (1000 cells/well) and after 24 hours transduced with lentiviral shRNAs. 72 hours after transduction, plates were treated or not with CBDCA for 16 hrs at the indicated concentrations. Cell viability was determined 24 hours after treatment using the CellTiter96 AQueous cell proliferation assay kit (Promega). In viability assays using platinum and GSK650394 combined treatment, EOC cells were seeded in 96-well culture plates ($4x10^3$ cells/well), the day after were treated with GSK650394 for 24 hours, and then with CBDCA at the indicated concentrations for 16 hours. Cell viability was determined 24 hours after the end of treatment using the CellTiter 96 AQueous cell proliferation assay kit (Promega).

5.6 Vectors, transfections and recombinant viruses

pDONR223 SGK1 (plasmid #23708 Addgene Inc. Cambridge, Massachusetts) and pDONR223 SGK2 (plasmid #23378 Addgene Inc. Cambridge, Massachusetts) were a gift

from William Hahn lab. Site directed mutagenesis was used to generate SGK2 dominant negative (SGK2 $^{T193A/S356A}$) mutant (primers are listed in Table 1) with commercial kit (QuikChange Site-Directed Mutagenesis Kit from Agilent). mRFP-GFP-LC3 was a gift from Tamotsu Yoshimori lab (plasmid # 21074 Addgene Inc. Cambridge, Massachusetts). Cells were transfected using FuGENE HD Transfection Reagent (Roche Applied Science, Indianapolis, Indiana), Lipofectamine 2000 (Invitrogen) (used for mRFP-GFP-LC3), or Oligofectamine (Invitrogen) (used for luciferase esiRNA and SGK1 esiRNA). Firefly luciferase esiRNA (EHUFLUC), used as control, and SGK1 esiRNAs (EHU035381), and pLKO for control and specific shRNAs (see Table 2) were purchased from Sigma-Aldrich Co.

5.7 Generation of stably SGK2-overexpressing cell population

Retroviral particles were produced in 293T17 cells transfected, using calcium phosphate method, with pLPC or pLPC SGK2 vectors and plasmids for packaging system pHIT456, pHIT60. The produced retroviral particles were collected from the culture medium of these cells after 48h and used to transduced OVCAR8 cells. Transduced cells were then selected in the presence of puromycin 0.75μg/ml.

5.8 Growth curve

OVCAR8 pLPC and pLPC SGK2 (30000 cells/well), TOV21G pLPC and pLPC SGK2 (40000 cells/well) and OVCAR8 pEGFP and pEGFP SGK1 cells (40000 cells/well) were plated in 6-well plates. Viable cells were counted daily in triplicate for 5-7 days, by trypan-blue dye exclusion method. pLPC and pLPC SGK2 OVCAR8 cells (40000 cells) were plated in 6-well plates (30000/well) and treated with GSK650394. Viable cells were counted daily in triplicate for 5 days, by trypan-blue dye exclusion method.

5.9 Immunofluorescence

For immunofluorescence, cells plated on coverslips and fixed in PBS 4%paraformaldehyde (PFA) or tissue sections were stained with primary antibodies, such as SGK2 (1:200), LAMP2 (1:200) (Santa Cruz Biotechnologies), p62 (1:200) (Abcam), and Ki67 (1:200) (Abcam). Propidium iodide (5μg/ml) was used for nuclear staining as reported (Berton et al. 2014). Stained cells were analysed using a confocal laser-scanning microscope (TSP8 Leica). Fluorescence intensity and protein co-localization were studied using the Volocity® software (PerkinElmer).

5.10 Electron microscopy

MDAH and TOV21G were plated on coverslips, treated with GSK 35µM for 36 hours, and fixed in 2,5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4. Post-fixation was conducted in 1% osmium tetroxide and 1,6% potassium ferricyanide for 2h at 4°C. The samples were dehydrated in alcohol and included in resin (Epon812). Ultrafine sections were obtained cutting the monolayers en face. The samples were stained with uranyl acetate and Reynold's lead citrate, and analysed with a transmission electron microscope (TEM, PhilipsEM400 at 100kV). >50 cells were evaluated for each experimental condition.

5.11 Preparation of Cell lysates, Immunoblotting, and Immunoprecipitation

Cell lysates were prepared using cold RIPA lysis buffer (150mM NaCl, 50mM Tris HCl [ph8], 1% Igepal, 0,5% sodium deoxycholate, 0,1% SDS*)* plus a protease inhibitor cocktail (Complete, Roche), 1 mM sodium orthovanadate, and 1 mM dithiothreitol as previously reported (Sonego et al. 2013). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad). For immunoblotting, equal concentrations of protein samples (60µg) were separated by 4–20% SDS-PAGE (Criterion precast gel; Bio- Rad) and transferred to nitrocellulose membranes (Hybond C; Amersham). Immunoprecipitations were performed using 1 mg of cell lysate in HNTG buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) plus 2µg of the indicated specific primary antibody and incubating overnight at 4°C. The immunocomplexes were precipitated by protein A and G agarose for another 2 hours at 4°C and separated on SDS-PAGE for western blot analysis. Immunoblotting were performed using the following primary antibodies: goat polyclonal anti-Vinculin (1:1000), mouse monoclonal LAMP2 (1:200) (Santa Cruz Biotechnology), rabbit monoclonal SGK2 (1:500), rabbit monoclonal SGK3 (1:500), rabbit monoclonal LC3B (1:1000), rabbit monoclonal Rab7 (Cell signalling), rabbit polyclonal SGK1 (1:500) (Millipore), mouse polyclonal SGK2 (used for IP), mouse monoclonal Cathepsin D (1:500) (Sigma Aldrich Co), rabbit monoclonal p62 (1:20000), mouse GRB2 (1:500) (Transduction Lab), mouse monoclonal GFP (1:500) (Roche). Antibodies were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for ECL detection (Biorad) or Alexa-conjugated secondary antibodies (Invitrogen) for Odyssey infrared detection (LI-COR Biosciences).

5.12 Protein stability

MDAH cells were treated or not with GSK for 16 hours (35μM) and then released in Cycloheximide (CHX, Sigma, 10μM) containing medium for 2, 3, 4, 6 or 8 hours. The expression of p62 was then evaluated by western blot.

5.13 qRT-PCR

Cells were treated as indicated and RNA was extracted at different time points using Trizol reagent (Invitrogen). Total RNA was quantified using the NanoDrop instrument (Thermo FisherScientific Inc., USA) and retro-transcribed using the AMV reverse transcriptase according to the manufacturer's instructions (Promega). Absolute quantification was evaluated by qRT-PCR, usingSYBR green dye-containing reaction buffer (Real SG Master Mix 5x, Experteam) and running the reactions in the MyiQ2 Two Color Real-time PCR Detection System (Biorad). Data normalization was performed using Pol2A and SdhA as housekeeping genes and relative expression was calculated using the mRNA concentration.

5.14 Tumour xenograft studies in nude mice

OVCAR8 pLPC and pLPC SGK2 xenografts were established by subcutaneously injection of $2x10^6$ cells in 0.2 ml PBS in flank (OVCAR8 pLPC on the right flank, OVCAR8 pLPC SGK2 on left flank) of three female athymic nude mice (Harlan Laboratories, 8 weeks-old), following validated procedures (Sonego et al, 2013). Tumour growth was followed for a month, tumour size was measured with a caliper. Tumour volume was calculated (0.5 x length x $width^2$). Animals were sacrificed after a month from the injection.

5.15 Statistical analyses

The computer software PRISM (version 4, GraphPad, Inc.) was used to make graphs in all statistical analyses. In all experiments, differences were considered significant when p was ≤0.05 and statistical significance was indicated with: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistical analyses included paired and un-paired t-tests as described in each figure.

Primer	Sequence 5'-3'
SGK2 T193A forward	GAGCCTGAAGACACCACATCCGCATTCTGTGGTACCCCTGAGTAC
SGK2 T193A reverse	GTACTCAGGGGTACCACAGAATGCGGATGTGGTGTCTTCAGGCTC
SGK2 S356A forward	CAAGTGCATTCCTGGGATTTGCTTATGCGCCAGAGGATGATGAC
SGK2 S356A reverse	GTCATCATCCTCTGGCGCATAAGCAAATCCCAGGAATGCACTTG

Table 1: Table 1 reports sequence data of the primers used for site-directed mutagenesis of SGK2 to obtain SGK2 dominant negative (SGK2 T193A/S356A) mutant.

NCBI Reference Sequence	shRNA	TRC number
NM_170693	SGK2 sh1	TRCN0000272924
	SGK2 sh2	TRCN0000272861
	SGK2 sh3	TRCN0000272863
NM_005627	SGK1 sh6	TRCN0000312569
	SGK1 sh7	TRCN0000040177
	SGK1 sh8	TRCN0000040175
	SGK1 sh9	TRCN0000009867
	SGK1 sh10	TRCN0000327644
NM_01357	SGK3 sh1	TRCN0000001517
	SGK3 sh2	TRCN0000001518
	SGK3 sh3	TRCN0000001519
	SGK3 sh4	TRCN0000001520
	SGK3 sh5	TRCN0000001521

Table 2: Table 2 reports the shRNAs targeting SGK1, SGK2 and SGK3 used in this work. NCBI Reference Sequence corresponds to GenBank accession number, while TRC indicates The RNA Consortium target

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