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MOLECULAR SIGNATURES OF PROGRESSION AND CHEMORESISTANCE IN EPITHELIAL OVARIAN CARCINOMA

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Molecular Signatures of Progression and Chemoresistance in Epithelial Ovarian Carcinoma

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ABSTRACT

Epithelial ovarian carcinoma (EOC) is a heterogeneous disease generally classified into five histopathological subtypes; low- and high-grade serous, endometrioid, clear cell and mucinous carcinomas. Although each subtype has distinct clinical and molecular characteristics, they are all treated with surgery and platinum/ taxane chemotherapy. Despite initial responsiveness a majority of patients relapse into platinum-resistant and disseminated disease. This, together with often late diagnosis, makes EOC the most lethal gynecological cancer. Dissemination is mainly abdominal, via exfoliated tumor cells in peritoneal ascitic fluid. The origin and phenotype of cells in malignant ascites is poorly understood. Tumor progression of carcinomas towards metastasis includes epithelial-to-mesenchymal-transition (EMT), where epithelial cells gain a mesenchymal morphology to facilitate invasion. Progression and chemoresistance have also been attributed to a small population of highly tumorigenic and chemoresistant cancer stem cells, or tumor-initiating cells (TICs). In addition, altered cellular energetics is a hallmark of cancer wherefore tumor-specific metabolic features are potential targets for overcoming chemoresistance.

In **Paper I** cell populations in malignant ascites were found to differ significantly with respect to protein expression levels of EMT and TIC markers. We identified two potential TIC profiles, highlighting a biological heterogeneity in ascitic tumor cell populations. The indicated presence of cancer-associated fibroblasts (CAFs) may further contribute to malignant properties. We found that CAF marker α-SMA expression was increased in clinical stage IV, compared to stage IIIC.

Paper II reveals that long-term repeated cisplatin treatment can select for and/or induce a multiresistant cell population with EMT and TIC features. Resistance could be linked to upregulation of VDAC and HK-II, which form an anti-apoptotic complex on mitochondria. Multiresistant cells were sensitive to the lactate/ pyruvate analogue 3-BP that dissociates this complex, and particularly sensitive to 3-BP when combined with cisplatin in low doses.

In **Paper III** expression of mitochondrial regulators PGC1α and TFAM was found to vary between EOC subtypes. For clear cell carcinomas (CCC) a profile consisting of low or undetectable levels of PGC1α, TFAM, ERα and low Ki-67 index was identified. This CCC profile, and also glycogen accumulation, was further linked to chemoresistance development *in vitro*.

In **Paper IV** we used ^{1}H NMR-based metabolomics to identify significant differences in the intracellular polar metabolome of parental and multiresistant EOC cell lines. Furthermore, we developed a tailored and reliable protocol for metabolic profiling of adherent cells, suitable for further characterization of metabolic alterations in EOC and other pathological conditions.

Taken together, this thesis identifies signatures of progression and chemoresistance in EOC and highlights the need for subtype-specific treatment.

LIST OF SCIENTIFIC PAPERS

I. **Wintzell M**, Hjerpe E, Åvall-Lundqvist E and Shoshan M. Protein markers of cancer-associated fibroblasts and tumor-initiating cells reveal subpopulations in freshly isolated ovarian cancer ascites *BMC Cancer* 2012;12:359

II. **Wintzell M**, Löfstedt L, Johansson J, Pedersen AB, Fuxe J and Shoshan M. Repeated cisplatin treatment can lead to a multiresistant tumor cell population with stem cell features and sensitivity to 3-bromopyruvate *Cancer Biol Ther* 2012;13:14,1454-1462

III. Gabrielson M, **Björklund M**, Carlson J and Shoshan M. Expression of mitochondrial regulators PGC1α and TFAM as putative markers of subtype and chemoresistance in epithelial ovarian carcinoma *PloS One* 2014; 9: e107109

IV. Engskog M[*], **Björklund M**[*], Haglöf J, Arvidsson T, Shoshan M and Pettersson C. Metabolic profiling of epithelial ovarian cancer cell lines: evaluation of harvesting protocols for profiling using NMR spectroscopy *Bioanalysis*- in press

[*]Equal contribution

RELATED PUBLICATION NOT INCLUDED IN THE THESIS

Björklund M, Roos J, Gogvadze V and Shoshan M. Resveratrol induces SIRT1- and energy-stress-independent inhibition of tumor cell regrowth after low-dose platinum treatment *Cancer Chemother Pharmacol* 2011;68:1459-67

CONTENTS

LIST OF ABBREVIATIONS

^{1}H	Proton
3-BP	3-bromopyruvate
α-SMA	α-smooth muscle actin
aa	Amino acid
ABC	ATP-binding cassette
ABCG2	ATP-binding cassette sub-family G member 2
AD	Adherent
ALDH	Aldehyde dehydrogenase
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ANT	Adenine nucleotide translocase
ARID1A	gene encoding AT-rich interactive domain-containing protein 1A (ARID1A)
ATP	Adenosine 5'-triphosphate
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
BRAF	gene encoding serine/threonine-protein kinase B-Raf
BRCA	Breast cancer susceptibility gene
CA125	Cancer antigen 125
CAF(s)	Cancer-associated fibroblast(s)
CCC	Clear cell carcinoma
CD	Cluster of differentiation molecule
CDH1	gene encoding E-cadherin
CIC(s)	Cortical inclusion cyst(s)
CK-8	Cytokeratine 8
CN	Chemonaïve
CR	Chemoresistant
CSC(s)	Cancer stem cells(s)
CTNNB1	gene encoding β-catenin
CV	Coefficient of variation
Da	Dalton
DNA	Deoxyribonucleic acid
E-cadherin	Epithelial cadherin
EC	Endometrioid carcinoma
EMT	Epithelial-to-mesenchymal transition
EOC	Epithelial ovarian carcinoma
EpCAM	Epithelial cell adhesion molecule
ERBB2	gene encoding receptor tyrosine kinase erbB-2 (HER2)
ERK 1/2	Extracellular-signal-regulated kinases 1/2
ERR	Estrogen-related receptors
ERα	Estrogen receptor α
ESR1	gene encoding ERα
EtBr	Etidium Bromide
FIGO	Federation Internationale de Gynecologie et d'Obstetrique
Glucose 6-P	Glucose 6-phosphate
GPC	Glycerophosphocholine
GSK3β	Glycogen synthase kinase 3β
HGSC	High-grade serous carcinoma
HK-II	Hexokinase II
HSMS	High resolution mass spectrometry
IC_{50}	Half-maximal inhibitory concentration
IL	Interleukin

ip	Intraperitoneal
iv	Intravenous
kDa	kilo Dalton
KRAS	gene encoding the GTPase KRas
LGSC	Low-grade serous carcinoma
MAPK	Mitogen-activated protein kinase
MC	Mucinous carcinoma
MeOH	Methanol
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MT-CO2	Cytochrome c oxidase subunit 2
mtDNA	Mitochondrial DNA
MTT	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide
MyD88	Myeloid differentiation factor 88
N-cadherin	Neural cadherin
NAD	Non-adherent
nDNA	Nuclear DNA
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NORDCAN	WHO Nordic cancer database
NRF	Nuclear respiratory factor
Oct-4	Octamer-binding transcription factor 4
OPLS-DA	Orthogonal projection to latent structures discriminant analysis
OSE	Ovarian surface epithelium
OxPhos	Oxidative phosphorylation
p53	Tumor protein p53
PCA	Principal component analysis
PDGF	Platelet-derived growth factor
PDGFRβ	Platelet-derived growth factor receptor β
PFI	Platinum-free interval
PFS	Progression-free survival
PGC1α	Peroxisome proliferator-activated receptor γ co-activator 1α
PI3K	Phosphatidylinositide 3-kinase
PIK3CA	gene encoding p110α, a catalytic subunit of class I PI3Ks
PPARGC1A	gene encoding PGC1α
PR	Progesterone receptor
PTEN	gene encoding phosphatase and tensin homolog (PTEN)
QC	Quality control
R^2	Linear regression coefficient
RB	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCM	Stem cell medium
SP	Side population
SRB	Sulforhodamine B assay
STAT3	Signal transducer and activator of transcription 3
STIC	Serous tubal intraepithelial carcinoma
SUMO	Small ubiquitin-like modifier
SUS-plot	Shared and unique structure plot
TCA	Tricarboxylic acid
TFAM	Mitochondrial transcription factor A

TGFβ	Transforming growth factor-β
TIC(s)	Tumor-initiating cell(s)
TLR4	Toll-like receptor 4
TP53	gene encoding p53
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZEB	Zinc finger E-box-binding homeobox

1 INTRODUCTION

Most cells in the metazoan body carry a complete genome, to retain the ability to grow and divide during lifespan of the organism. Retained proliferation and programed cell death allows maintenance, including wound repair and replacement of cells, important for organism survival. Although tissue homeostasis is tightly regulated, various genetic and environmental mechanisms can alter the structure and content of the genome. As a result, mutated genes cause abnormal cell phenotypes, often with alterations in cellular growth programs, that give rise to large populations of cells, i.e. tumors, that no longer follow the rules for tissue homeostasis. Tumors can further be divided into two broad categories depending on their ability to invade nearby tissue; benign tumors that grow locally and do not invade, and malignant tumors that invade and cause metastatic tumors in distant organs. The majority of tumors arising in humans are benign and harmless to their host, except for rare cases where the tumor mass put pressure on vital organs. Some, but not all benign tumors, further develops into dysplasias with abnormal cells and major effects on the overall tissue architecture. Additional degrees of abnormality are found in neoplasias that can be pre-malignant or malignant. Malignant neoplasias are collectively called cancer[1], that caused approximately 8.2 million deaths worldwide in 2012 (Ferlay et al., 2014).

Cancer is not one, but several diseases that can be broadly divided into subgroups based on the cellular origin of the tumor. The great majority of malignant tumors, carcinomas, arise in epithelial tissue. Non-epithelial cancers include sarcomas derived from mesenchymal cells in various connective tissues, hematopoietic malignancies[2] in blood-forming tissues, and neuroectodermal tumors originating from cells in the central and peripheral nervous system. Irrespective of tumor origin, most malignant tumors acquire the same set of functional capabilities during tumor progression, i.e. the development from normal to malignant. These common characteristics, "the hallmarks of cancer", includes the ability to; *sustain proliferative signaling*, *evade growth suppressors*, *resist cell death*, *induce angiogenesis*, *enable replicative immortality* and *activate invasion and metastasis*, *avoid immune destruction* and *deregulate cellular energetics* (Hanahan and Weinberg, 2000, 2011). In addition, two consequential characteristics of neoplasia, *genomic instability* and *tumor-promoting inflammation*, assist acquisition of the other hallmarks (Hanahan and Weinberg, 2011). Knowledge of the molecular mechanisms behind each hallmark leads to increased insights in tumor progression and makes it possible to identify new therapeutical targets.

Although some types of cancer are curable, especially if detected in an early stage, a major clinical challenge is resistance to treatment. If treatment fails to kill all cancer cells, the remaining cell population can give rise to new, resistant tumors that eventually cause death of the patient. This is the case for ovarian carcinomas, where development of chemoresistance in

[1] Sometimes the term cancer is used more loosely to include all types of abnormal growth

[2] Includes leukemias with malignant cells that freely moves through the circulation as dispersed, single-cells instead of forming classical tumors.

addition to late-stage diagnosis make it the most lethal of gynecological cancer in the Western world (Lengyel, 2010). Unfortunately, the survival rates have improved very little in the last 30 years (Engel et al., 2002; Vaughan et al., 2011). Therefore, the work presented in this thesis aims to study tumor progression and chemoresistance in ovarian carcinomas, in order to provide insights of underlying molecular events that eventually can lead to development of new treatment strategies.

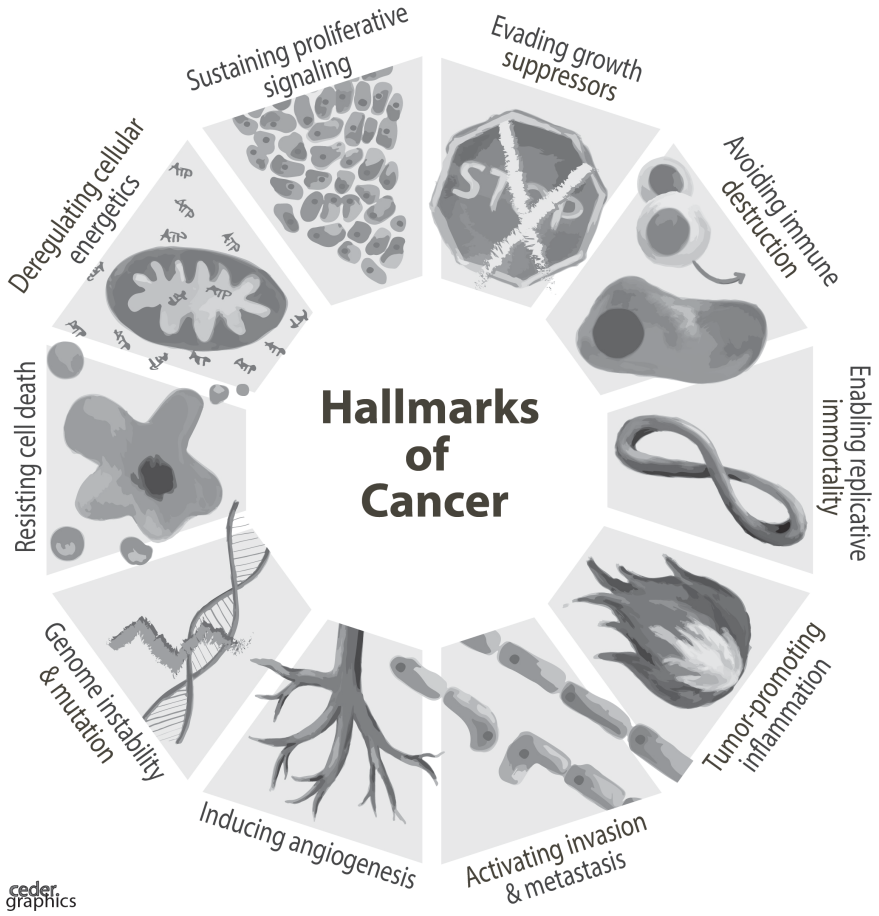

Figure 1. The hallmarks of cancer. Overview of common characteristics of malignant neoplasias, including two emerging hallmarks, avoiding immune destruction and deregulating cellular energetics, and two enabling characteristics, genome instability and mutation and tumor-promoting inflammation. Adapted from Hanahan, D., Weinberg, R.A., 2011. *Hallmarks of cancer: the next generation*. Cell 144, 646–674. Reprinted with permission from Elsevier.

1.1 EPITHELIAL OVARIAN CARCINOMA

1.1.1 Epidemiology

Epithelial ovarian carcinoma (EOC) is the seventh most common malignancy in women, with an incidence of 239 000 cases, causing 152 000 deaths worldwide in 2012 (Ferlay et al., 2014). It affects women of all ages, although uncommon before age of 30, with a peak in incidence in women 65-74 years old. Incidence rates are highest in Northern Europe (Permuth-Wey and Sellers, 2009) with approximately 10 cases per 100 000 women in the Nordic countries (Engholm et al. NORDCAN database, version 6.1, 2014). The incidence in Sweden has been decreasing over the past decade and is now down to approximately 700 new cases per year[3], mainly due to protective effects of oral contraception and reduced usage of hormone replacement in menopausal women (Collaborative Group on Epidemiological Studies of Ovarian Cancer et al., 2008; Mørch et al., 2009). The mortality has also decreased somewhat, but not at the same rate as the incidence (Figure 2).

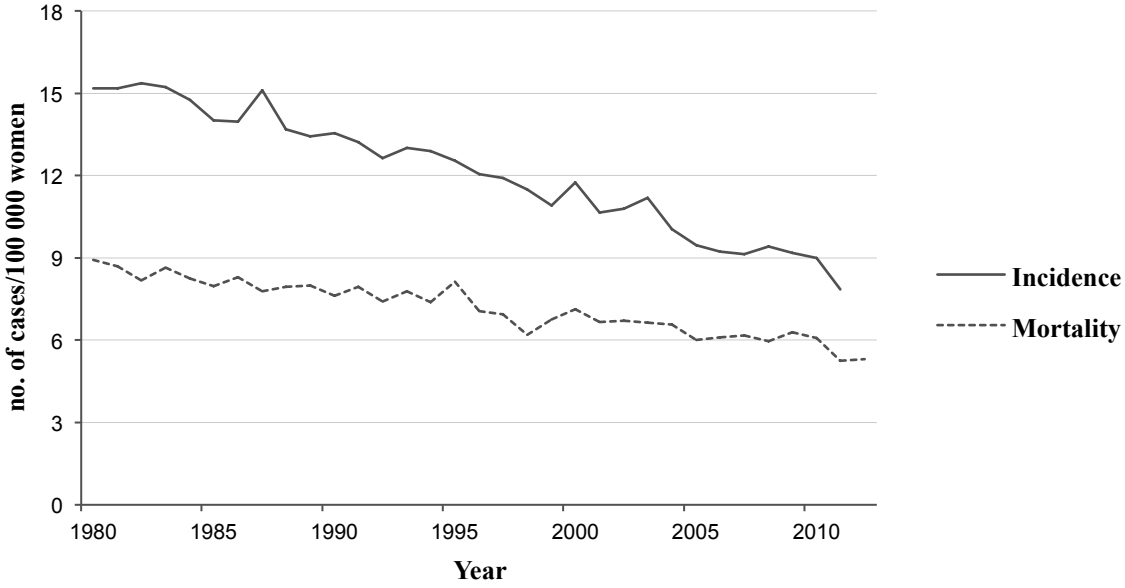

Figure 2. Ovarian cancer incidence and mortality. Incidence and mortality for ovarian cancer per 100 000 women in Sweden 1980-2011. All ages are included, age-standardized rate according to world standard population. Source; NORDCAN database, available from http://www.ancr.nu, accessed on 14/10/2014.

Approximately 90% of the malignant ovarian tumors are of epithelial origin but international statistics sometimes include rare cases of tumors arising in ovarian stroma or germ cells.

In this thesis, the term EOC also includes tumor material from patients diagnosed with carcinomas originating from the fallopian tube or peritoneum, since these are prognostically and clinicopathologically indistinguishable from those originating from the ovary.

[3] Including patients diagnosed with fallopian tube carcinomas.

1.1.2 Pathogenesis and risk factors

EOC tumors are classified into different subtypes based on morphological appearance according to WHO histopathological standards, including serous, endometrioid, clear cell, mucinous and undifferentiated tumors (International Agency for Research on Cancer and World Health Organization, 2014). Additional classification, based on molecular genetics, divides EOC into two broad categories designated types I and II (Vang et al., 2009; Kurman and Shih, 2011). Type I tumors, that are relatively stable genetically, include low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas. Type II consists of high-grade serous, high-grade endometrioid and undifferentiated/mixed tumors, all genetically highly unstable.

In general, the use of oral contraceptives (Purdie et al., 2003), parity and breastfeeding (Hunn and Rodriguez, 2012) decrease the risk of developing any subtype of EOC, while the use of menopausal estrogen treatment increases the risk (Mørch et al., 2009). Genetic factors, like *BRCA1* or *BRCA2* mutations and Lynch-syndrome[4] increase the risk (Gayther, 2012) and carriers are recommended prophylactic surgical removal of ovaries and fallopian tubes. Other risk factor are subtype specific and are further described for each subtype.

The origin and pathogenesis of EOC have confused investigators for decades, although ovulation for long has been considered an important factor for EOC development. Ovulation causes damage to the ovarian surface and activates local tissue repair in a cyclic process that increase the risk for malignant transformation. A conventional theory suggests that malignant neoplasms originate from the ovarian surface epithelium (OSE) and/or cortical inclusion cysts (CICs), which are invaginations of epithelium in the ovarian stoma. However, recent studies strongly suggests that tumors develop in Müllerian duct-derived epithelia of the fallopian tube and endometrium and involve the ovary secondarily (Kurman and Shih, 2011).

Serous tubal intraepithelial carcinomas (STICs) that closely resembles ovarian high-grade serous carcinomas were first described in women with a genetic predisposition to ovarian cancer (Piek et al., 2001). The model suggests that implantation of malignant cells from the fimbriated distal fallopian tube to the ovary give rise to high-grade serous tumors. 61% of pelvic high-grade serous carcinomas (including those of the ovary, fallopian tube and primary peritoneal) have been reported to involve STICs (Przybycin et al., 2010). Another possibility is that benign tubal epithelial cells implant on the, by ovulation disrupted, ovarian surface to form CICs (Kurman and Shih, 2010) that later progress to serous carcinomas. In addition, endometriotic lesions are precursors of the endometrioid and clear cell carcinomas (Nezhat et al., 2008). These lesions derives from the endometrium (lining of the uterus) that establish outside the uterine cavity as a result of retrograde menstruation, a condition known as endometriosis (Bulun, 2009).

[4] Genetic disorder with defects in DNA mismatch repair genes, which leads to microsatellite instability.

1.1.2.1 Serous carcinomas

Serous carcinomas account for 75% of EOC (Köbel et al., 2010) and are further divided into low- and high-grade tumors, with distinct clinical and molecular features. Almost all serous tumors are high-grade serous carcinomas (HGSC), hence this is the most common EOC diagnosis.

HGSC may arise in the fallopian tube epithelium, either directly from a carcinoma in the fallopian tube or from tubal epithelium implanted in the ovary forming a CICs (Nik et al., 2014). Mutation in the *TP53* gene as well as post-translational induced dysfunction of p53 is a hallmark for HGSC, found in close to all cases (Ahmed et al., 2010; Cancer Genome Atlas Research Network, 2011). Another common features in this subtype is BRCA pathway mutations, found in 51% cases in a cohort of 489 HGSC cases (Cancer Genome Atlas Research Network, 2011). This is in line with women carrying BRCA-mutations have increased risk to develop EOC, particular HGSC (Lakhani et al., 2004). Other signaling pathways that are altered are retinoblastoma (RB), phosphatidylinosidide 3-kinases (PI3K)/Akt and Notch (Cancer Genome Atlas Research Network, 2011). HGSC are thus a genetically heterogeneous disease with high chromosomal instability (Bowtell, 2010).

Low-grade serous carcinomas (LGSC) represent 3-5% of all EOC cases (Köbel et al., 2010). *TP*53 mutations are uncommon and they have lower levels of chromosomal instability than HGSC (Vang et al., 2009; Lim and Oliva, 2013). Common mutations are those of *KRAS*, *BRAF* and *ERBB2* oncogenes (Singer et al., 2003; Vang et al., 2009), all upstream of the mitogen-activated protein kinase (MAPK), resulting in activation of MAPK signaling and proliferation (Hsu et al., 2004). Two thirds of LGSC harbor these mutations that can also be found in serous borderline tumors, i.e. tumors with semi-malignant potential (Bonome et al., 2005). They seem to share common molecular features and tumor specimens of LGSC often contain areas with borderline malignant potential (Malpica et al., 2004), suggesting that borderline serous tumors might transform into LGSC. Unlike in HGSC, a high body mass index (BMI) increases the risk of LGSC and serous borderline tumors (Olsen et al., 2013).

1.1.2.2 Endometrioid carcinomas

Approximately 10% of EOCs are endometrioid carcinomas (EC) (Köbel et al., 2010). They develop slowly from transformation of endometriotic lesions, hence endometriosis increases the risk of developing EC (McCluggage et al., 2002; Nezhat et al., 2008; Pearce et al., 2012). Subtype-specific risks are Lynch syndrome as well as high BMI (Olsen et al., 2013; Chui et al., 2014). Frequent mutations are those in *CTNNB1* (Palacios and Gamallo, 1998), *PTEN* (Obata et al., 1998), *KRAS* (Cuatrecasas et al., 1997), *ARID1A* and in the gene coding for the catalytic subunit of class I PI3Ks, *PIK3CA* (Wiegand et al., 2010; Yamamoto et al., 2011; Samartzis et al., 2013).

1.1.2.3 Clear cell carcinomas

Clear cell carcinomas (CCC) account for 10-12% of all EOC cases (Köbel et al., 2010). For unknown reasons, this subtype has a higher prevalence in Japan, where 15-25% of EOCs are CCC (Yoshikawa et al., 2000). Similar to EC, the majority of CCC originate from endometriotic lesions (Nezhat et al., 2008). Lynch syndrome and endometriosis increase the risk of developing CCC (McCluggage et al., 2002; Pearce et al., 2012; Chui et al., 2014). Clear cell tumors are normally p53 wild-type and have low chromosomal instability. Mutations in *ARID1A* and *PIK3CA* genes are reported in 40-50% of cases (Anglesio et al., 2011; Yamamoto et al., 2011; Ayhan et al., 2012) and low expression of PTEN is a common feature (Hashiguchi et al., 2006).

1.1.2.4 Mucinous carcinomas

Mucinous carcinomas (MC) that arises slowly from mucinous borderline lesions accounts for 3% of EOCs (Köbel et al., 2010). The cell of origin is still unknown. *KRAS* mutations and overexpression of *ERBB2* is common (Gemignani et al., 2003; Gilks, 2010; Anglesio et al., 2013). Subtype-specific risk factors include high BMI and smoking (Collaborative Group on Epidemiological Studies of Ovarian Cancer et al., 2012; Olsen et al., 2013).

1.1.3 Clinical staging and disseminated disease

Staging of ovarian cancer is done according to FIGO guidelines (Prat and FIGO Committee on Gynecologic Oncology, 2014), found in Table 1. The 5-year disease specific survival decreases with higher stage, from more than 90% in stage I to less than 20% in stage IV (Heintz et al., 2006). Unfortunately, the majority of cases are diagnosed at a late stage (stage III or IV), when dissemination is already at hand. The late stage discovery is due to non-specific symptoms, such as abdominal swelling, urinary and bowel dysfunction, loss of appetite, pain and fatigue (Bankhead et al., 2005). Possibly due to slower progression, the non-high-grade serous subtypes are detected in early stage (stage I-II) (Köbel et al., 2010) and thus have better prognosis, except for CCCs (stage II-IV) that have poor prognosis due to chemoresistance (du Bois et al., 2003; Takano et al., 2006; Winter et al., 2007).

Dissemination is mainly abdominal, either by direct extension from tumors to neighboring organs or via exfoliated tumors in peritoneal fluid, ascites (Lengyel, 2010). Malignant ascites, frequently associated with the HGSC subtype, cause implantation metastasis in omentum, on liver and intestinal surfaces. Invasion in the peritoneum and adhesion to the peritoneal mesothelium is facilitated by CD44 (Gardner et al., 1996), β-integrins (Wagner et al., 2011) and cancer antigen 125 (CA125) (Gubbels et al., 2006) expressed on the ovarian cancer cell surface. Exfoliated tumor cells can reach the pleura through the diaphragm, causing pleural effusions. Dissemination through hematologic vasculature is rare while involvement of pelvic and/or para-aortic lymph nodes are seen in many cases (Morice et al., 2003). Distant metastasis to the lung, liver and bone are primarily seen in patients living with EOC for several years.

Table 1. 2013 FIGO staging classification for cancer of the ovary, fallopian tube and peritoneum.	
Stage I	Tumor confined to ovaries or fallopian tube(s)[a]
IA	Tumor limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IB	Tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IC	Tumor limited to one or both ovaries or fallopian tube(s) with any of the following:
IC1	Surgical spill intraoperatively
IC2	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
IC3	Malignant cells in the ascites or peritoneal washings
Stage II	Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer[b]
IIA	Extension and/or implants on uterus and/or fallopian tubes and/or ovaries
IIB	Extension to other pelvic intra-peritoneal tissues
Stage III	Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes
IIIA	Positive retroperitoneal lymph nodes and/or metastasis to the retroperitoneal lymph nodes
IIIA1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven)
IIIA1(i)	Metastasis ≤ 10 mm in greatest dimension
IIIA1(ii)	Metastasis > 10 mm in greatest dimension
IIIA2	Microscopic extra-pelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes
IIIB	Macroscopic peritoneal metastasis beyond the pelvis ≤ 2 cm in greatest dimension, with or without metastasis to the retro-peritoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
IIIC	Macroscopic peritoneal metastasis beyond the pelvis > 2 cm in greatest dimension, with or without metastasis to the retro-peritoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
Stage IV	Distant metastasis excluding peritoneal metastases
IVA	Pleural effusion with positive cytology
IVB	Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)[c]

[a] It is not possible to have stage I peritoneal cancer

[b] Dense adhesions with histologically proven tumor cells justify upgrading apparent stage I tumors to stage II

[c] Extra-abdominal metastases include transmural bowel infiltration and umbilical deposit

The pathophysiology of malignant ascites is not fully understood but factors that contribute to the onset of ascites include lymphatic obstruction by tumor cells, excess vascular permeability and other tumor-specific effects such as excess metalloproteinase production (Kipps et al., 2013). Ascites is thus a dynamic reservoir containing cytokines, chemokines and growth factors that can affect tumor growth and progression through different mechanism (Mills et al., 1988; Puiffe et al., 2007; Matte et al., 2012). Even though it is evident that EOC creates a highly immunosuppressive environment in the peritoneal cavity (Wertel et al., 2011), high levels of inflammatory cytokines is found in malignant ascites. Interleukin (IL) 6 and -8, were found at higher levels in ascites than in serum of EOC patients, and increased levels correlated with a poor initial response to paclitaxel treatment (Penson et al., 2000). IL-6 can promote ovarian tumor growth, migration and invasion (Obata et al., 1997), and correlates with chemoresistance (Wang et al., 2010; Cohen et al., 2013). In accordance with these findings, high levels of IL-6 in ovarian cancer ascites is associated with shorter progression-free survival (Lane et al., 2011). Another factor found in high levels in malignant EOC ascites is vascular endothelial growth factor (VEGF) that stimulates the production of ascites via increased vascular permeability (Herr et al., 2012). Importantly, VEGF also enhances tumor growth and elevated VEGF ascites levels correlates with poor clinical outcome (Rudlowski et al., 2006).

The origin and phenotype of cells in malignant ascites is poorly understood. Tumor cells can be present as single cells, loose aggregates or compact spheres, and combinations of the different growth-patterns are common (Hudson et al., 2008). Their origin is possibly both primary and metastatic tumors. Other non-tumoral cell types, are also present in ascites that thus contains a complex heterogeneous mixture of cells (Kipps et al., 2013). Further studies are needed to better understand how the cellular and soluble components in malignant ascites contribute to advance-stage EOC.

1.1.4 Treatment

Standard treatment is debulking surgery, including removal of the ovaries, uterus, fallopian tubes, omentum and all visible tumor lesions. Surgery is often followed by chemotherapy, typically platinum-based chemotherapy together with paclitaxel[5]. For selected patients with stage IIIC-IV EOC neoadjuvant chemotherapy followed by interval debulking is the preferred strategy, especially in the case of comorbidities or extensive non-removable tumor(s). Even though Sweden relative other European countries have the highest 5-year age-standardized relative survival, 44.1% (De Angelis et al., 2014), there is an urgent need for new treatment strategies. One promising agent is bevacizumab, a monoclonal antibody targeting VEGF. Recently two randomized phase III trials, GOG 218 and ICON 7, reported significant

[5] Combinations of either cisplatin or carboplatin with paclitaxel are equally effective in terms of progression-free survival and overall survival (du Bois et al., 2003). Carboplatin is less toxic, and is therefore considered a better option (Ozols et al., 2003).

prolonged progression-free survival (PFS) when bevacizumab was added to standard chemotherapy and continued as maintenance therapy (Burger et al., 2011; Perren et al., 2011). In Sweden, the coming and revised National guideline for epithelial ovarian cancer recommends the addition of bevacizumab for the high-risk group with advanced EOC. Several ongoing randomized trials evaluate targeted therapies directed towards specific subgroups.

Chemotherapy can also be administrated via intraperitoneal (ip) infusions, which facilitates higher local drug concentrations at sites of the disease. Compared to intravenous (iv) administration of cisplatin-paclitaxel, ip treatment with the same combination resulted in longer median survival but did also cause high toxicity in patients (Armstrong et al., 2006; Jaaback et al., 2011). Due to the toxicity and lack of consistent results from trials, ip administration is not widely used in Sweden or other European countries.

Patients usually respond well to initial treatment but approximately 75% of EOC patients will relapse into incurable disease within three years (Gonzalez-Martin, 2013). Recurrence treatment is based on the time interval from the last course of platinum-based chemotherapy until relapse, called platinum-free interval (PFI)[6]. A PFI of at least six months is considered a platinum sensitive recurrence and patients typically respond also to second line platinum-based treatment. Relapses within six months are considered platinum resistant and additional chemotherapy treatment is palliative. Several different agents can be used as mono-treatment including metronomic cyclophosphamide, weekly paclitaxel, pegylated liposomal doxorubicin, oral etoposide and topotecan but the response rates are low, with 10-15% patients responding (Gonzalez-Martin, 2013). Bevacizumab has also been evaluated in platinum-sensitive and platinum-resistant recurrent EOC, with positive results. When added to carboplatin-gemcitabine bevacizumab significantly prolonged PFS compared to carboplatin-gemcitabine alone (Aghajanian et al., 2012). In addition, bevacizumab plus any standard treatment for platinum-resistant disease increased the PFS in recurrent platinum-resistant EOC, recently reported in the AURELIA trial (Pujade-Lauraine et al., 2014).

The efficacy of chemotherapy is routinely monitored via CA125 levels in serum. Elevated CA125 levels is seen in approximately 80% of advanced EOC (Díaz-Padilla et al., 2012) and is therefore used as a tool to detect EOC as well as recurrent disease, even though elevated levels can be caused also by benign conditions. CA125 levels post-treatment can predict treatment outcome, where a rapid decrease implicates a good treatment response while a slow decrease or stable CA125 indicates poor outcome with chemoresistance (Gadducci et al., 2004; Rocconi et al., 2009). Except for CA125, very few EOC biomarkers have been established and there is currently no routine marker to distinguish the different subtypes or for treatment guidance.

[6] PFI are clinical guidelines, thus some patients with a PFI < 6 months can still be sensitive for platinum and those with a PFI ≥ 6 months can be platinum-resistant.

1.2 TUMOR PROGRESSION

1.2.1 Epithelial-to-mesenchymal transition

Malignant tumors have the potential to form metastases at distant sites in the body and an important step in tumor progression is the process when tumor cells acquire the ability to invade nearby tissue. In most epithelial tissues, a thin layer of epithelia is separated from the underlying stroma by a basement membrane. Tumors formed in the epithelium are considered benign as long as they grow only on the epithelial side, while carcinomas that breach the basement membrane have gained motility and invasiveness. To invade, epithelial cells change their morphology and gene expression to a morphology and transcriptional program characteristic for mesenchymal cells, a process known as epithelial-to-mesenchymal transition (EMT). EMT was first discovered as a feature of embryogenesis (Bolender and Markwald, 1979) and is, together with the reverse process of mesenchymal-to-epithelial transition (MET), crucial for the formation of organs and tissues during embryonic development. In the adult body, these transitions normally occur in the process of wound healing (Thiery et al., 2009).

EMT is characterized by the loss of the adherens junction protein epithelial cadherin (E-cadherin), that is cleaved at the plasma membrane and further degraded (Yilmaz and Christofori, 2009). The loss of epithelial junction results in reorganization of the actin cytoskeleton and loss of the apical-basal polarity (Huang et al., 2012). E-cadherin is often replaced by the mesenchymal adherens junction protein neural cadherin (N-cadherin), forming weaker cell-cell bonds that favor cell motility (Thiery, 2002). Downregulation of epithelial intermediate filaments, cytokeratins, and upregulation of the mesenchymal counterpart vimentin is another event during EMT (Savagner, 2010). Cells often upregulate their synthesis and release of matrix metalloproteinase (MMP) 2 and -9 to degrade components of basement membrane to further support invasion (Brinckerhoff and Matrisian, 2002). Transcriptional regulation of EMT includes repression of the gene encoding E-cadherin, *CDH1*, via several transcription factors that binds directly at the promoter such as Slug, Snail, ZEB1 and -2 or indirectly via Twist (Smit et al., 2009; Fuxe et al., 2010). Slug (Guo et al., 2012), Snail and Twist (Mani et al., 2008) have been shown to link EMT to the concept of cancer stem cells (See section 1.2.3).

The tumor-associated stroma actively contributes to tumor progression, and the stromal involvement occurs early in epithelial transformation. Tumor cell secretion of growth factors, such as VEGF, transforming growth factor-β (TGFβ) and platelet-derived growth factor (PDGF) activates the stroma. Activated stromal cells, including fibroblasts, inflammatory cells and endothelial cells stimulates tumor proliferation and invasion (Mueller and Fusenig, 2004).

Tumor cells that have undergone EMT are thus more mesenchymal and can penetrate the basement membrane and enter the vasculature. Metastases formed in distant organs often revert to a more epithelial phenotype, via MET (Thiery, 2002), implicating a epithelial-

mesenchymal plasticity. It is a dynamic process, and it is evident that tumor cells can go through a partial EMT (Klymkowsky and Savagner, 2009).There is also a heterogeneity within tumors, as demonstrated in colon carcinoma where both primary tumor and its corresponding liver metastasis had mixed populations of epithelial-mesenchymal phenotype with epithelial characteristics in the center and mesenchymal characteristics at the invasive front (Brabletz et al., 2005).

1.2.2 EMT in EOC

In the normal ovary, E-cadherin is expressed in CICs while the ovarian surface epithelium lacks this expression. It was previously believed that early steps in tumor formation included differentiation of OSE to take on characteristics of Müllerian duct-derived epithelia. Such differentiation included a MET process since benign adenomas and borderline tumors express E-cadherin (Sundfeldt et al., 1997; Davies et al., 1998; Ahmed et al., 2007). However, it is now suggested that EOC originates from implantations of fallopian tube epithelium and endometrium, both Müllerian duct-derived (Kurman and Shih, 2011). Fallopian tube epithelium can also give rise to CICs (Nik et al., 2014) which may explain their altered cadherin profile compared to the ovarian surface epithelium.

Some studies report that E-cadherin expression is reduced in advanced primary tumors compared to benign and borderline tumors (Ahmed et al., 2007; Hudson et al., 2008), while Davidson et al (2000) showed that E-cadherin is significantly increased in metastatic ovarian lesions and ascites compared to the respective primary tumors One can suggest that EMT/MET occurs several times during tumor progression, resulting in heterogeneous tumors with both epithelial and mesenchymal characteristics. One confounding factor for these type of studies is the difficulty to define the primary tumor in advanced-stage disease. In addition, the exfoliated cells in malignant ascites may arise from budding of single cells and/or multicellular aggregates from primary and/or metastatic tumors into the peritoneal fluid, which represent a unique route for dissemination. It is thus possible that the E-cadherin expression in such aggregates may be advantageous for overcoming anoikis.

Further studies are needed to better understand the epithelial-mesenchymal plasticity in EOC and how it is related to patient survival. Verhaak et al (2013) studied ovarian HGSC, divided into four gene expression signatures; differentiated, immunoreactive, mesenchymal, and proliferative, previously described in (Cancer Genome Atlas Research Network, 2011). In addition, they also classified survival in good and poor expression profiles and combined the different signature-strategies to identify patients with better or worse prognosis. Differentiated and immunoreactive signatures had a more favorable prognosis while the mesenchymal signature was found in tumors with lower median survival and increased platinum resistance (Cancer Genome Atlas Research Network, 2011; Verhaak et al., 2013).

The tumor-associated stroma and its role in EOC progression is an important topic that has gained attention in recent years. Several studies now point out cancer-associated fibroblasts (CAFs) as key players in EOC progression. For instance, CAFs are associated with advanced-

stage disease (Zhang et al., 2011) and promotes EOC growth and metastasis *in vivo* (Cai et al., 2011).

1.2.3 Tumor-initiating cells

So-called cancer stem cells (CSCs) are hypothesized to underlie tumor progression as well as therapy resistance. The theory claims that a small fraction of tumor cells, CSCs, is responsible for initiation of disease and relapses in addition to cause tumor heterogeneity. Such subpopulations have been described for several cancer types, including ovarian, but to what extent these findings are clinically significant is still not clear.

CSCs were first described by Lapidot et al (1994), who isolated a highly tumorigenic stem cell population in acute myeloid leukemia that could reinitiate the disease in mice. However, in the majority of cancers where CSCs have been identified, the origin of this subpopulation is not necessarily a normal stem cell, and in most of the cases the origin is unknown (Dean et al., 2005). The term tumor-initiating cells (TICs) to describe this subpopulation is increasingly used[7], in particular as TICs are highly chemoresistant and are believed to initiate relapse. Similar to normal stem cells TICs are capable of asymmetric self-renewal, i.e., also give rise to more differentiated cancer cells (Alison et al., 2012). The differentiated cells are more proliferative and will quickly make up the major tumor burden and respond to treatment with chemo- and radiotherapy. An alternative scenario is that TICs develop due to external stimuli, such as therapy (illustrated in Figure 3). Regardless of origin, if it is possible to identify and isolate a cell population that survives initial treatment, then targeting of these cells would most likely improve treatment outcome.

It is generally agreed that TICs have certain characteristics *ex vivo*;

- Increased tumorigenicity when injected in xenograft models, where TICs can establish tumors from very few cells compared to non-TICs/mixed populations of same origin.

- Unlimited self-renewal, i.e. that TICs can divide numerous times while still maintaining an undifferentiated state.

- Pluripotency in terms of giving rise to new TICs and to differentiated cancer cells.

- Spontaneous formation of spheres in serum-free stem cell medium (SCM) when cultured *in vitro*.

- Resistance to chemo- and radiotherapy.

[7] In addition to CSCs and TICs, this subpopulation of cells are in the literature sometimes referred to as cancer-initiating cells, therapy-resistant cells or as being ”stem cell-like”.

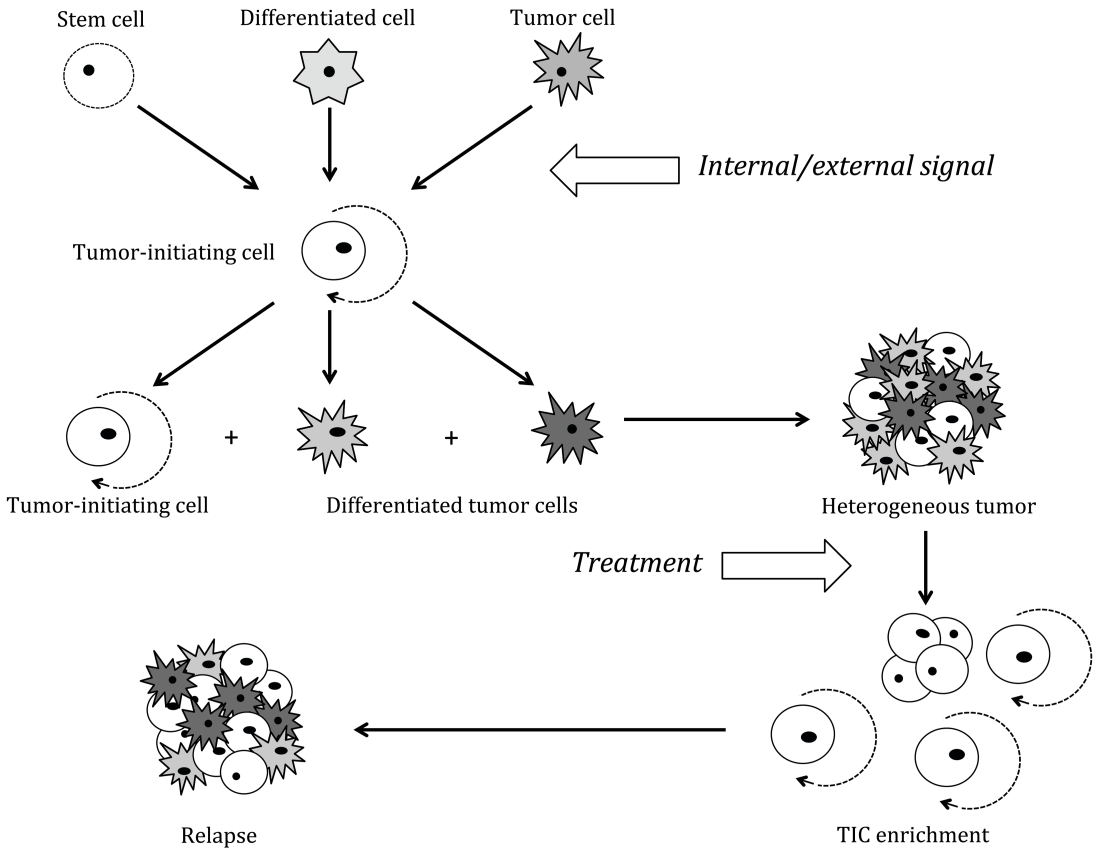

Figure 3. Tumor-initiating cells. TICs may arise from stem cells, partly differentiated cells or tumor cells depending on internal and/or external stimuli. Due to pluripotency, TICs give rise to new TICs as well as differentiated cells which results in heterogeneous tumors. Differentiated cells are responsive to treatment, while TICs survive and are able to form new tumors and cause disease relapse.

1.2.4 TICs in EOC

A common strategy to investigate TICs is to sort them out from cell lines or primary samples using flow cytometry. No single marker clearly identifies ovarian TICs; instead, combinations of markers are used and a variety of TIC profiles are suggested in the literature. There is thus a possibility that several different TIC populations exist and cell sorting for only one of them fails to cover this diversity. Some markers are based on functional assays, such as side population (SP) enrichment to sort out cells expressing ATP-binding cassette (ABC) transporters, and the ALDEFLUOR assay that detects aldehyde dehydrogenase family (ALDH) enzyme activity (Shah and Landen, 2014). Commonly used markers and suggested TIC profiles are discussed below.

1.2.4.1 Cell surface markers

Cluster of differentiation (CD) molecules are cell surface markers, and sorting of EOC TICs is commonly done based on CD44, CD117, CD133 and CD24. Additional surface proteins used are ATP-binding cassette sub-family G member 2 (ABCG2) and epithelial cell adhesion molecule (EpCAM).

CD44, a hyaluronate receptor involved in cell adhesion, migration and cell-cell interactions is frequently used as a marker of both somatic stem cells and TICs (Gardner et al., 1996; Garson and Vanderhyden, 2014). In EOC tumors, it is reported that CD44 expression defines a highly invasive and chemoresistant TIC population and correlates with shorter PFS in early stage disease (Alvero et al., 2009; Steffensen et al., 2011). However, some studies claim that $CD44^+$ cells are less aggressive and that the expression can predict a favorable prognosis (Sillanpää et al., 2003; Latifi et al., 2012). CD44 is regulated both post- transcriptionally and post-translationally but detection of CD44 is performed using antibodies reactive with most isoforms (Zöller, 2011), which may explain the inconsistent results.

CD117, also known as c-Kit, is a type III tyrosine kinase receptor that is activated upon binding its ligand stem cell factor (SCF) and initiates signal transduction via several pathways, including those of PI3K/Akt and MAPK (Lennartsson and Rönnstrand, 2012). Activated CD117 mediates cell survival, proliferation and migration and CD117 is frequently mutated and/or upregulated in various types of cancer (Liang et al., 2013). Using EOC tissue and ascites in a xenograft model, Luo et al (2011) showed that $CD117^+$ xenograft-derived cells had TIC features, including self-renewal, high tumorigenic potential, and chemoresistance.

Prominin-1, or CD133, is a transmembrane glycoprotein with unknown function (Grosse-Gehling et al., 2013). It is a common marker to define EOC TIC populations and increased expression is associated with poor survival (Baba et al., 2009; Curley et al., 2009; Zhang et al., 2012a). However, CD133 has been questioned as a TIC marker (Bapat, 2010) mainly due to the unknown roles of splice variants and glycosylation forms which together with different antibody clones for detection cause inconsistent results (Bidlingmaier et al., 2008; Hermansen et al., 2011).

The cell adhesion molecule CD24 is another questioned marker. Breast cancer TICs are defined as $CD44^+/CD24^-$ (Marsden et al., 2009) while pancreatic TICs are suggested to $CD44^+/CD24^+$ (Li et al., 2007a), implying a tumor-specific role for CD24. Gao et al (2010) showed that in EOC the $CD24^+$ fraction of cells derived from tumor tissue was chemoresistant and capable of self-renewal. When injected into immuno-compromised mice, the $CD24^+$ cells resulted in tumor formation while the $CD24^-$ did not (Gao et al., 2010). However, profiles including both $CD44^+/CD24^-$ (Meng et al., 2012) and $CD44^+/CD24^+/EpCAM^+$ (Wei et al., 2010; Meirelles et al., 2012) have been suggested to define EOC TIC populations.

ABCG2 is the major ABC transporter protein responsible for efflux of a variety of drugs (Zhou et al., 2001; Stacy et al., 2013) as well as the fluorescent dye Hoechst 33342 dye, used for SP enrichments. SPs from EOC cell lines and ascites are shown to be highly tumorigenic, chemoresistant and capable of self-renewal (Szotek et al., 2006; Hu et al., 2010; Dou et al., 2011) and ABCG2 is therefore considered to be a TIC marker in EOC.

1.2.4.2 Intracellular markers and TIC profiles

ALDH enzymes are important for detoxification of aldehydes as they catalyze the oxidation of aldehydes to carboxylic acids. High expression is reported for both normal stem- and progenitor cells and TICs (Marcato et al., 2011). Enzymatic activity can be measured by the ALDEFLUOR assay which is frequently used to identify such populations. ALDH1A1 is identified as the isoform responsible for stemness features (Marcato et al., 2011). ALDH1A1 expression and ALDH enzymatic activity were significantly higher in chemotherapy-resistant EOC cell lines as compared to their corresponding chemosensitive parental cell lines, and ALDHA1A^{+} cells were more tumorigenic then the ALDH1A1^{-} cells when injected in mice (Landen et al., 2010). Interestingly, the authors showed that siRNA-mediated downregulation of ALDH1A sensitized resistant cells to chemotherapy both *in vitro* and *in vivo* using a xenograft model.

It has been reported that only CD44^{+} cells express ALDH1A1 (Steffensen et al., 2011). Another intracellular marker that is increased in CD44^{+} cells is myeloid differentiation factor 88 (MyD88) (Alvero et al., 2009). MyD88 is an important component in Toll-like receptor 4 (TLR4) signaling pathway and its activation leads further to activation of the transcription factor nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) (Akira and Hoshino, 2003). Paclitaxel is a known activator of TLR4 (Kelly et al., 2006) and treatment led to a significant increase of NFκB activity in CD44^{+}cells but not in CD44^{-}cells which instead underwent apoptosis, suggesting a role for MyD88 in paclitaxel resistance (Alvero et al., 2009).

Two transcription factors important for maintenance of pluripotency in embryonic stem cells, Nanog and octamer-binding transcription factor 4 (Oct-4) (Shi and Jin, 2010), are commonly used for detection of EOC TICs. Nanog expression correlates with poor clinical outcome in serous EOC (Lee et al., 2012) and increased Oct-4 levels are related to high stage and high grade tumors (Peng et al., 2010). Of importance, alternative splicing results in several isoforms of Oct-4 of which Oct-4A is responsible for pluripotency properties of embryonic stem cells (Wang and Dai, 2010).

Several profiles using combinations of markers, most of them including expression of CD44, have been suggested to define TICs in EOC cell lines and tissue, based on tumorigenicity, sphere formation *in vitro*, chemotherapy resistance and, in some cases, poor clinical outcome. Profiles include CD44^{+}/CD24^{-} and CD44^{+}/CD24^{+}/EpCAM^{+} (Wei et al., 2010; Meirelles et al., 2012; Meng et al., 2012). Furthermore, CD44^{+}/MyD88^{+} (Alvero et al., 2009), CD44^{+}/CD117^{+} (Zhang et al., 2008) and ALDH1A1^{+}/CD133^{+} (Silva et al., 2011; Kryczek et al., 2012) profiles have all been reported do define potential TIC populations.

1.2.4.3 Summary

Possibly reflecting the heterogeneity of EOC, there is no consensus on one defining EOC TIC profile. Most likely, several TIC populations exist and efforts should be made to identify subtype-specific TIC profiles. It should also be noted that it is unknown whether the timing,

i.e. before or after treatment, for TIC isolation is of importance. If the hypothesis that TICs remain after treatment is true, then a larger proportion of TICs would be found directly after treatment rather than before. One can speculate that tumors caused by TICs at first have a TIC-rich heterogeneous cell population, but due to higher proliferation in the differentiated cells the TIC proportion will decrease as the tumor grows. This theory is supported by the finding that TIC levels, defined as $CD44^+$ cells, inversely correlated with FIGO stage and tumor grade (Steffensen et al., 2011). Although uninvestigated, the same theory should be possible to apply to relapsing tumors.

1.3 ALTERED CELLULAR ENERGETICS

1.3.1 Warburg…and more!

Altered cellular energetics of cancer cells was first described in the 1920s by Otto Warburg, who discovered that proliferating tumor cells converted the majority of their glucose carbon to lactate, even in the presence of oxygen (Warburg et al., 1924). Warburg hypothesized that this phenomenon arose from mitochondrial defects causing decreased capacity to convert glucose to carbon dioxide. His finding, known as "the Warburg effect", has been observed in numerous cancers which in turn has led to the clinical use of ^{18}F-deoxyglucose positron emission tomography (FDG-PET) for detection of tumors. The use of glycolysis instead of oxidative phosphorylation (OxPhos) might seem inefficient since aerobic glycolysis of one glucose molecule generates only two adenosine 5'-triphosphate (ATP) molecules, while the net result from tricarboxylic acid (TCA) cycle and OxPhos is 36 ATP. However, increased glucose uptake allows redirection of glycolytic intermediates into various biosynthetic pathways to meet the demand for macromolecular synthesis in proliferating cells (Ward and Thompson, 2012).

In contrast to Warburg's hypothesis, the aerobic glycolysis used by most tumor cells is not caused by defective or damaged mitochondria. Although tumors do have the ability to use OxPhos to generate ATP from glucose, mitochondrial metabolism is instead partly reprogrammed to sustain proliferation. In addition to glucose, cancer cells rely on glutamine as their source of carbon. The glutamine is used to provide TCA cycle intermediates that further feed to biosynthetic pathways. The use of aerobic glycolysis is thus linked to glutamine dependence to maintain the TCA cycle (DeBerardinis et al., 2007, 2008). A simplified overview of important metabolic pathways in proliferating tumor cells is shown in Figure 4.

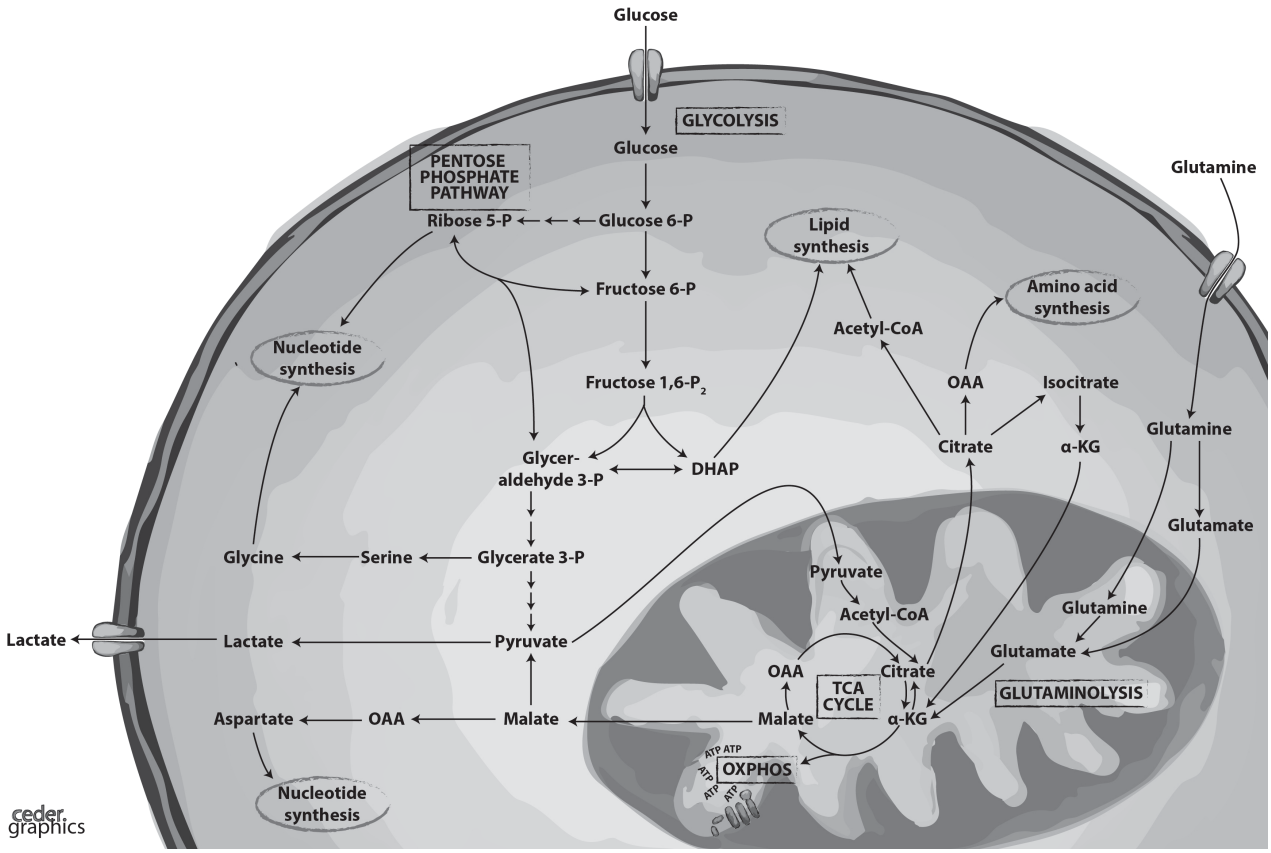

Figure 4. Metabolic features of malignant cells . Simplified overview of important pathways and biosynthesis of amino acids, lipids and nucleotides in proliferating tumor cells. Abbreviations: α-KG, α-ketoglutarate; DHAP, dihydroxyacetone phosphate; OAA, oxaloacetate.

Altered cellular energetics is programmed and regulated by several oncogenes and tumor suppressor genes with well-known roles in sustained proliferation. Signaling pathways like PI3K/Akt MAPK, and NFκB enhance the transcription of a number of genes that encode mediators of glycolysis and glutaminolysis (Levine and Puzio-Kuter, 2010). The metabolome of a tumor cell thus mirrors the net result of complex intracellular signaling. Metabolic profiling is made possible by metabolomics, an emerging field in cancer research (see section 1.3.2).

The identification of tumor-specific metabolic alterations is a promising field for the development of new therapeutical strategies. Several important metabolic enzymes have been described as possible drug targets and energy modulating substances have been shown to efficiently eradicate tumor cells when used as single agents or in combination with standard chemotherapeutic drugs (reviewed in Shoshan, 2012 and Zhao et al., 2013).

Out of many key players in metabolic pathways, the work in this thesis has focused on the enzyme hexokinase II (HK-II), the transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α) and its downstream target mitochondrial transcription factor A (TFAM) wherefore they are discussed below.

1.3.1.1 Hexokinase II

The first step of glycolysis, when glucose is phosphorylated, is facilitated by hexokinase enzymes. The isoenzyme HK-II is expressed in low levels in normal cells but is frequently upregulated in malignant cells (Pedersen et al., 2002; Mathupala et al., 2006). HK-II enhances glycolysis via its enzymatic activity, but also through its localization to mitochondria where it binds the voltage-dependent anion channel (VDAC). The ATP produced in OxPhos is transported out from the mitochondrial via adenine nucleotide translocase (ANT) associated with VDAC and supplies mitochondrial HK-II with ATP directly from the ATP synthase (Mathupala et al., 2006). The HK-II/VDAC complex has dual roles, as it also inhibits the intrinsic apoptosis pathway (Pastorino et al., 2002). Mitochondrial binding of HK-II is promoted via PI3K/Akt signaling. Conversely, high levels of the end product of HK-IIs enzymatic activity, glucose 6-phosphate (Glucose 6-P), has been reported to dissociate the complex (Gottlob et al., 2001).

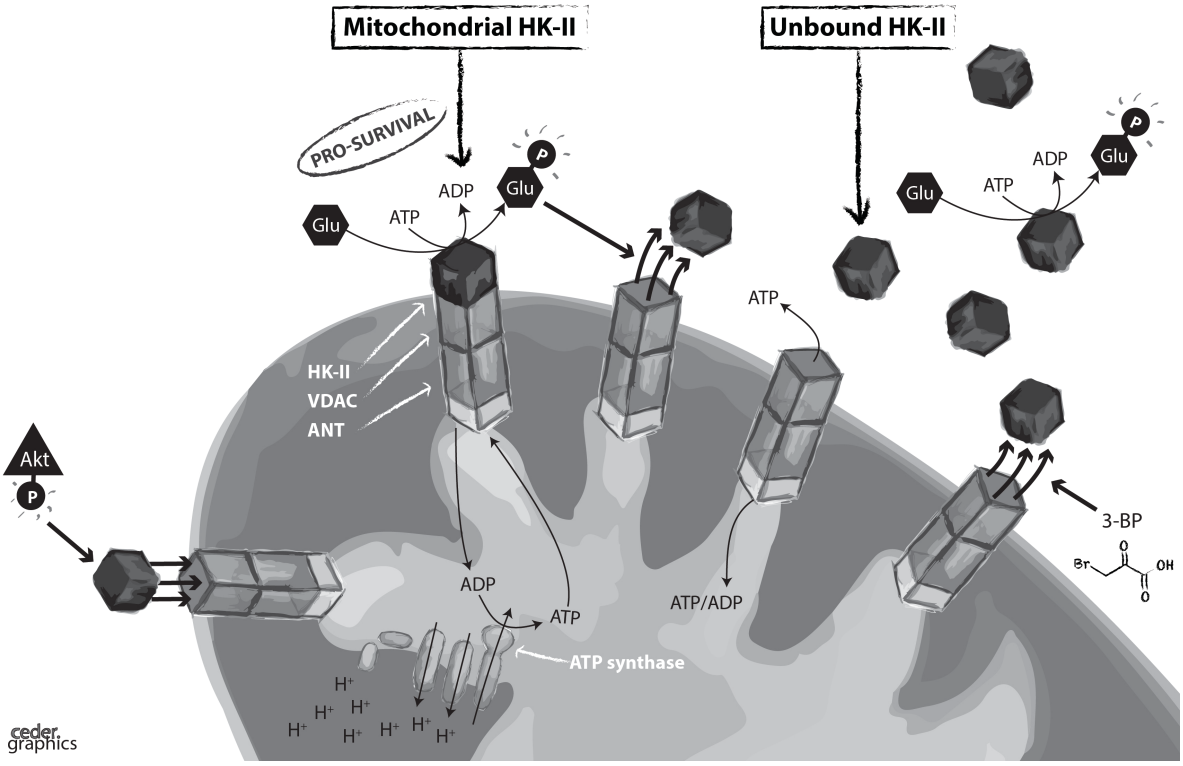

Figure 5. Dual role of hexokinase II. HK-II binds to VDAC, located in the OMM. Via interaction with ANT, in the IMM, ATP produced by the ATP synthase is directly supplied to HK-II that use ATP to convert glucose to glucose 6-phospate. In addition, mitochondrial HK-II inhibits apoptosis. Activated Akt promotes the formation of this pro-survival complex while high levels of glucose 6-phospate cause dissociation of HK-II. In addition, dissociation can also be achieved by 3-BP treatment. Abbreviations: HK-II, hexokinase II; VDAC, voltage-dependent anion channel; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; ANT, adenine nucleotide translocase; Glu, glucose; Glu-6-P, glucose 6-phosphate; ATP, adenosine 5'-triphosphate; ADP, adenosine 5´-diphosphate; 3-BP, 3-bromopyruvate.

Mitochondrial bound HK-II is far more often seen in cancer cells than in normal cells (Mathupala et al., 2009), wherefore the HK-II/VDAC complex is a potential target for cancer therapy. One agent that has been suggested to dissociate HK-II from the mitochondrial complex is the lactate/pyruvate analogue 3-bromopyruvate (3-BP) (Chen et al., 2009; Pereira

da Silva et al., 2009), reported to have significant anticancer effects in *in vivo* models of hepatocarcinomas but low levels of toxicity in normal cells (Geschwind et al., 2002; Ko et al., 2004). In 2013, the US Food and Drug Administration (FDA) approved 3-BP for Phase I clinical trial in patients with primary and/or metastatic liver cancer[8]. The use of 3-BP in EOC has not been investigated and few studies investigate the role of HK-II in this disease. However, a recent study (Suh et al., 2014) shows that high expression of HK-II was associated with chemoresistance and decreased PFS in EOC tumors.

1.3.1.2 PGC1α and TFAM

The transcriptional coactivator PGC1α is a major coordinator of metabolism and mitochondrial function (Austin and St-Pierre, 2012), as well as an important regulator of antioxidant defense (Valle et al., 2005; St-Pierre et al., 2006). PGC1α effectors include nuclear respiratory factor 1 and 2 (NRF1, NRF2) (Wu et al., 1999), which in turn regulate TFAM expression (Virbasius and Scarpulla, 1994). TFAM is needed for transcription of mitochondrially encoded OxPhos proteins, but also regulates the structure and copy number of mtDNA (Kanki et al., 2004; Kaufman et al., 2007). PGC1α may also directly regulate mtDNA transcription (Aquilano et al., 2010).

Very little is reported on the role of PGC1α in cancer in general. In EOC, lower levels of PGC1α have been reported in tumor samples compared to normal ovaries, and PGC1α overexpression *in vitro* led to apoptosis via downregulation of Bcl-2 and upregulation of Bax (Zhang et al., 2007a). There are no reported results on the role of PGC1α in chemoresistance. However, the role of the PGC1/estrogen-related receptors (ERR) axis is known to regulate cancer cell metabolism (Deblois et al., 2013), in turn known to affect treatment response (Zhao et al., 2013).

There are few reports on TFAM and its role in cancer. However, partial loss of mtDNA has been shown to increase invasivity and progression in cancer cells originating from lung (Amuthan et al., 2002), prostate (Naito et al., 2008; Moro et al., 2009) and breast (Naito et al., 2008) tissue. As TFAM is a required for normal replication and transcription of mtDNA its downregulation might be important for tumor progression. This is in part confirmed in colon carcinoma cell lines and tumor biopsies where truncating mutations of TFAM caused mtDNA reduction and mitochondrial instability (Guo et al., 2011).

1.3.2 Metabolomics

In the era of ”omics” it is now possible to study biological systems on several levels, including genomic variances (genomics), gene expression (transcriptomics), protein abundance (proteomics) and metabolite concentrations (metabolomics). The metabolic composition, i.e. the metabolome, is defined as all low-mass small molecules[9] in a given

[8] Announced at http://presciencelabs.com/prescience-labs-investors/press.php

[9] Molecules <1500 Daltons (Da).

system (Halama, 2014). The metabolome of a cell results from its genetic characteristics, regulation of gene expression, protein abundance, posttranslational modifications and environmental influences, hence the metabolome is a direct readout of the phenotype (Griffin and Shockcor, 2004).

The complete cell metabolome includes all the intracellular metabolites, the endo-metabolome, as well as all the metabolites surrounding the cell, the exo-metabolome. The exo-metabolome, a result from cellular uptake and excretion, can be examined in biofluids and are useful for the identification of disease-related biomarkers. However, a more accurate reflection of the metabolic state of cells can be found in their intracellular metabolome, wherefore cell-based metabolomics is an emerging field (León et al., 2013).

There are two different strategies to study the metabolome, targeted and untargeted. Untargeted metabolomics, also described as global metabolomics, normally consists of unbiased analysis of all the possible metabolites in a given biological sample and is usually used for hypothesis generation. In contrast, targeted metabolomics focuses on a fraction of the metabolites or a metabolic pathway for more detailed studies applied to examine a hypothesis (Halama, 2014). Numerous analytical techniques have been developed for these studies, most of them based on proton (^{1}H) nuclear magnetic resonance (NMR) or high resolution mass spectrometry (HRMS) (Vermeersch and Styczynski, 2013). Typically, metabolomic analysis generates huge amounts of data and requires the use of appropriate chemometric and statistical techniques to evaluate biological relevant data.

To answer an biological question using metabolomics, the workflow includes *experimental design*, *sample processing*, *measurement*, *data processing*, *data interpretation* and *result validation* (León et al., 2013). Knowing how to proceed in each step of the workflow is important since any undesired alteration in the metabolome may lead to misleading results. Sample processing for intracellular metabolomics of any cell model include culture conditions, harvesting technique and metabolite extraction. Unfortunately, no standard protocols exist for these procedures which makes it difficult to compare data between different laboratories.

1.3.2.1 Metabolomic studies in EOC

A number of metabolomic investigations of EOC have been performed, most of them with a primary focus to identify diagnostic or prognostic biomarkers, using urine or serum in patients with early stage disease (Odunsi et al., 2005; Zhou et al., 2010a; Chen et al., 2011; Zhang et al., 2013; Ke et al., 2014). In addition, intracellular metabolic profiling of normal ovarian tissue, primary and metastatic tumors (Fong et al., 2011) as well as metabolic patterns of borderline versus invasive carcinoma (Denkert et al., 2006) has also been reported, both using fresh frozen biopsies from untreated tissues. One recent study investigated the proteome and metabolome of cell-free malignant EOC ascites and compared it to cirrhosis-related ascites (Shender et al., 2014). Taken together, these untargeted studies identify a large

number of metabolome differences but their impact on tumor progression and outcome in terms of survival needs to be investigated in more detail.

Recently, targeted metabolomics was used to link metabolic dependence and invasiveness in EOC (Yang et al., 2014). Metabolic profiling and metabolite tracing using isotope labeled glucose and glutamine revealed increased dependence on glycolysis in low-invasive cell lines while highly invasive cell lines were more dependent on glutamine. Furthermore, glutamine was found to regulate invasiveness by regulating the activity of signal transducer and activator of transcription 3 (STAT3). This study nicely demonstrates how targeted metabolomics can be used as a complement to standard *in vitro* methods, and similar future studies will hopefully increase our understanding of metabolic features in EOC and how it is linked to disease progression, TICs and chemoresistance.

2 AIMS OF THE THESIS

The overall aim was to study ovarian carcinoma with focus on tumor progression and chemoresistance. All studies were performed in EOC cell lines, tumor material and ascites. The specific aims of each paper were:

Paper I: To examine different cell populations in malignant ascites by protein profiling for markers of EMT, TICs, metabolism and CAFs.

Paper II: To investigate long-term effects of cisplatin treatment in terms of EMT and TIC features as well as mitochondrial alterations in the treatment surviving cell population, and to target these resistant cells.

Paper III: To study the role of mitochondrial regulation by examining the expression of PGC1α and TFAM in a tissue microarray as well as in cell lines, and relate the expression to tumor subtypes and treatment response.

Paper IV: To evaluate protocols for harvesting adherent cells prior to ^{1}H NMR-based metabolomics and to detect differences in the polar metabolome of parental versus multiresistant cells.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Protein markers of cancer-associated fibroblasts and tumor-initiating cells reveal subpopulations in freshly isolated ovarian cancer ascites

In this study malignant ascites from 22 patients were collected (see **Paper I**, Table 1 for clinicopathological characteristics). The majority was high-grade serous carcinomas and all patients were diagnosed with advanced stage of disease (FIGO stages IIIC or IV). Ascites were removed from the patients via therapeutic paracentesis whereafter cells were pelleted and separated on a discontinuous gradient. The tumor cell fractions were then plated and subjected to further analysis. Ascitic samples contained one, two or all three of either single cells, loose sheet-like aggregates or spheres, defined as compact spherical aggregates in which individual cells could not be discernible. When plated in cell cultures, single cells and loose aggregates quickly attached to the surface and formed monolayers, wherefore they were entitled M-type cells. In contrast, spheres did not attach to the surface and could be dispersed only by passage through a fine-mesh filter. After passaging, these so-called S-type cells rapidly formed new spheres, showing that these cells were proliferative.

M- and S-type cells differed significantly with respect to protein levels of markers of EMT, as E-cadherin expression was higher in S-type samples, while vimentin expression was associated with M-type (**Paper I**, Figure 1b). Hence, we concluded the M- and S-type cells constitute two separate populations. Expression of CAF markers α-smooth muscle actin (α-SMA) and platelet-derived growth factor receptor β (PDGFRβ) was also analyzed and results demonstrate significantly higher expression levels of α-SMA among M-type cells. In addition, all nine samples expressing the CAF marker PDGFRβ were of M-type (**Paper I**, Figure 2). Interestingly, CAFs may originate not only from fibroblasts but also from malignant or normal epithelial cells that have undergone EMT (Kalluri and Zeisberg, 2006). In line with the findings that EOC CAFs are associated with advanced-stage disease and can promote growth and metastasis of EOC *in vivo* (Cai et al., 2011; Zhang et al., 2011), we found that clinical stage IV ascitic samples showed significantly higher expression of α-SMA, compared with stage IIIC samples (**Paper I**, Figure 2b).

High expression of TIC markers and potentially high levels of TICs were demonstrated in this study, based on expression of ABCG2, CD44, CD117, EpCAM, Nanog and Oct-4A. CD44 was found in nine samples, all M-type, and its expression correlated strongly with that of vimentin, and an inverse correlation was found with expression of E-cadherin. M- and S-type did not differ with regard to ABCG2 and EpCAM, in contrast to CD44 (**Paper I**, Figure 3a, Table 2 and 3). CD117 was found in four samples and was not further analyzed.

When investigating transcription factor Oct-4A, the nuclear isoform of Oct-4 responsible for pluripotency properties of embryonic stem cells (Wang and Dai, 2010), we noted a ≈ 90 kDa band in addition to the ≈ 40 kDa band corresponding to the 360 aa protein Oct-4A in western

blot analyses. Since small ubiquitin-related modifier (SUMO)-1 modification (sumoylation) of Oct-4 had previously been found to increase the stability and function of the protein (Wei et al., 2007), we sought to investigate if SUMO-Oct-4A could be detected. Using immunoprecipitation to pull down SUMO-1 and Oct-4A we could confirm that the ≈ 90 kDa band corresponds to SUMO-Oct-4A in EOC cell line SKOV-3 (**Paper I**, Figure 3b), and confirmed the findings in EOC cell lines A2780 and CaOv-4. Sumoylation is a post-translational modification where small SUMO proteins are covalently bound to proteins to modify their function. Similar to ubiquitination, sumoylation is directed by an enzymatic cascade involving an activating enzyme complex (E1), a conjugating enzyme (E2) and a ligase (E3), resulting in an isopeptide bond between the carboxyl terminus of the SUMO protein and specific lysine residue(s) in the target protein (Saitoh and Hinchey, 2000; Wang and Dasso, 2009). Interestingly Ubc9, the only E2 enzyme catalyzing SUMO conjugation (Zhang et al., 2007b), is upregulated in ovarian carcinoma (Mo et al., 2005) but sumoylated Oct-4A has not been investigated. Thus, our study is the first to show presence SUMO-Oct-4A in EOC.

SUMO-Oct-4A was observed at varying levels in 24/27 ascitic samples while unmodified Oct-4A was found in 17/27 samples and expression levels of both were significantly higher in M-type samples compared to S-type (**Paper I**, Figure 3c and 3d). In contrast, M- and S-type cells did not differ with respect to expression levels of the transcription factor Nanog. However, we found a strong positive correlation between Nanog and EpCAM in the material, independently of sample type (**Paper I**, Figure 3d, Table 2 and 3).

We hypothesized that spheres would be more glycolytic as a result from adaptation to internal hypoxia and show lover levels of mitochondrial proteins. However, expression of β-F1-ATP synthase, reported to be downregulated in cancer cells reflecting low mitochondrial respiration and worse prognosis in breast, lung and head-and-neck cancer (López-Ríos et al., 2007; Willers et al., 2010), and TFAM, which correlates with cellular mitochondrial content (Montoya et al., 1997), was similar in M-and S-type samples. Nevertheless, there was a positive correlation between β-F1-ATPsynthase and Nanog expression as well as with EpCAM, independently of sample type. Hence, we concluded that the $Nanog^{high}$ /$EpCAM^{high}$ population also expresses high levels of β-F1-ATPsynthase.

Previous studies have suggested that spheres from malignant ascites represent a subpopulation of highly malignant cells responsible for disease progression, reviewed in (Shield et al., 2009). This assumption is mainly supported by *in vitro* work on artificial spheroids, often on TIC-enriched spheroids cultured in SCM. Our initial hypothesis was therefore that spontaneous ascitic spheres would harbor TICs. However, our results show that S-type populations were low in TIC markers CD44 and Oct-4A compared to M-type populations. Moreover, they did not express CAF markers α-SMA and PDGFRβ and were more epithelial, and thus represent a less invasive phenotype than M-type.

M-type samples could be divided into two subtypes; one that resembled the S-type profile but apparently lacked some factor required for sphere formation, and one that was $CD44^{pos}$, Oct-

4A^{high} and expressed CAF markers. We also identified Nanog^{high} /EpCAM^{high} samples that represent a TIC subset which may be either M- or S- type and which is separate from the CD44^{pos} and Oct-4A^{high} subset observed only in M-type samples (summarized in Figure 6). Studies on primary EOC tissues demonstrate that CD44 expression defines a highly invasive and chemoresistant TIC population (Zhang et al., 2008; Alvero et al., 2009). We therefore suggest that the particular subset of M-type cells expressing CD44 and Oct-4A represents the more tumorigenic ascitic cell population. Supporting this, expression of Oct-4 was reported to be expressed at higher levels in EOC tumors than in normal ovarian and fallopian tube epithelium, and to correlate with advanced FIGO stage and high clinical grading (i.e. low differentiated tumors) (Peng et al., 2010; Zhang et al., 2010).

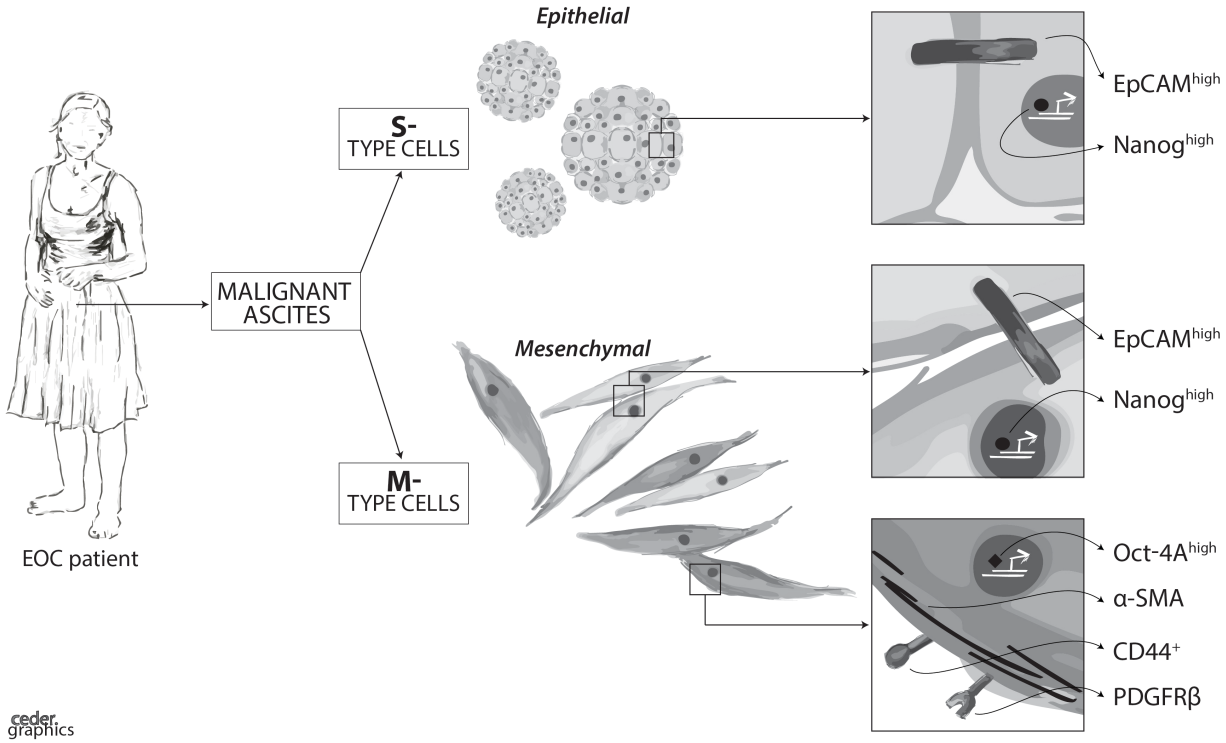

Figure 6. Cell populations in malignant ascites. . Based on our results, we concluded that M- and S-type cells in malignant EOC ascites constitute separate cell populations, where the monolayer-forming single M-type cells were mesenchymal while the sphere forming S- type cells were epithelial. A subset of M-type samples was positive for CD44, were high in Oct-4A and exclusively expressed CAF markers α-SMA and PDGFRβ. An additional Nanog^{high} /EpCAM^{high} subset of TICs was identified and could be found in both M- and S-type samples.

There are contradictory conclusions regarding populations in malignant ascites. In a very similar report, published two months after our study, Latifi et al (2012) isolated and characterized EOC ascitic cells and compared adherent (AD) and non-adherent (NAD) cells for expression of EMT and TIC markers. Results showed that AD cells were higher in vimentin, N-cadherin, CD44, MMP-2 and MMP-9, and had low or no expression of E-cadherin, EpCAM, CA125, Oct-4 and STAT3. Injected into mice[10], NAD cells were more

[10] Cells from three patients.

tumorigenic than AD cells. It should be noted, however, that NAD cells were first grown as adherent monolayers, which may cause selection and alter their phenotype, before ip injection. Interestingly, compared to the original patient ascites, cells from mouse xenograft ascites showed decreased levels of EpCAM and CA125 and an increase in CD44 expression. In addition, comparison of AD and NAD cells from five chemonaïve (CN) patients and five chemoresistant[11] (CR) patients showed that EpCAM, Oct-4 and STAT3 levels were increased in CR-NAD cells while CD44, MMP9 and Oct-4 expression were increased in CR-AD cells (Latifi et al., 2012). CR cells in general were thus higher in expression of TIC markers, in line with the potentially high levels of TICs found in our study where all but two patients were in relapse with resistant disease. Altogether, this suggests that treatment might select for and/or induce expression of TIC markers.

Nevertheless, the sphere-forming S-type cells in our study are most likely chemoresistant since chemotherapeutic drugs do not penetrate such multicellular structures (Fayad et al., 2009; Tunggal et al., 1999). It is difficult to evaluate the drug response in single cells suspensions/monolayers compared to spheres *in vitro*. One strategy, used by Latifi and colleagues, is to mechanically disrupt spheres and analyze the drug response in sphere-forming cells in a single cell format. This might however change their phenotype and cause confusing results. Latifi et al reported that the AD cells were more sensitive to cisplatin, but this is possibly a trivial finding considered that they showed more rapid proliferation, compared to NAD cells.

One important factor that may contribute to conflicting results is how to separate tumor cells from malignant ascites. We used a gradient separation technique, first described by Bjørge et al (Bjørge et al., 2005). When investigating similar preparations of ascites samples the proportion of tumor cells were found to be on average 77% (Hjerpe et al., 2014). Latifi and colleagues removed red blood cells but did not enrich for tumor cells using gradient separation, a protocol that most likely decreases the proportion of tumor cells.

There also seems to be a difference in the definition of spheres. Unlike Latifi et al, we did not classify loose aggregates as spheres since they readily attached to the plastic and formed monolayers when grown under standard conditions. However, there is a possibility that the aggregates would act differently if plated on low-attachment plates in serum-free medium, the culture condition used by Latifi. The fact that they could culture their NAD cell population as monolayers after dispersing spheres by pipetting clearly indicates that NAD cells are not equivalent to our S-type cell populations. Moreover, the authors emphasized that there was an extensive variation in morphology and size of ascitic spheres between different samples and also within each patient sample. One in this context interesting study (Strauss et al., 2011) identified subpopulations of cells in EOC biopsies and ascites that were in a transitory epithelial/mesenchymal (E/M) hybrid stage, with cells that simultaneously expressed

[11] Defined as recurrent disease within 6-20 month of first line treatment.

epithelial and mesenchymal markers. A particular E/M-hybrid that was low in E-cadherin and high in CD44 expression, similar to our $CD44^{pos}$ M-type cells, was highly enriched for tumor-forming cells *in vivo* and displayed TIC-associated features. The authors suggest that E/M hybrids show high plasticity, are able to differentiate into different lineages under certain conditions and show capacity for self-renewal (Strauss et al., 2011).

To summarize, we show that several populations of potential TICs exist in malignant ascites. This may have practical implications for TIC isolation based on cell sorting, since using a particular set of markers may result in a particular subset of TICs. The results also imply a biological heterogeneity and epithelial-mesenchymal plasticity that may underlie the diversity of results found in the literature. We found high levels of sumoylated Oct-4A that most likely represent a stabilized form of the protein. In addition, we demonstrate the likely presence of CAFs in ascites and show that α-SMA expression was significantly higher in stage IV samples compared with stage IIIC.

3.2 PAPER II

Repeated cisplatin treatment can lead to a multiresistant tumor cell population with stem cell features and sensitivity to 3-bromopyruvate

The antineoplastic effect of cisplatin is based on its DNA damaging effects, in particular via DNA adduct formation, but it can also induce apoptosis via non-nuclear target(s) (Mandic et al., 2003). Cisplatin has also been shown to bind mtDNA (Olivero et al., 1995; Podratz et al., 2011), and since mtDNA mutations have been shown to promote tumor progression (Brandon et al., 2006; Lu et al., 2009) we sought to investigate whether long-term exposure to cisplatin would cause mitochondrial defects and also if resistance could be associated with EMT and/or TIC features.

To do so, resistant sublines from the EOC cell lines A2780 and SKOV-3 were generated by treatment for several passages with increasing concentrations of cisplatin (up to 10μM). Surviving cells were allowed to recuperate until near-confluence between treatments. The resulting resistant phenotype was confirmed and stable over at least 10 passages without cisplatin. A similar protocol for long-term treatment with ethidium bromide (EtBr) that binds to mtDNA and destroys it, causes partial loss of mtDNA and has been shown to increase invasivity and progression in several cancer types (Amuthan et al., 2002; Naito et al., 2008; Moro et al., 2009). We therefore used EtBr to create mtDNA deficient SKOV-3 cells, i.e. SKOV-3-ρ_0 cells, as a control for damaged mtDNA.

In the resistant subline of A2780 cells, A2780-R, the 2-fold increase in platinum resistance, expressed as half-maximal inhibitory concentration (IC_{50}), could be related to upregulation of ABCG2. Since upregulation of this transporter has been reported to decrease the response to cisplatin (Herraez et al., 2012) and to be predictive of poor response to platinum-based chemotherapy (Kim et al., 2009), these cells were not further investigated. In contrast, the resistant subline SKOV-3-R, did not express ABCG2 and the resistance could not be linked to increased levels of anti-apoptotic Bcl-2 protein family members, Bcl-2 and Bcl-XL. These

cells were highly crossresistant also to the mitotic inhibitor paclitaxel and the antimetabolite 5-fluorouracil (**Paper II**, Figure 1a and Table 1).

Compared to the parental cell line, SKOV-3-R cells presented a more mesenchymal morphology. In accordance with having undergone EMT, they showed downregulation of E-cadherin, upregulation of Snail and Twist and showed increased motility towards a serum attractant (**Paper II**, Figure 2). Expression of EOC TIC markers CD44, CD117 and ALDH1A1 were upregulated in SKOV-3-R, whereas levels of Nanog and Oct-4A were unaltered (**Paper II**, Figure 3a and S2). While parental cells did not survive prolonged culturing in SCM the resistant cells detached and, within 72 h, formed spheres that could be cultured for at least 30 days. Moreover, spheres could be dispersed and re-plated to grow as spheres in SCM or as monolayer cells in standard medium, indicating that the cells were capable of self-renewal (**Paper II**, Figure 3d).

Immunocytochemistry revealed that the proportion of cells positive for E-cadherin decreased from approximately 40% in parental SKOV-3 to 10% in SKOV-3-R. In parallel, an increase in CD117 expression was seen both as a larger proportion of positive cells and as greater expression in a subset of SKOV-3-R cells (**Paper II**, Figure 3b and 3c). These results highlight the complexity of resistance development that can be due to drug-mediated selection and/or induction of genetic/phenotypic alterations.

SKOV-3-ρ_0 cells were mtDNA deficient and thus lack the mitochondrial-encoded OxPhos proteins. Despite the loss of mtDNA, SKOV-3-ρ_0 cells retained a mitochondrial content comparable to that of SKOV-3 parental cells, detected as mitochondrial mass (**Paper II**, Figure 1b and 1c). In contrast to multiresistant SKOV-3-R, SKOV-3-ρ_0 cells showed no or little increase in resistance to platinum-based drugs, no morphological alterations or altered expression of EMT markers compared to parental cells (**Paper II**, Table 1, Figure 2a and 2b). CD117 was slightly upregulated in SKOV-3-ρ_0 cells, while the other investigated TIC markers were unchanged (**Paper II**, Figure 3a), suggesting that CD117 might be regulated via mtDNA status. We noted that SKOV-3-ρ_0 cells survived for a prolonged time in SCM compared to parental SKOV-3 cells. However, if this was a consequence of CD117 upregulation or of OxPhos depletion, causing highly glycolytic cells that thrive in the high glucose SCM, was not further investigated. Regarding the expected malignant progression in ρ_0 cells, one can speculate that while partial loss of mtDNA causes progression, the complete lack of mtDNA might have low impact or even reverse effects on tumor progression. This is supported by the finding that OxPhos Complex III is required for metastatic disease (Weinberg et al., 2010).

Although our initial hypothesis that cisplatin causes damage and/or downregulation of mtDNA leading to tumor progression was refuted, we found mitochondrial alterations in SKOV-3-R cells. Resistant cells showed a tendency towards increased mtDNA:nDNA ratio and significantly increased mitochondrial mass. This was supported by higher levels of the mitochondrial proteins VDAC and cytochrome c (**Paper II**, Figure 1b, 1c and 1d). While cytochrome c is required for electron transfer from OxPhos Complex III to Complex IV,

VDAC regulates both mitochondrial metabolism and cell death, partly by forming an anti-apoptotic complex via binding to HK-II (Pastorino et al., 2002; Mathupala et al., 2006). We therefore investigated the levels of HK-II, and found that it was slightly upregulated in SKOV-3-R cells and, as expected in OxPhos depleted cells, even more so in SKOV-3-ρ_0 (**Paper II**, Figure 1e).

While we investigated the effects of long-term exposure to cisplatin in terms of EMT and TIC features, similar findings were reported (Latifi et al., 2011). The authors showed that cisplatin, at higher concentrations and for shorter time periods than we used[12], can induce EMT and expression of TIC markers in the EOC cell line OVCA433 as well as in cell populations expanded from clinical samples. Our results thus support their findings that cisplatin induces classical EMT profiles, including upregulation of Snail and Slug and increased motility, and upregulation of TIC markers Oct-4, Nanog, CD44 and CD117. However, our data show that lower and indeed clinically more relevant doses of cisplatin can lead to EMT and TIC-marker upregulation, and that the resulting multiresistant phenotype is stable even in the absence of the drug. SKOV-3-R cells thus represent a steady-state model rather than a model for induced responses to treatment.

Targeting multiresistant TICs is a major treatment challenge (Alison et al., 2012). Since CD117 was upregulated in SKOV-3-R we examined the effects of CD117 inhibition on resistance. CD117 can be targeted by imatinib mesylate that inhibits CD117 and other tyrosine kinase receptors by blocking the active site of the kinase. The IC_{50} of imatinib mesylate was lower in SKOV-3-R than in SKOV-3, but the concentration needed to prevent growth was clinically irrelevant (>20μM), since plasma levels likely do not exceed approximately 5μM (Druker et al., 2001) (**Paper II**, Table 2). Subsequently we examined if imatinib mesylate at 5 and 10μM could potentiate cisplatin at 2-5μM, but found no effect on growth alone or in combination treatment using clinically relevant doses. Nevertheless, the increased sensitivity to imatinib mesylate in SKOV-3-R indicated a partial dependence on CD117. Latifi and colleagues reported that inhibition of extracellular-signal-regulated kinases 1/2 (ERK 1/2)[13], located downstream of receptor kinases, inhibited the cisplatin-induced motility and vimentin levels in OVCA433. Cells treated with 16.5μM cisplatin for 4 + 3 days were shown to activate ERK1/2, motility and increased levels of vimentin, responses that could be avoided by co-treatment with ERK1/2 inhibitor U0126 (Latifi et al., 2011). We could, however, not detect any inhibition of motility or altered morphology in SKOV-3-R using the CD117 specific inhibitor ISCK03, that blocks phosphorylation of CD117 and consequently downstream phosphorylation of ERK1/2. This might be a result of the different protocols used, since cisplatin is well known to activate ERK1/2, and combination treatments with ERK inhibitors have been found to potentiate cell death (Brozovic and Osmak, 2007).

[12] Doses from 3.3 to 33μM cisplatin for 3 days up to a month.
[13] Also known as MAPK1 and MAPK3

ERK1/2 activation might be important in acute response to treatment but less so in a stable phenotype when the drug is not present.

Several calorie restriction mimetic phytochemicals have been reported to efficiently target TICs, i.e. epigallocatechin gallate (Tang et al., 2012), quercetin (Zhou et al., 2010b) and resveratrol (Shankar et al., 2011). We have earlier shown that resveratrol can potentiate cisplatin in regrowth experiments (Björklund et al., 2011), and therefore examined the effect of these compounds in SKOV-3-R. Of importance when investigating substances that modulate energy metabolism, such as resveratrol, is to choose a proper assay to assess drug treatment response. Since many of the most frequently used methods to measure cell proliferation are based on monotetrazolium salts, e.g. MTT and similar assays that depend on reduction via NAD(P)H-dependent oxidoreductase enzymes (Berridge et al., 2005), changes in metabolic state of the cells may cause misleading results. We therefore quantified total cellular protein using Sulforhodamine B assay (SRB) (Skehan et al., 1990) to determine IC_{50} and regrowth capacity following drug treatment. However, using the SRB assay will not provide information regarding cell status. Cells might be early apoptotic or senescent but still contribute to the read-out via their cellular proteins. As a consequence, it is difficult to evaluate short-time responses to treatment but a preferable method when studying longer time points. Hence, we treated cells for 72h to estimate IC_{50} values, and for regrowth experiments cells were treated for 48h followed by another 72h in drug-free medium.

Compared to the parental cells, SKOV-3-R showed increased resistance to all three phytochemicals and none of them could potentiate the effect of cisplatin in regrowth experiments (**Paper II**, Table 2 and Figure S4). Due to the upregulation of HK-II in SKOV-3-R, we examined the effect of 3-BP which exerts its antitumoral action at least in part via dissociation of HK-II from mitochondria (Mathupala et al., 2006; Chen et al., 2009). SKOV-3-R cells were sensitive to 3-BP, and combinations of 3-BP and cisplatin were highly antiproliferative in both the parental and resistant cell line, as demonstrated in regrowth experiments (**Paper II**, Table 2 and Figure 4a).

To further investigate the roles of glucose and HK-II in resistance we used glucose-free standard medium supplemented with galactose. In the absence of glucose, cells can generate glucose 6-P from galactose via the Leloir pathway, which thus enables evaluation of glucose and HK-II function without compromising glycolysis. In addition, it has been shown that galactose medium causes dissociation of HK-II from mitochondria, without altering the protein expression (Shulga et al., 2010). The growth rates of SKOV-3 and SKOV-3-R cells over 72h in galactose medium were essentially unaltered compared to growth rates in standard medium containing 11 mM glucose. Cells cultured in galactose medium were resistant to 3-BP (**Paper II**, Table 2), which further supports the role of mitochondrial HK-II as a 3-BP target. When sensitivity to cisplatin was evaluated in galactose medium and compared with the response in standard medium, SKOV-3 showed a non-significant reduction while SKOV-3-R were significantly sensitized to cisplatin (**Paper II,** Figure 4b), indicating that glucose and/or HK-II was required for cisplatin resistance.

To recapitulate, we show in this study that long-term repeated cisplatin treatment can lead to a multiresistant cell population with stem cell features. Multiresistant cells showed increased motility and had undergone EMT. Since resistance could be related to upregulation of VDAC and HK-II, forming a anti-apoptotic complex on the outer mitochondrial membrane, resistant cells were sensitive to the lactate/ pyruvate analogue 3-BP, which dissociates HK-II from this complex. The effect of 3-BP was enhanced by co-treatment using cisplatin in low doses. As dissociation of HK-II from mitochondria re-sensitized resistant cells to cisplatin it is suggested that resistance, at least in part, depends on HK-II.

3.3 PAPER III

Expression of mitochondrial regulators PGC1α and TFAM as putative markers of subtype and chemoresistance in epithelial ovarian carcinoma

Based on the findings that mitochondrial content was higher in the multiresistant cell line SKOV-3-R, derived from the EOC cell line SKOV-3 (previously described in **Paper II**) we sought to investigate markers of mitochondrial regulation, PGC1α and TFAM, in these cells. In addition, expression of these markers were evaluated and related to tumor subtypes in a clinical cohort consisting of 53 primary tumor samples. Clinicopathological features of the cohort can be found in **Paper III**, Table 1. We found no significant difference in median age between the subtypes, while significant differences were observed for proliferation marker Ki-67, progesterone receptor (PR) and estrogen receptor α (ERα), as well as for the distribution across histological stages.

We found that both mRNA and protein expression of the genes coding for PGC1α and TFAM (*PPARGC1A* and *TFAM*, respectively) were significantly decreased in SKOV-3-R compared to parental SKOV-3 cells (**Paper III**, Figure 1). In the clinical cohort, immunohistochemical staining for PGC1α was cytoplasmic and could be detected in 42/53 tumors, i.e. in 79% of the samples. Expression varied significantly across all EOC subtypes. Of note 8/11 samples negative for PGC1α expression were CCC tumors. We found no significant correlation between PGC1α and Ki-67 index, PR, age or histological stage. In contrast, there was a significant positive correlation between PGC1α and ERα. Expression of TFAM also varied significantly across all EOC subtypes. Positive cytoplasmic staining of TFAM was detected in 35 tumors while undetectable in 18 tumors. Of the HGSC tumors, 18 were positive and three negative, contrasted by CCC with three positive tumors and eleven tumors with undetectable levels. There was no significant correlation between TFAM expression and PR, age or histological stage. However, similar to PGC1α, there was a significant positive correlation between TFAM and ERα expression. In addition, there was a significantly higher Ki-67 index in TFAM positive compared to TFAM negative tumors (**Paper III**, Figure 2), possibly reflecting a role for TFAM in highly proliferative cells. Data on PGC1α and TFAM expression for all EOC subtypes can be found in **Paper III**, Table 2.

As TFAM is regulated in part by PGC1α (Virbasius and Scarpulla, 1994; Finck and Kelly, 2006; Ventura-Clapier et al., 2008), we investigated the co-expression of these two proteins.

EOC tumors were categorized into four groups depending on their expression of PGC1α and TFAM; PGC1α$^-$/TFAM$^-$, PGC1α$^-$/TFAM$^+$, PGC1α$^+$/TFAM$^-$ and PGC1α$^+$/TFAM$^+$. The PGC1α/TFAM expression varied significantly across the EOC subtypes. Co-expression of PGC1α/TFAM was found in 33 samples of which 16 were HGSC. Within the HGSC subtype, 76% of the tumors were double-positive for PGC1α/TFAM. In contrast, lack of both PGC1α and TFAM was observed in nine tumors. Of these nine, all but one were CCC (**Paper III**, Table 3). In addition, there was a significant difference in median Ki-67 index between the four groups, where positive TFAM expression was associated with high Ki-67 index independently of PGC1α expression. (**Paper III**, Table 3 and Figure S3).

In order to investigate if undetectable levels of PGC1α and TFAM correlated with decreased mitochondrial content we evaluated the expression of VDAC as a positive control for presence of mitochondria. Expression of VDAC was found in all samples and was equal across all EOC subtypes (not shown). Since TFAM is important for transcription of mtDNA (Kaufman et al., 2007), we also assessed protein expression of the mitochondrial encoded protein cytochrome c oxidase subunit 2 (MT-CO2). Of 51 tumors stained for MT-CO2, 47 were positive and four were negative, whereof three tumors were also TFAM negative. However, there was no significant correlation between expression of MT-CO2 and TFAM (**Paper III**, Table S1). In addition, we did not observe any correlation between PGC1α, VDAC and MT-CO2 expression wherefore we concluded that the loss of PGC1α and/or TFAM did not lead to loss of mitochondrial content.

To further investigate the decreased TFAM levels found in CCC tumors we assessed expression of the Lon protease that regulates TFAM expression via degradation (Matsushima et al., 2010; Lu et al., 2013). Cytoplasmic Lon staining was found in 38 tumors and the expression was differently distributed across all EOC subtypes. However, there was no significant correlation between expression of TFAM and Lon (**Paper III**, Table S2) wherefore we concluded that undetectability of TFAM was not due to Lon-mediated degradation.

Mitochondrial biogenesis has in several reports been linked to expression and localization of estrogen and its receptor ERα (Tcherepanova et al., 2000; Klinge, 2008). ERα is expressed at different levels in the EOC subtypes, with lowest expression in CCC (Köbel et al., 2008; Sieh et al., 2013). This was further confirmed by our data, where 12/14 CCC tumors were ERα negative. We also found a positive association between ERα expression and co-expression of PGC1α/TFAM, clearly reflected by the finding that all PGC1α$^-$/TFAM$^-$ CCC samples were ERα negative. Our results thus demonstrate that the expression of PGC1α and TFAM varied significantly between HGSC and CCC, and that co-expression correlated with that of ERα and Ki-67 index.

SKOV-3 cells have been reported to have a 32-basepair deletion in exon 1 of ERα transcript and are thus ERα positive but estrogen-insensitive (Hua et al., 1995; Lau et al., 1999). However, to further investigate the link between regulation of ERα expression, as such, and PGC1α/TFAM, levels of ERα were analyzed on mRNA and protein levels in SKOV-3

parental and resistant cells. Multiresistant SKOV-3-R cells showed significantly decreased mRNA expression of the *ESR1* gene and accordingly decreased ERα protein expression compared to parental SKOV-3 cells (**Paper III**, Figure 4a and 4b). Additionally, since accumulation of glycogen characterizes CCC (Ohkawa et al., 1977), we analyzed the intracellular glycogen levels in parental and resistant cells. SKOV-3-R were found to accumulate significantly higher levels of glycogen compared to parental cells (**Paper III**, Figure 4c). Taken together, the development of resistance *in vitro* resulted in cells with a CCC phenotype, even though they are not of CCC origin.

CCC, with the characteristic accumulation of intracellular glycogen and poor response to chemotherapy, represents a distinct clinicopathological subtype of EOC which requires new treatment strategies (Anglesio et al., 2011). Our findings support this notion and we further suggest that this phenotype is present not only in chemoresistant primary tumors, but also in treated, relapsed tumors, possibly also in non-CCC cases. Interestingly, it has been shown that ovarian serous carcinomas altered their morphology and resembled CCC after neoadjuvant chemotherapy (McCluggage et al., 2002). Additionally, in a mouse model for treatment with trastuzumab, SKOV-3 tumor xenografts were post-treatment found to be heterogeneous with large areas of ERα- negative CCC morphology (Faratian et al., 2011). Similar findings are presented in our study, as the multiresistant SKOV-3-R cells had acquired the CCC phenotype, compared to parental SKOV-3 cells.

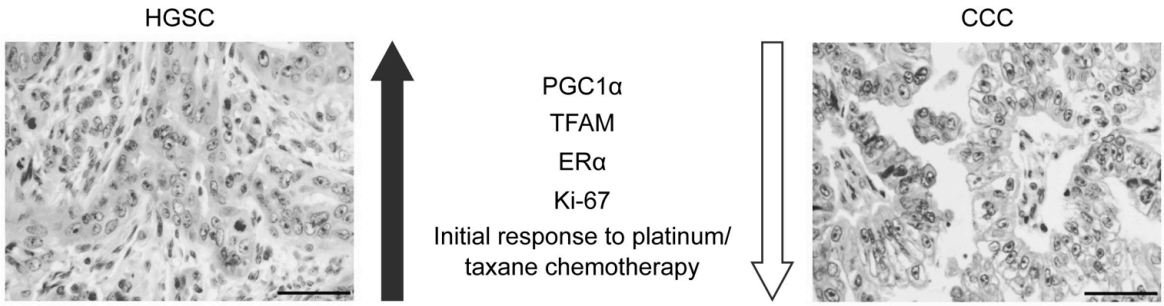

Figure 7. Suggested profiles in HGSC and CCC. Ovarian HGSC express PGC1α, TFAM, ERα, have a high Ki-67 index and are more responsive to initial platinum-based chemotherapy. A contrary profile is found in ovarian CCC, with low/undetectable levels of PGC1α, TFAM, ERα, low Ki-67 index and less response to initial chemotherapy. Hematoxylin/eosin stainings of representative HGSC and CCC tumors, magnification 400x, scale bar shows 50μM.

In summary, we identified a profile in CCC tumors consisting of undetectability of PGC1α/TFAM, and low levels of ERα and Ki-67 index. This was contrasted by HGSC tumors that were characterized by a converse state of PGC1α, TFAM and ERα expression as well as a high Ki-67 index (summarized in Figure 7). We propose that the CCC profile, and likely also glycogen accumulation, is representative not only of CCC but also chemoresistance. Altogether, our data provide insights into the development of chemoresistance as well as the diversity of subtypes in EOC and corroborate the need to develop subtype specific treatment strategies.

3.4 PAPER IV

Metabolic profiling of epithelial ovarian cancer cell lines: evaluation of harvesting protocols for profiling using NMR spectroscopy

Metabolomics is a promising approach to explore the phenotype of cancer cells since the metabolome reflects all small molecules (<1500 Da) in a biological system (Halama, 2014). However, well-designed analytical protocols are needed to accurately gain biologically relevant data (Teng et al., 2009). Although the use of *in vitro* cell models is emerging in the fields of metabolomics, no current standard for the workflow exists. Metabolomic investigations aim to analyze snapshots of ongoing biological processes, hence the harvesting method used should immediately stop all enzymatic activity and thereby any changes in metabolite levels to fulfill this aim. Leakage of intracellular metabolites is one source of error, and has been described for the use of organic solvents when harvesting cells (Sellick et al., 2009). Trypsin/EDTA treatment has also been reported to cause metabolite leakage compared to a cell scraping technique (Bi et al., 2013). Nevertheless, trypsin/EDTA is frequently combined with cell counting to later normalize the data to number of cells analyzed. However, alternative normalization strategies have been suggested (Silva et al., 2013), often in parallel samples to the ones further processed for separation techniques. A quick and straightforward harvesting protocol that allows quantification of cell amounts in samples used for analysis would be ideal.

Several harvesting techniques have been reported in the literature of which either harvesting with cold organic solvent (often methanol), or by addition of water combined with rapid liquid nitrogen freezing are the most common ones (Teng et al., 2009; Bi et al., 2013; León et al., 2013). In the present study, we aimed to compare these two methods in adherent ovarian cancer cell lines by detection of the polar fraction of the metabolome using ^{1}H NMR spectroscopy.

To further understand how alterations in cellular metabolism are linked to progression and chemoresistance, our objective was also to characterize the polar metabolome and detect differences between SKOV-3 parental and the multiresistant sub line SKOV-3-R, previously described in **Paper II** and **III**. We thus used SKOV-3 and SKOV-3-R to evaluate harvesting protocols in terms of reproducibility and ability to classify metabolite differences in the metabolomes of these cells. Moreover, we identified significant differences in the polar metabolome between the two cell lines.

For each cell line, six biological replicates were harvested in parallel using the two different techniques, cold methanol (MeOH) or MilliQ freeze/thaw, generating 24 samples in total. In order to study variation during extraction and data acquisition, three samples per protocol and cell line were divided in two prior to metabolite extraction, generating additional technical replicates. Two pooled quality control (QC) samples, one for the MeOH protocol and one for the MilliQ freeze/thaw, were also created by pooling equal volumes of cell extracts. The sample set for analysis thus consisted of 38 samples; nine samples per cell line and protocol

plus two QC samples. Except for the different harvesting protocols, all additional sample handling such as culturing of cells, metabolite extraction and NMR analysis were performed in an identical manner. In order to evaluate protein concentration as a normalization strategy, an insignificant volume was removed from samples harvested with MilliQ freeze/thaw to assess protein concentration using a spectrophotometric assay. Protein concentration could not be measured in samples harvested using MeOH due to protein precipitation.

A low variability in biological replicates is essential for metabolomic studies since it indicates high reproducibility, hence increases the probability to detect small differences between samples. Here, experimental reproducibility was evaluated by determination of the coefficient of variation (CV) for each bin in the NMR spectra. Averaged CVs were lower for MilliQ freeze/thaw harvesting compared to MeOH in both cell lines, hence the MilliQ freeze/thaw method generated data with lower experimental variability than the MeOH protocol. Regardless of harvesting protocol, the parental SKOV-3 cells exhibited considerable lower variance than SKOV-3-R, indicating that resistance development increased the heterogeneity between biological replicates.

When comparing the two harvesting techniques, all data were normalized to total NMR intensity, and multivariable data analysis was performed using both unsupervised and supervised statistical procedures. Unsupervised principle component analysis (PCA) models, generated for both cell lines in order to identify differences in metabolic profiles resulting from the harvesting method used, indicated minor sample clustering. Thus, we could conclude that both harvesting protocols resulted in very similar metabolite profiles in SKOV-3 and SKOV-3-R. However, S-line plots[14] from supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) models revealed minor differences in metabolite profiles, based on the choice of harvesting protocol. Levels of creatine and glycine differed significantly between harvesting protocols in SKOV-3, while only glycine levels were significantly altered in SKOV-3-R (data not shown, for p-values see **Paper IV**).

Unsupervised PCA models were also used to evaluate the harvesting techniques. Both MeOH and MilliQ freeze/thaw harvesting displayed distinct clustering of SKOV-3 and SKOV-3-R according to cell line, indicating that they display different metabolic profiles (**Paper IV**, Figure 1). OPLS-DA was used to evaluate the classification power of models for the different harvesting protocols. For each harvesting technique, the sample set was randomly divided into a training set consisting of 2/3 of the samples and a test set with the remaining 1/3 samples. While the training sets were used to create models of the differences between SKOV-3 and SKOV-3-R, the test sets were used to evaluate the predictive power of the generated models. It was possible to correctly classify all samples, suggesting that both protocols can be used to examine differences in the metabolome of SKOV-3 and SKOV-3-R (**Paper IV**, Figure 2).

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[14] S-line plots are used to visualize the OPLS-DA model. It combines the covariance and correlation in a scatter plot and can be used for extraction of putative biomarkers.

S-line plots from OPLS-DA models revealed differences in the polar metabolome between the two cell lines. Major alterations found in SKOV-3-R were decreased levels of creatine, glycine, lactate, leucine and myo-inositol and increased levels of glutamate and glycerophosphocholine (GPC), compared to the metabolome of SKOV-3. Subsequent t-testing showed that all the presented alterations, except for lactate, were significant and consistent for both harvesting techniques (**Paper IV**, Figure 3). Similarities, and dissimilarities, between two multivariate models can be displayed in a shared and unique structure plot (SUS-plot), where similarities are found on the positive diagonal while dissimilarities are displayed on the negative diagonal. Here, the similarity in detection of metabolite differences between MeOH and MilliQ freeze/thaw harvesting protocols was confirmed, since the differences in metabolic profiles between cell lines are found on, or close to, the positive diagonal line (**Paper IV**, Figure 4).

The differences found here in the polar metabolome between parental SKOV-3 and the multiresistant subline SKOV-3-R, that harbors TIC features, are in accordance with recent metabolic studies. Glutamate, increased in SKOV-3-R, has been found at higher levels in EOC primary tumors, and even more so in omentum metastasis, compared to normal tissue (Fong et al., 2011). Decreased levels of glycine in SKOV-3-R are in line with low glycine levels detected in lung cancer TICs due to upregulation of glycine decarboxylase (GLDC) (Zhang et al., 2012b). Regarding GPC, upregulated in SKOV-3-R, it was reported that high levels were associated with worse prognosis in pre- and post-treatment biopsies from breast cancer patients (Cao et al., 2012). However, the comparison of the metabolomes of SKOV-3 and SKOV-3-R was here used to statistically evaluate harvesting techniques, not to prove any biological significance. Hence, further investigation of the altered metabolites of SKOV-3-R cells is needed to evaluate how these alterations affect the resistant phenotype.

In this study, two common cell harvesting methods, cold MeOH and MilliQ freeze/thaw, were evaluated with focus on reproducibility and model classification power. Since both protocols were shown to identify the same alterations between SKOV-3 and SKOV-3-R, we conclude that either protocol is appropriate for cell-based metabolomic investigations. Nevertheless, MeOH and MilliQ freeze/thaw provides different prospects for further protocol refinements. Firstly, the use of an aqueous solvent, instead of an organic one, allows a more rigid control of pH during metabolite extraction. Thus, of importance for targeted metabolomics, this makes it possible to control which classes of metabolites that are retained in the aqueous phase during liquid-liquid extraction and influence the recovery of these metabolites. In addition, MilliQ freeze/thaw protocol allows quantification of protein and/or DNA using standard techniques in harvested samples useful for normalization of data. Here, normalization to protein concentrations was performed in MilliQ freeze/thaw samples to investigate the differences between the normalization strategies. Evaluation of protein concentration versus total NMR intensity revealed four clearly deviating samples, and a linear regression coefficient (R^2) for the remaining samples equal to 0.84. The correlation was lower than expected, possibly reflecting the low precision of the spectroscopic method used to assess protein concentration compare with the high precision NMR. However, observed

differences in metabolic profiles of SKOV-3 and SKOV-3-R showed a high degree of similarity, regardless of normalization strategy used (data not shown).

To summarize, we identified significant alterations in the intracellular polar metabolome of SKOV-3-R involving decreased levels of creatine, glycine, leucine and myo-inositol and increased levels of glutamate and glycerophosphocholine, compared to SKOV-3 cells. Furthermore, two harvesting protocols, MeOH and MilliQ freeze/thaw, were statistically evaluated and compared. The MilliQ freeze/thaw protocol showed increased reproducibility and allows for more options during metabolite extraction and data normalization, thus represents a tailored and reliable protocol for metabolic profiling of adherent cells.

4 CONCLUSIONS

I. Several populations of potential TICs exist in malignant EOC ascites, highlighting a biological heterogeneity and epithelial-mesenchymal plasticity in these tumors. The likely presence of CAFs may further contribute to this heterogeneity and expression of CAF marker α-SMA were increased in advanced-stage disease. Furthermore, transcription factor Oct-4A can be sumoylated in EOC, although the clinical significance of this finding needs further evaluation.

II. Long-term repeated cisplatin treatment can select for and/or induce a multiresistant cell population with TIC and EMT features. Resistance partly depends on upregulation of VDAC and HK-II, forming an anti-apoptotic complex on the mitochondria. The lactate/ pyruvate analogue 3-BP, that dissociates this complex, is thus a treatment candidate to target these cells.

III. Ovarian CCC and HGSC have different profiles with regard to expression of mitochondrial regulators PGC1α and TFAM. The undetectability of these markers in CCC is associated with low levels of ERα and decreased Ki-67 index, in clear contrast to HGSC that express PGC1α, TFAM and ERα and have high Ki-67 index. The CCC profile, and likely also glycogen accumulation, is representative not only of CCC but also chemoresistance. These findings provide insights into the development of chemoresistance, demonstrate subtype diversity and stress the need for subtype specific treatment strategies.

IV. The MilliQ freeze/thaw harvesting protocol is optimal for metabolic profiling of adherent cells since it is highly reproducible and allows for optional refinements of metabolite extraction and data normalization. Moreover, significant alterations in the intracellular polar metabolome of SKOV-3-R cells involves decreased levels of creatine, glycine, leucine and myo-inositol and increased levels of glutamate and GPC, compared to SKOV-3 cells. These alterations are compatible with reported features of TICs but the biological significance of these alterations remains to be investigated.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Firstly, this thesis provides insights into the biology of EOC ascites. We reported that several populations of potential TICs and likely also CAFs exist in malignant ascites. Secondly, we show that cisplatin can induce and/or select for TIC properties and that chemoresistance can be linked to metabolic alterations. We also demonstrate EOC subtype-specific profiles with regard to expression of mitochondrial regulators. In addition, we established a tailored and reliable protocol for metabolic profiling that can be used to further investigations of such mitochondrial/metabolic phenotypes.

CAF identification (**Paper I**) was based on commonly used CAF markers α-SMA and PDGFRβ. However, we did not evaluate if this expression was found in tumor cells or in a contaminating cell population. CAF markers were only expressed in the vimentin^{pos} M-type samples. In order to distinguish between tumor cells and fibroblasts one could analyze expression of cytokeratin 8 (CK-8) to distinguish tumor cells and fibroblasts, since EOC cells have a vimentin^{pos}/CK-8^{pos} profile in contrast to vimentin^{pos}/CK-8^{neg} fibroblasts (personal communication; Professor Arne Östman). Furthermore, one might also study the ability of cell-free ascites fluid to induce CAF markers in tumor cells and fibroblasts. Indeed, our unpublished data revealed that cell-free ascites from samples used in **Paper I** could induce expression of α-SMA in hTERT-immortalized foreskin fibroblasts, but not in the EOC cell line SKOV-3. While cell-free ascites has been shown to induce growth, invasion and sphere-formation in cancer cell lines (Puiffe et al., 2007; Carduner et al., 2014), the effect on fibroblasts remains uninvestigated. An ideal cell-model for such studies would be normal or immortalized fibroblasts from the ovary, fallopian tube or peritoneum. Functional studies may also be performed, for instance to examine if ascites-treated fibroblasts can stimulate tumor cell growth. Antibody arrays and/or enzyme-linked immunosorbent assay (ELISA) can then be used to correlate levels of selected cytokines, chemokines and growth factors to increased CAF marker expression and functional read-outs. Such studies would improve our understanding of the pathophysiology of malignant ascites.

The presence of different populations expressing TIC markers highlights a biological heterogeneity and epithelial-mesenchymal plasticity in EOC that warrants further studies. Cell sorting for one or two markers will fail to cover this diversity. In general, since TICs are suggested as targets for future therapy, research should aim to identify differences between TICs and normal stem cells to avoid fatal side effects of such treatment. TICs have been suggested to sustain specific metabolic alterations (Scatena, 2012; Pecqueur et al., 2013; Ito and Suda, 2014) which might comprise possible therapeutic targets. Our results regarding the TIC-like SKOV-3-R cells (**Paper II-IV)** support the notion of particular alterations, including decreased glycine levels.

In **Paper II** we showed that cisplatin can select for and/or induce a highly multiresistant cell phenotype with EMT and TIC features. Similar findings have been reported not only for cisplatin (Latifi et al., 2011) but also paclitaxel (Kajiyama et al., 2007; Craveiro et al., 2013)

and for cisplatin-paclitaxel combination treatment (Abubaker et al., 2013). In contrast, there are no reports on carboplatin-induced TIC and/or EMT phenotypes in EOC, even though sorted TIC populations in general are resistant also to this drug. Nevertheless, carboplatin was found to induce self-renewal and pluripotency in hepatocellular carcinoma cells (Hu et al., 2012). We addressed the question and established carboplatin-resistant EOC sublines using a similar protocol as in **Paper II** with long-term treatment for several passages and increasing doses. Similar to SKOV-3-R cells, the carboplatin resistant subline SKOV-3-CaR went through EMT and showed increased motility and invasivity (unpublished data). However, in the SKOV-3-CaR cells, this was not associated with TICness, suggesting that these two events are not always linked and that the underlying differential signaling can be investigated.

The SKOV-3-CaR cells showed similar mitochondrial alterations as the SKOV-3-R cells, i.e. increased mtDNA:nDNA ratio and mitochondrial mass (**Paper II** and unpublished data), and we therefore sought to investigate mitochondrial regulation in these cells. We found that expression of PGC1α and TFAM were downregulated in these cells, even though we were expecting the opposite. Expressions of these markers were also assessed in an EOC cohort (**Paper III)**. We discovered that ovarian CCC and HGSC have different expressional profiles of mitochondrial regulators PGC1α and TFAM. Our findings should be validated in a bigger cohort and, importantly, further linked to clinical parameters, such as platinum-free interval, progression-free survival and overall survival. Other molecular events in the suggested profiles can also be evaluated, such as p53 status (commonly mutated in HGSC, wild-type in CCC). The CCC profile was further linked to development of resistance *in vitro* since the SKOV-3 sublines acquired a CCC phenotype even though they are not of CCC origin and p53 mutated. These cell lines thus represent a good model for further evaluation of PGC1α and/or TFAM to define their role in chemoresistance and tumor progression.

Additional alterations in SKOV-3-R cells were revealed when measuring mitochondrial respiration (using Seahorse XF, in collaboration with Professor Pierre Sonveaux, unpublished data). Although basal respiration was unchanged, resistant cells had increased reserve respiratory capacity, which has been linked to increased ability to withstand oxidative stress (Hill et al., 2012). Since PGC1α is an important regulator of antioxidant defense (Valle et al., 2005; St-Pierre et al., 2006), we hypothesized that reactive oxygen species (ROS) levels would be altered in these cells. However, this was not the case. One can speculate that ROS regulation is directed in a different manner in resistant cells and interesting candidates to study would be NRF1, NRF2 and PGC1β.

We also addressed if the mitochondrial alterations in resistant sublines, were due to mtDNA mutations by sequencing the mitochondrial genomes of two EOC cell lines and their resistant sublines. Unpublished results showed no mutations that could explain the phenotype, and we concluded that the mitochondrial alterations were not driven by damaged/dysfunctional mtDNA.

Investigation of PI3K/Akt signaling in these cells would also be interesting. Activated Akt inhibits the transcriptional activity of PGC1α (Li et al., 2007b) as well as that of glycogen

synthase kinase 3β (GSK3β). Inhibition of GSK3β has been linked to induction of EMT (Kao et al., 2014) and also increases the activity of glycogen synthase. This might be linked to glycogen accumulation and additional alterations related to the CCC profile/chemoresistant phenotype. Interestingly, activated Akt also promotes mitochondrial HK-II (Gottlob et al., 2001), found to be important for resistance in **Paper II**.

The role of HK-II in resistance, EMT and TICness remains to be investigated in more detail. In **Paper II**, we show that multiresistant cells sensitive to 3-BP treatment and suggested that it was due to targeting of mitochondrial-associated HK-II. However, 3-BP has additional targets (Shoshan, 2012b) and other drugs that cause HK-II dissociation, such as the antifungal azole derivatives clotrimazole and bifonazole, could be used to further investigate this mechanism of resistance. Evaluation of drug response, as well as TICness, would benefit from *in vivo* models in addition to *in vitro* cell culture procedures.

HK-II, PGC1α and TFAM are all important for the regulation of cellular energetics. Metabolomics will be a useful tool to characterize how these markers affect the metabolome. For instance, the glycogen accumulation seen in the CCC phenotype is an interesting metabolic alteration that needs further investigation. Parallel samples to the ones used for ^{1}H-NMR analysis in **Paper IV** were analyzed using HRMS and evaluation of data is currently ongoing. These data provide insights regarding therapy-induced chemoresistance. Additional studies using targeted metabolomics will be used for studies on specific metabolic pathways in resistant cells as well as in PGC1α siRNA-knockdowns.

In summary, this thesis provides insights in metabolic alterations of chemoresistance and TIC phenotypes in EOC, and further supports the need to develop subtype- and/or phenotype-specific treatment strategies.

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