An ovarian cancer stem cell study: Regulation of cell stemness and the role of cancer stem cell-related markers in patient outcome

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ABBREVIATIONS

ABC	ATP-binding cassette
APC	Allophycocyanin
ATCC	American Type Culture Collection
BMI	Body mass index
CA 125	Cancer antigen 125
CA 19-9	Carbohydrate antigen 19-9
CAFs	Cancer-associated fibroblasts
CCC	Clear cell carcinoma
CEA	Carcinoembryonic antigen
CR	Complete remission
CSCs	Cancer stem cells
CXCL7	Chemokine C-X-C motif ligand 7
DEGs	Differentially expressed genes
DHT	5α-dihydrotestosterone
DMSO	Dimethyl sulfoxide
E2	Beta-estradiol
ECL	Enhanced chemiluminescence
EMT	Epithelial Mesenchymal Transition
EOC	Epithelial ovarian cancer
EtBr	Ethidium Bromide
FBS	Fetal bovine serum
FCM	Flow cytometry
FIGO	International Federation of Gynecology and Obstetrics
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
gDNA	Genomic deoxyribonucleic acid
HE4	Human epididymal protein 4
HGF	Hepatocyte growth factor
HGSC	High-grade serous carcinoma
HRT	Hormone replacement therapy
ICC	Immunocytochemistry
ICC	Immunocytochemistry
IF	Invasive front
IHC	Immunohistochemistry

IL-6	Interleukin 6
LGSC	Low-grade serous carcinoma
MDR	Multidrug resistance
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
nDNA	Nuclear deoxyribonucleic acid
OCP	Oral contraceptive pills
OS	Overall survival
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline-tween 0.05%
PCR	Polymerase chain reaction
PE	Hycoerythrin
PFI	Progression-free interval
PFS	Progression free survival
PVDF	Polyvinylidene difluoride
RNA-seq	RNA sequencing
RPMI	Roswell Park Memorial Institute
RRSO	Risk-reducing salpingo-oophorectomy
SCF	Stem cell factor
SHBG	Sex hormone-binding globulin
SP	Side population
SP	Side population
TME	Tumour microenvironment
TNFalpha	Tumour necrosis factor alpha
TVUS	Transvaginal ultrasound
TZ	Transitional zones
WB	Western Blotting
WHO	World Health Organization

LIST OF PAPERS

I. **Huang R**, Wang J, Zhong Y, Liu Y, Stokke T, Trope CG, Nesland JM, Suo Z. Mitochondrial DNA deficiency in ovarian cancer cells and cancer stem-like probabilities. (Submitted)

II. **Huang R**, Ma Y, Holm R, Trope CG, Nesland JM, Suo Z. (2014) Sex hormone-binding globulin (SHBG) expression in ovarian carcinomas and its clinicopathological associations. PLoS ONE 8: e83238.

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IV. **Huang R**, Li X, Holm R, Trope CG, Nesland JM, Suo Z. The expression of aldehyde dehydrogenase 1 (ALDH1) in ovarian carcinomas and its clinicopathological associations: a retrospective study. (Submitted)

1 INTRODUCTION

1.1 Ovarian cancer

Ovarian cancer includes three types by the origin: epithelial ovarian cancer, germ cell tumour and stromal cell tumour. Epithelial ovarian cancer (EOC), also called ovarian carcinoma, typically begins in the epithelial cells on the surface of an ovary which holds a proportion of 85% to 90% in ovarian cancers [1]. Germ cell tumours develop in the germ cells of an ovary and happen more frequently for women aged from 10 to 29 (http://www.cancer.net/). Stromal cell tumour usually develops in the connective tissue cells and is a rare type of ovarian cancer.

In recent decades new evidence suggests at least some of ovarian cancer actually begins in special cells in the fallopian tube which locates near the ovary and may transfer to the surface of the ovary in the early cancer process. The term 'ovarian cancer' in this thesis is used to describe epithelial cancers that begin in the ovary, the fallopian tube and the peritoneum, which is the same meaning of ovarian carcinoma or EOC.

1.1.1 Epidemiology

Ovarian cancer is the fifth to seventh most common cancer in women [2-5], and it has the highest mortality rate among gynaecologic malignancies [5, 6]. According to the cancer statistics of GLOBOCAN 2012, 2.39 million new cases were diagnosed wordwide and 1.52 million deaths of ovarian cancer occurred [3]. In European countries, ovarian cancer is the ninth most common cancer in women with 65.5 thousands of new cases and 42.7 cases of death in 2012 [7]. The incidence of ovarian cancer is higher in white women compared to African-American women, but the African-American women have a more poor survival and higher mortality [8]. The racial disparities have increased over time [9], partly due to differences in treatment, such as receipt of surgery [9, 10]. The incidence of ovarian cancer in Norway is around the average level of the European countries, but the mortality is much higher than the average level (Figure 1, EUCAN 2012).

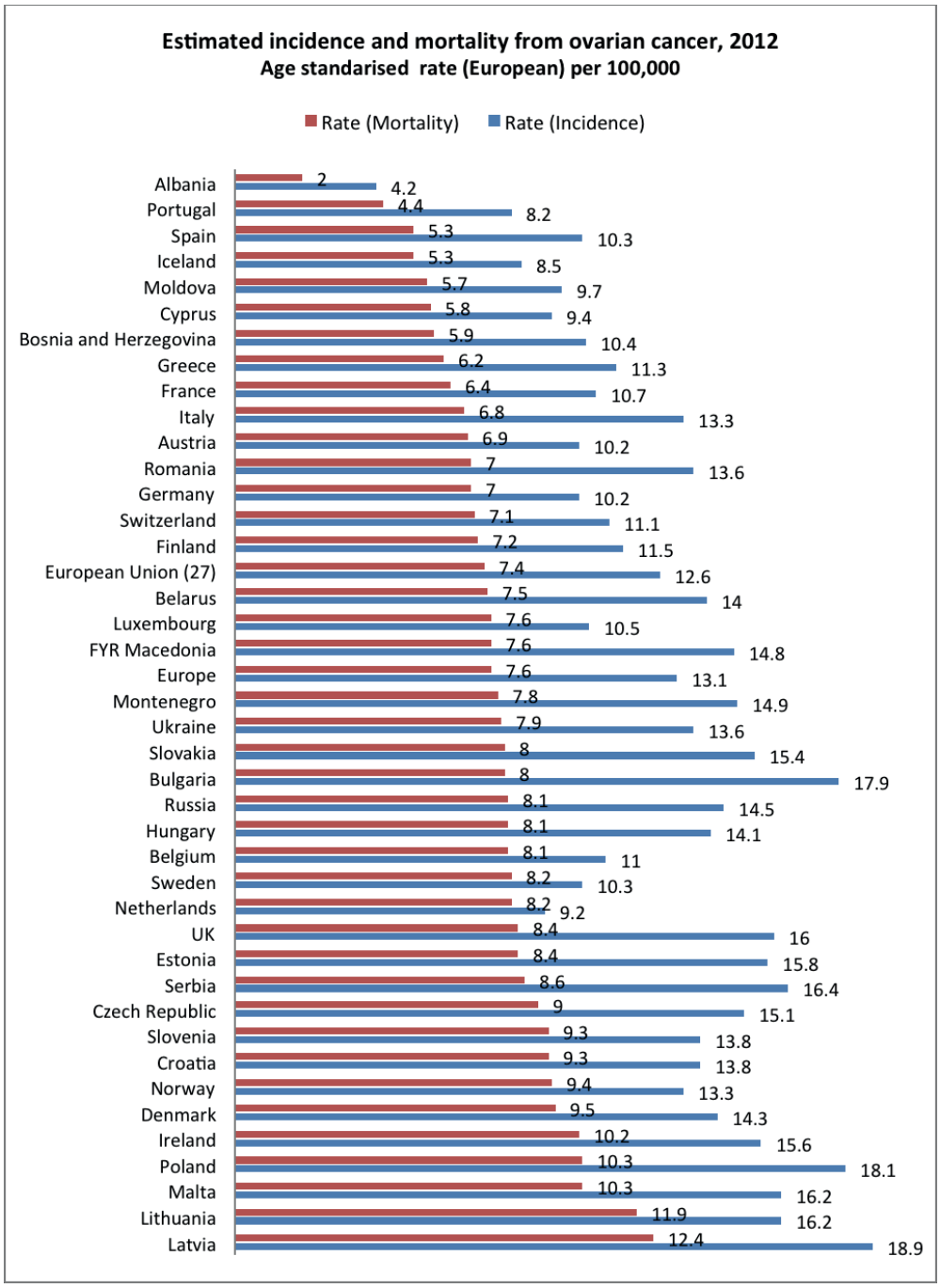

Figure 1. Estimated incidence and mortality rate from ovarian cancer, Europe 2012 (Reproduced by the statistics from EUCAN 2012)

1.1.2 Etiology

We still do not acknowledge the specter of gene mutations leading to ovarian cancer [5], and more studies are warranted to better understand this deadly disease. Several risk factors which may contribute to the incidence of ovarian cancer have been high-lightened and the risk factors for different subtypes of ovarian cancer may differ from each other, especially among the EOCs [11][12].

1.1.2.1 Age

The median age at diagnosis for ovarian cancer is around 63 years old, and thus, most ovarian cancers develop after menopause. It is most frequent on women ages at 55-64 years old.

1.1.2.2 Obesity

Compelling evidence shows that obese women who have a higher body mass index (BMI) have a higher risk to develop ovarian cancer [13-15], and may have poor clinical outcomes including overall survival (OS) [16]. Ovarian cancer patients with diabetes mellitus have a poor progression free survival (PFS) and OS [17]. However, some other studies point out that obesity may increase the risk of getting ovarian cancer, but does not affect the long-term survival outcomes [18, 19].

Obesity is a physiological state associated with alterations in hormone, especially estrogen production and metabolism [20, 21], therefore, this factor may have a similar mechanism with a risk factor hormone which will be discussed later in this part.

1.1.2.3 Reproductive history

Full term pregnancy is inversely associated with ovarian cancer risk [12]. Each pregnancy may lead to a 10%-16% reduction of ovarian cancer risk [22]. A large cohort study on 64,185 Japanese women covered from 1988 to 2009 showed that nulliparous and nullipregnant women did get an increased risk of ovarian cancer [23]. Women giving the first birth at late age have an elevated risk of ovarian cancer [24-26]. The time interval between first and last birth seems not influence the risk of ovarian cancer

[27]. Strong evidence shows that breast-feeding, especially a longer duration of breastfeeding may decrease the risk of ovarian cancer [28-30].

1.1.2.4 Gynaecologic surgery

Tubal ligation is reported to reduce the risk of ovarian cancer [31], particularly when conducted before the age of 35 years [32]. Unilateral oophorectomy was associated with a 30% lower risk [32]. Hysterectomy is associated with lower risk of ovarian cancer, especially for nonserous types [32]. Prophylactic bilateral oophorectomy at time of hysterectomy is more effective to reduce the risk of ovarian cancer, but it is still largely controversial whether it is beneficial for young women, particularly for those at low-risk of ovarian cancer [33].

1.1.2.5 Hormone

Women have a lower risk of ovarian cancer if they have taken or currently take oral contraceptive pills (OCP) [12, 34, 35]. Application of OCP for more than three years may lead to a 30%-50% reduction of ovarian cancer risk [36]. Treatment with progestin alone or in combination with estrogen may decrease the prevalence of ovarian cancer, and ovulation may reduce the risk of ovarian cancer [37].

Large cohort studies show that postmenopausal women who have used hormone replacement therapy (HRT) have an obviously increased risk of both the incidence and the mortality of ovarian cancer [12, 38, 39]. The impact of HRT for the development of ovarian cancer may be slightly different for the multiple types, but the existed evidence is consistent for the two most common types serous and endometrioid tumours. This risk was similar in European and American prospective studies and for oestrogen-only and oestrogen-progestagen therapies [40, 41].

The use of drugs to treat infertility (gonadotropin releasing hormone antagonists or clomiphene) may also increase the risk of ovarian cancer, and it is thought to be associated with high concentrations of estrogen stimulation [42]. It is still discussed whether there is any association with the use of fertility drugs and ovarian cancer risk, and more studies are warranted [43, 44].

1.1.2.6 Family history

The risk for a woman to get ovarian cancer is increased if she has a family history, that is to say, her sister or mother or daughter has or has had ovarian cancer [45, 46].

1.1.2.7 Molecular predictors

Some ovarian cancers are a part of family cancer syndromes resulting from inherited mutations of certain genes including the oncogenes HER2, C-myc, K-ras, Akt, and the tumour suppressor gene p53, among which mutations of BRCA1 and BRCA2 are highlighted [47]. This syndrome is linked to a high risk of many cancers, among which breast cancer and ovarian cancer are highlighted [48, 49]. Based on this the development of poly (ADP-ribose) polymerase (PARP) inhibitors was prompted as a treatment for BRCA mutation associated ovarian cancer [48-50].

1.1.3 Symptoms

Ovarian cancer may cause several signs and symptoms, particularly when it has been spread beyond the ovaries. The most common symptoms include: unusual bloating, unusual pelvic or abdominal pain, pressure in the abdomen, trouble eating or feeling full quickly, lack of energy, and urinary symptoms such as urgency or frequency [51-53]. Other symptoms of ovarian cancer can include: fatigue, upset stomach, back pain, pain during sex, constipation, menstrual changes, abdominal swelling with weight loss, but these symptoms can be caused by other cancers and benign diseases as well. When they are caused by ovarian cancer, they tend to be persistent and represent a change from normal. (American cancer society, http://www.cancer.org/).

1.1.4 Screening

When ovarian cancer is found early at a localized stage, the five-year survival rate is over 94%. Approximate seventy-five percent of women with ovarian cancer are diagnosed at advanced-stage (III or IV) [54]. Women ignoring their symptoms were significantly more likely to be diagnosed with advanced disease [55]. There has been a lot of research to develop an effective screening test for

ovarian cancer, but without wide-spead use. However, early diagnosis has become urgent, especially for high risk women with BRCA1/BRCA2 mutations.

A pelvic examination is routinely in used diagnosis of ovarian cancer and other gynaecologic cancers [56]. Several studies in various countries have recommended bimanual pelvic examination to be an annual screening test for ovarian cancer [57, 58]. However, it is not accurate as a screening method for ovarian cancer and to distinguish benign from malignant diseases [59]. In a typical screening population, the positive predictive value of an abnormal pelvic examination is approximately 1%.

The blood test of cancer antigen 125 (CA 125) and ultrasound are two of the most common tests to screen for ovarian cancer at present. Ultrasonography of the abdomen and pelvis is usually the first imaging investigation recommended for women in whom ovarian cancer is suspected [60]. Transvaginal ultrasound (TVUS) has improved the visualisation of ovarian volume and morphology, thus is accurate in detecting abnormalities in ovarian [61-63], but is less reliable in differentiating benign from malignant ovarian tumours [64]. Therefore, serum biomarkers such as CA 125 are often used together with TVUS to identify ovarian cancer in high risk population. However, serum CA 125 blood test is not specific for ovarian cancer. In other words, people may get an increased serum CA 125 level even when they have other benign diseases instead of ovarian cancer, and not all patients diagnosed with ovarian cancer have increased serum CA 125 levels [60].

For BRCA1/2 mutation carriers, Risk-reducing salpingo-oophorectomy (RRSO) may lower the mortality from ovarian and tubal cancers [65]. Nevertheless, women at average risk for ovarian cancer, using TVUS and CA-125 for screening may lead to more intense repeated testing, more surgeries and more psychological morbidity [66-68], and the mortality caused by ovarian cancer may be not decreased [68-70]. Therefore, the routine use of TVUS or the CA-125 blood testing to screen for ovarian cancer is not widely in use[67]. Better ways to screen for ovarian cancer are being explored.

1.1.5 Diagnosis

Ovarian cancer is often diagnosed by symptoms (see above), clinical assessment, laboratory testing and other examinations. Few or no symptoms may be found for patients with ovarian cancer confined to the ovary, making diagnosis of early stage ovarian cancer very challenging [60]. Symptoms are most commonly seen in advanced stage (stage III or IV), which compromised approximate seventy-five percent ovarian cancer [54].

Measurement of serum CA 125 is routinely in use to help diagnosis if ovarian cancer is suspected. However, CA 125 lacks accuracy and specificity [60]. Serum carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19–9) and human epididymal protein 4 (HE4) levels are sometimes measured when it is difficult to identify whether an ovarian mass is of gastrointestinal origin, or a primary ovarian tumour [71]. In these situations, colonoscopy and gastroscopy may be considered as well, particularly when CA 125/CEA ratio is ≤25 [60]. However, these markers are all inadequate to diagnose ovarian cancer. Therefore more studies are warranted to identify better biomarkers for diagnosis and prognosis [72].

Ultrasound, especially TVUS, may be considered as the first imaging investigation for suspected ovarian cancer patients. Computed tomography (CT) scans are routinely used to determine the extent of disease and to aid in surgical planning. Confirmed diagnosis of ovarian cancer is made by histopathological diagnosis of surgical samples considering all the above examination results [60].

1.1.6 Histology and molecular classification

According to World Health Organization (WHO) classifications, EOC can be classified into several subtypes according to the histological features including serous, endometrioid, clear cell (CCC), mucinous, Brenner (transitional cell), undifferentiated, unclassified and mixed epithelial tumours [73, 74]. Among the above histological subtypes, serous carcinoma is the most common type which composed up to 80% of EOC [60].

Recently some researchers divide ovarian carcinomas into five main types that account for over 95% of cases, based on histopathology and molecular genetic alterations: high-grade serous carcinoma (HGSC) (70%), endometrioid carcinoma (10%), CCC (10%), mucinous carcinoma (3%), and low-grade serous carcinoma (LGSC) (<5%) [75]. HGSC and LGSC were not classified in the initial clinical data in this thesis.

In addition to histological subtypes, ovarian carcinomas can be subclassified into the following types based on the degree of differentiation (differentiation grade): well differentiated (G1), moderately differentiated (G2), poorly differentiated (G3) and Grade which cannot be assessed (Gx) [76]. In the papers involved in this thesis, G1, G2 and G3 were enrolled for clinical data analyses while Gx and other missing data were excluded.

Molecular testing is becoming broadly recommended nowadays for early detection, prevention and therapeutic strategies. EOC has recently been classified into Type I and Type II tumours according to their clinical behaviours and molecular differences. Type I tumours typically grow slowly and are therefore often confined to the ovary. They are less sensitive to standard chemotherapy, which include LGSC, low grade clear cell, endometrioid, and mucinous cancers. Type I tumours are characterized by specific mutations, including KRAS, BRAF, ERBB2, CTNNB1, PTEN, PIK3CA, ARID1A, and PPP2R1A [77]. BRAF and KRAS somatic mutations are relatively common in these tumours, particularly in low grade serous, mucinous types [78, 79], which may have important therapeutic indications. On the other hand, type II tumours are clinically aggressive and are often widely metastatic when diagnosed. This type includes HGSC, high grade endometrioid cancers, malignant mixed carcinomas and undifferentiated tumours. Type II tumours rarely have the above mutations shown in type I tumours [77, 80], but they display high levels of genomic instability including mutation or methylation of BRCA genes and a very high frequency of TP53 mutations [77, 81]. PIK3CA and RAS signaling pathways are altered in 45% of the cases [80]. More molecular studies are being studied.

Extensive molecular studies contribute to novel diagnostic options and personalized treatment for EOC patients. In a recent study, EOC was divided into five distinct subtypes according to the

molecular subtypes, and each subtype displayed significantly different gene expression patterns, deregulated pathways and patient prognoses, verified using independent datasets [82]. For instance, Stem-A subtype, which reveals a poor prognosis in patients, was found to be involved in tubulin processes including the pathway and expression of related genes TUBGCP4 and NAT10. This subtype was indeed turned out to be more sensitive to inhibitors of tubilin polymerization like vincristine and vinorelbine [82]. It is envisaged that more molecular studies are required for the development of powerful diagnosis and therapies in EOC.

1.1.7 Staging

Ovarian cancer is staged surgically and pathologically. The FIGO staging classification, which was used in the current thesis, was first implemented in 1988 by Rio de Janeiro [76]. It remains most powerful method today [60, 83], although there is some slight modification recently [84].

Table 1. FIGO staging classification 1988 version (Rio de Janeiro) [76]

Stage I	**Growth limited to the ovaries**
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surface; capsule intact
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumour on the external surfaces; capsules intact
Ic[a]	Tumour either Stage Ia or Ib, but with tumour on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage II	Growth involving one or both ovaries with pelvic extension
IIa	Extension and/or metastases to the uterus and/or tubes
IIb	Extension to other pelvic tissues
IIc[a]	Tumour either Stage IIa or IIb, but with tumour on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage III	Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum
IIIa	Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histological proven extension to small bowel or mesentery
IIIb	Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2cm in diameter; nodes are negative
IIIc	Peritoneal metastasis beyond the pelvis >2cm in diameter and/or positive retroperitoneal or inguinal nodes
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV

[a]In order to evaluate the impact on prognosis of the different criteria for allotting cases to stage Ic or IIc, it would be of value to know whether rupture of the capsule was spontaneous, or caused by the surgeon and whether the source of malignant cells detected was peritoneal washings or ascites.

1.1.8 Treatment

Surgery followed by platinum-based chemotherapy is the standard treatment, although individualised assessment and management may take place. Early disease may be successfully treated with surgery alone; advanced disease may require chemotherapy and other complex treatments [85, 86]. Platinum/taxane chemotherapy has long been considered as the standard regimen for advanced disease [87]. For most patients, either with newly diagnosed or with recurrent disease, surgery would be optimal recommended if it is practicable. The aim of surgery for early ovarian cancer is to remove the tumour and to undertake adequate staging classification, which will provide prognostic information and define whether chemotherapy is needed [60].

New treatments such as targeting therapy are developing and becoming more and more popular in clinical treatments. For example, bevacizumab has shown promising improvement of ovarian cancer outcomes [85].

Nevertheless, approximately 85% of EOC patients who achieve CR following first-line therapy will develop recurrent disease [54]. One factor that may help with the treatment planning is the primary progression-free interval (PFI). Patients can be divided into those who are platinum-sensitive (PFI > 6 months) vs platinum-resistant (PFI ≤ 6 months) [88]. The OCEANS study in women with platinum-sensitive recurrent disease showed that the addition of bevacizumab to carboplatin/gemcitabine chemotherapy increased toxicity with no improvement in OS, despite an improvement in response rate and PFS [89]. Combination therapy without bevacizumab could generally be appropriate for recurrent diseases, while single agent bevacizumab can be reserved for subsequent use [90].

1.1.8 Prognosis

The prognosis of ovarian cancer patients is predicted by many factors, such as surgical residues, ascites, FIGO staging, histological classifications, genetic changes, etc. In addition, plenty of biomarkers are being tested both in the serum and tumour tissues to improve prediction of the potential prognosis for EOC patients. For instance, while some studies showed that serum CA 125 level may have predictive role in EOC survivals [91, 92], other studies found that the preoperative serum level of

a novel molecular marker HE4 seemed to play more predictive role than the traditional marker CA 125 for the platinum response and the survivals of EOC patients [93, 94].

1.1.9 Hypothesis of ovarian cancer origins

It is still an enigma how the normal ovarian epithelial cells change and develop a tumour. Several hypotheses are developed including the traditional "incessant ovulation hypothesis" and a recent developed "incessant menstruation hypothesis".

1.1.9.1 The incessant ovulation hypothesis

This hypothesis accounts ovarian cancer formation and progression for the processes of repetitive wounding during ovulation and the subsequent postovulatory wound repair. During these processes, total proliferations are active and an increased number of mutations may occur in the ovarian surface epithelial cells [95]. Progesterone, which promotes to clear the transformed epithelial cells in these processes [96], is increased during pregnancy and the application of OCP which are both protective factors for ovarian cancer.

1.1.9.2 The incessant menstruation hypothesis

Most serous cancers are supposed to originate from precursor lesions at the end of fimbriate tube, whereas most endometrioid and clear cell cancers may derive from atypical endometriosis.

Ovarian cancer, especially serous, endometrioid and CCC subtypes, may be developed because of iron-induced oxidative stress derived from retrograde menstruation. Erythrocytes in menstrual floating in the tube may be haemolyzed by pelvic macrophages, and the deposition of iron may generate a genotoxic effect of reactive oxygen species and become carcinogens [96, 97]. This hypothesis may explain why OCP and tubal ligation particularly decrease the risk of serous and endometrioid cancer but not mucinous cancer, and why endometrioisis-asscociated cancers develop more frequently in the ovary than at extragonadal sites[96].

1.2 Cancer stem cells (CSCs)

Cancer stem cells (CSCs), or tumour stem cells are a subpopulation of tumour cells with the properties of self-renewal and tumourigenecity [98, 99], and they may stay dormant in an appropriate niche where they are not recognized by current chemotherapy and other anti-tumour therapies [100-102]. However, under some specific condition they are activated and recruited into variable tissues, where they play key roles in chemoresistance, relapse and metastasis [103, 104]. Due to these properties, CSCs are thought to be the roots of cancer and the metastatic seeds, and becomes one of the promising targets to prevent cancer relapse and vastly improve cancer survival probability [101, 105, 106], whereas it is still such a challenge to target them because of their complex biology and unstable status [107-109].

1.2.1 Properties of CSCs

CSCs, like other stem cells, may have the properties of multilineage differentiation potential, self-renewal [110], slow cycling [111] and long-living. During cell division, a stem cell produces one (asymmetric division) or two (symmetric division) daughters that retain the capacity for self-renewal, ensuring that the stem cell population is maintained or expanded for long-term colonel growth, and as a result, self-renewal is the key and unique biological process of stem cell [110]. It is reported that BMI-1 gene is a crucial regulator of self-renewal, which can be targeted as a CSC-targeting therapeutic method in colorectal cancer [112]. CSCs were also reported to be anti-apoptosis, and more intriguingly, their capability of sphere formation and tumourigenesis were enhanced after apoptosis [113].

The difference between CSCs and stem cells mainly lies on the capacity of tumour-initiating/tumour propagating [114], and the capability to recapitulate the heterogeneity of all cell types observed in the primary lesions they are derived from, when transplanted into immune-deficient mice [115]. These capabilities make it possible to originate recurrences at distant organ sites in cancer patients.

Considering the clinical importance, CSCs are often insensitive to the currently existed anticancer treatment including chemotherapy, radiotherapy and others [110], and as a result able to survive when most of the tumour cells eliminated [112]. In addition, this property of treatment-resistance may be passed on to their daughter generations and progeny.

In addition, CSCs were proposed to be orchestrated hierarchically, supported by some observations that purified CSCs can quicly reform a balanced culture containing both CSCs and differentiated tumour cells (Figure 2A) [116]. However, the hierarchical concept was doubted and challenged by some other studies, showing that purified CSCs and differentiated tumour cells with expression of CSC and differentiation makers can return to the equilibrium that contains all tumour statuses [117-121].

1.2.3 Origin of CSCs

The existence of somatic CSCs was theoretically raised almost 40 years ago and inspired by the theory of haematopoietic stem cells [122]. However, few publications about CSCs were published in the 1980' (searched by PubMed). The theory became hot in the 1990' and was controversial until recently, when evidence accumulated confirming their existence and their potential role in tumour originating, progression, treatment and consequences.

The presence of CSC may explain why cancer therapy initially seems effective initially but the patient gets a recurrence later [123-126]. It is believed now that most of differentiated tumour cells may be eradicated during the treatment, but some specific cells (CSCs) which can smartly escape from the existent cancer therapies and survive by hiding in their niche, or by some immune and molecular mechanisms. Moreover, to be more threatening, these cells may be activated and generate new tumour under some appropriate conditions [124].

The existence of CSCs is becoming more convincible especially after recently the tumourigenetic cells which have the properties of cancer stem cells and their propagated cells can be tracked and displayed clearly *in vivo* [127-129].

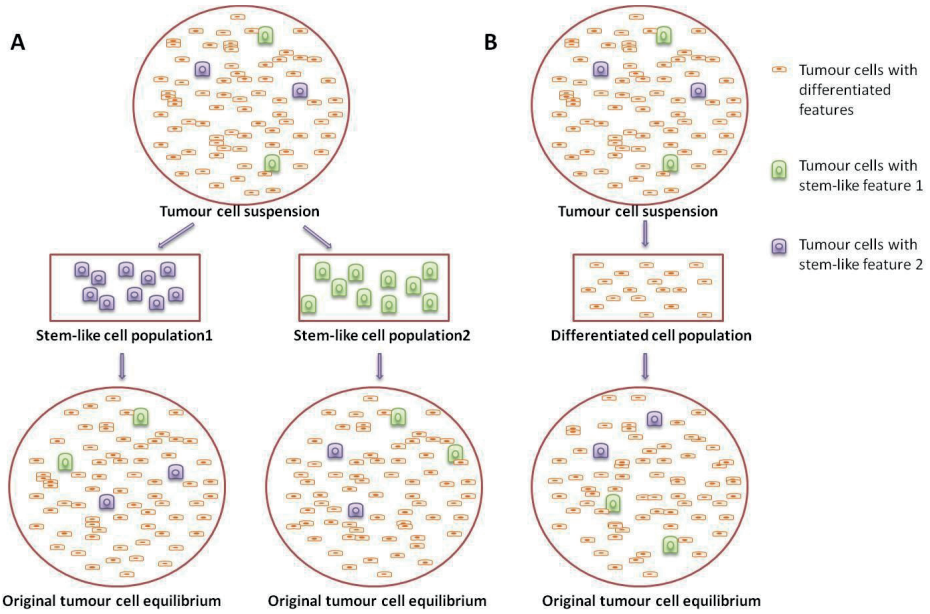

Figure 2. Cell models for cancer cell and cancer stem-like cells. (A) Tumour is a heterogeneous disease. When tumour cells are sorted by CSC markers, such as CD44, CD133, CD90, CD117 and side population, variable so called 'cancer stem-like cell' populations (purple and green cells) can be obtained, but when these cells are cultured in a given medium, these 'purified' populations all tend to return to an equilibrium status like the original tumour cell population, which contains similar proportions of each cell populations. (B) It was found recently in breast cancer cells that the more differentiated tumour cells can not only proliferate but also spontaneously turn to stem-like cells, and generate equilibrium status as well.

The origin of CSCs is still obscure, but there are some speculations. Piyush and coworkers sorted breast cancer cell lines SUM159 and SUM149 into three subpopulations: basal differentiation, stem-like or luminal types by fluorescence-activated cell sorting (FACS), and the "purified" subpopulations were send back to culture separately. Each subpopulations grew rapidly toward equilibrium proportions which includes all the three original cell subtypes [117]. This experiment indicates that not only cancer stem-like cells can differentiate into multilineages of cancer cells, but tumour cells can dedifferentiate into cancer stem-like cells as well. More other studies supported and verified this (Figure 2B) [118-121]. It has been proposed that normal stem cells or progenitor cells may change to neoplastic stem cells through several steps when acquire heritable change such as a somatic mutation [126]. Chaffer and Weinberg recently illustrated this hypothesis in details [130]. It is found that this process may be promoted by some factors such as hepatocyte growth factor (HGF), chemokine C-X-C motif ligand 7 (CXCL7) and cytokines interleukin 6 (IL-6) which are derived from mesenchymal cells [131].

1.2.2 The CSC niche

Normal stem cells often require input from their microenvironment to achieve an optimal balance between self-renewal, activation and differentiation [132, 133], similar with the CSCs. The CSCs also need a favourable microenvironment which is called CSC niche to protect themselves from being eliminated from anticancer treatment or other injuries. Studies have shown that the microenvironment may play a key role in the regulation of CSCs. For example, CSCs in glioma often locate near endothelial cells which are found to stimulate the stemness through Notch and diffusible factors [134, 135]. The associations of CSCs and the microenvironment will be more discussed later in this chapter.

It was proposed that at least some of the CSC niches may located in the transitional zones (TZ) where two different types of epithelial cells meet resulting in the appearance of a distinct abrupt transition [136]. Many epithelial cancers originate here, such as the cornea-conjunctiva junction, esophagogastric junction, gastro-duodenal junction, endo-ectocervix junction, ileocecal junction, and anorectal junction [136]. For ovarian cancer, recent investigations have demonstrated that some primary ovarian tumours (particularly serous, endometrioid, and CCC types), which traditionally were thought to originate from the ovary, actually originate in the fallopian tube and the endometrium, and involve the ovary secondarily [75]. Recent studies showed that the junction area in between the ovarian and the fallopian may be where the ovarian CSC niche located [102].

1.2.4 Identification of putative CSCs

Therefore, to characterize CSCs, both molecular and functional assays are being used. Molecular markers will be discussed in the following part. The functional assays are usually set based on their properties which were just discussed. They include clonogenic activity in soft agar, sphere formation efficiency in non-adherent cultures, and the limiting dilution tumourigenicity assay which was accepted as standard [126]. In some cases, the presence of an activated self-renewal pathway is thought to be important [137, 138] and intrinsic drug and/or apoptosis resistance may also be a characteristic [139].

Side population (SP) discrimination assay is a method using flow cytometry (FCM) to detect stem cells and CSCs based on the dye efflux properties of ATP-binding cassette (ABC) family of transporter proteins expressed within the cell membrane [140]. SP is another recommended test which is widely used for identification of the putative CSC populations [141-145]. Sorting method by SP population has been considered simple and effective in cancer stem cell research [146]. However, SP phenotype is not exclusive to stem cells and it is not universal in all cancer types [147]. The procedure of SP population detection is always being optimized for more specific and sensitive results [140].

1.2.5 CSCs related markers

To achieve cures on cancers, CSC targeting therapy has become a promising way nowadays [126]. Clinically, it is still challenging to quickly identify the CSCs inside tumour or elsewhere by a functional assay, which makes CSC specific markers more important. Therefore, targeting CSC specific markers is one of the most important and easily achievable ways to identify putative CSCs, although it is accepted that molecular assays are not sufficient to define CSCs and functional studies are necessary with the limiting dilution tumourigenetic assays [126]. A number of markers have been proved to be useful for isolation of enriched CSCs in many, if not all, types of solid tumours, including CD90, CD133, CD117, CD20, CD24, CD44, ALDH1, ABCG2, EpCAM and others [148]. They may be useful to identify different cancer types, but none of these markers are universally positive for all cancer types [148]. The identical markers for ovarian CSCs are listed in Table 2. CD117 and ALDH1 which are further studied in this thesis are introduced as following.

CD117, also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit, is a transmembrane cytokine receptor expressed on the surface of hematopoietic stem cells and other cell types. It is normally phosphorylated and activated by binding to its ligand stem cell factor (SCF). It is a widely-used stemness marker for recognition of cancer stem cells in various tumours, including ovarian carcinoma, endometrial cancer, osteosarcoma [149-151] and others.

Aldehyde dehydrogenase 1 (ALDH1) originally acts as a metabolic enzyme, which is localized in the cytoplasm, to catalyse dehydrogenation of aldehydes. In another way, it is regarded as a cancer stem

cell (CSC) marker in a variety of cancers [152], including EOC [153], lung cancer[154, 155], rectal cancer [156] and others [157, 158].

Table 2 Stemness markers for ovarian CSCs

Markers	Properties	References
CD133	Tumour initiation, asymmetric cell division, recapitulation of tumour heterogeneity	[159, 160]
ALDH1	Tumour initiation, asymmetric cell division, recapitulation of tumour heterogeneity, high sphere forming efficiency	[161, 162]
CD117	Tumour initiation, self-renewal, differentiation, chemoresistance	[163]
CD24	Quiescence, self-renewal, differentiation and tumourigenesis	[164]
SP	Tumour initiation, chemoresistance, stem cell gene expression	[165, 166]
$ABCG2^{+}$/SP	Tumour initiation, self-renewal, chemoresistance	[167]
$CD44^{+}/CD117^{+}$	Propagate the original tumours from patients, stem gene expression, anti-apoptosis, chemoresistance	[168, 169]
$CD133^{+}/ALDH1^{+}$	Tumour initiation in mice, recapitulation of tumour heterogeneity	[170, 171]
$CD44^{+}/MyD88^{+}$	Tumour initiation, recapitulation of tumour heterogeneity, high sphere forming efficiency , self-renewal	[172]
$CD44^{+}/CD24^{-}$	Tumour initiation, self-renewal, differentiation, drug resistance	[173]

1.2.6 Ways to induce cancer stem-like cells

1.2.6.1 Hypoxia

Oxygen level in the TME may regulate the stemness status of CSCs. It has been proved in our group that hypoxia microenvironment may increase the stem-like properties of prostate and ovarian cancer cells [174, 175]. SP and the expression of variable stem cell markers were enhanced when the cells were treated with hypoxia environment. What's more intriguing, cells cultivated at hypoxic condition grew relatively slowly with extended G0/G1 phase, but they showed significantly higher proliferation and infiltration capability and significant more colonies and spheres were generated, if they were brought back to normoxia after pre-treated under 1% O_2 for 48 hrs [174, 175].

1.2.6.2 Cytokines

Some cytokines in the TME, either secreted by tumour cells or stromal cells or some distal cells, may stimulate and influence the stemness properties of tumour cells and CSCs [176]. SCF and granulocyte colony-stimulating factor (G-CSF) were found in our group to induce stem-like properties in prostate

cancer cell lines and they may have synergistic effect when cells were exposed to them simultaneously [177]. Tumour necrosis factor alpha (TNFalpha), a major proinflammatory cytokine, was also shown to enhance CSC properties, such as sphere formation ability, expression of stem cell related genes, chemoresistance, radioresistance and tumourigenicity [178]. Treatment with IL-6, a multifunctional cytokine, may enrich the properties of lung cancer stem-like cells, including sphere formation and cell proliferation [179].

1.2.6.3 Mitochondrial deoxyribonucleic acid (mtDNA) blocking

Human mitochondrial DNA (mtDNA) is a 16.6 kb circular double-stranded DNA containing 37 genes, including 2 ribosomal RNAs, 22 transfer RNAs and 13 protein-encoding RNAs [180]. The mtDNA encodes proteins for all subunits of respiratory complexes I, III, IV and V, a part of complex II [181, 182]. Unlike nDNA, mtDNA exists in each cell with several hundreds to more than 10 thousand copies. The copy number of mtDNA in cells is dependent on various internal or external factors associated with ATP demand, e.g. exercise, hypoxia, and steroid hormones stimulation [183]. Both genetic disorders and chemical treatments drive reduction of mtDNA copy number and lead to insufficient synthesis of respiratory chain complexes [181]. Ethidium bromide (EtBr) is a known agent to inhibit mtDNA replication with a negligible effect on nDNA, and therefore is generally used to generate mtDNA-deficient models [181, 184-186]. Pyruvate and uridine are essential nutrients for cultured mtDNA-deficient cells to survive [187].

In the previous study in our laboratory, EtBr is used with the supply of pyruvate and uridine in the cultural medium of prostate cancer cell lines PC-3 and DU145. It turned out that the mtDNA replication was blocked by EtBr and the expression of stemness related genes including Oct3/4, Nanog, CD44, and ABCG2 were increased in both cell lines [188], indicating that the stem cell properties may be upregulated by mtDNA dysfunction.

1.2.6.4 Sex hormone stimulation

Due to the similar effects of estrogen or testosterone exposure on their progression, breast, prostate and ovarian cancers are sometimes grouped together and called sex hormone-related cancers. For these

cancers, sex hormone stimulation may play a role in the change of stem cells and therefore contribute to carcinogenesis. Breast cancer cells treated with beta-estradiol (E2) *in vitro* may generate more colonies than the controls cells treated with charcoal [189]. Repeated sex hormone stimulation in the course of menstrual cycles may impinge on stem cells and the stem cell niche, and therefore drive an early event in breast carcinogenesis [190]. Laboratory work in our group has revealed that prostate cancer cell lines LNCaP and PC-3 cultivated with 5α-dihydrotestosterone (DHT), an active form of androgen, exhibited higher clonogenic potential and higher expression levels of stemness related factors CD44, CD90, Oct3/4 and Nanog [191]. Sex hormone-binding globulin (SHBG) is a carrier protein mainly synthesized in the liver and secreted into the circulating system where appropriate steroids may bind with it and make different functions. SHBG expression in hormone related-cancer cells may drive the cancer progression. Thus, we reported that the expression of SHBG in the DHT treated prostate cancer cells was simultaneously upregulated with the cancer stemness related markers. Moreover, gain and loss functioning tests showed that the induction of Oct3/4, Nanog, CD44 and CD90 by DHT was correspondingly blocked when the SHBG gene was blocked by SHBG siRNA knock-down [191].

2 AIMS OF STUDY

Microenvironment changes of cancer cells may increase the stemness of cancer cells, which have been well established in our laboratory using hypoxia treatment, cytokine treatment and androgen treatment, respectively, in prostate cancer cell lines and ovarian cancer cell lines. Moreover, androgen treatment may induce the stemness of prostate cancer cells through SHBG protein. Ovarian cancer, as another gonadal malignancy whose development and progression is believed to be affected by female and male hormones, the expression of SHBG in the cancer cells and the potential significance become intensely interested. MtDNA deficiency shows promising to induce prostate cancer stemness, and it is warranted to be tested in ovarian cancer cell lines. The molecular mechanism seems interesting. On the other hand, the expression of CSC related markers, such as SHBG, CD117 and ALDH1, and their potential predictive significance in ovarian cancer patients are of great value for ovarian CSC study.

This project is aiming at the following aspects:

1. To study the biological and stemness changes of ovarian cancer cells with the treatment of a mtDNA blocking agent EtBr.
2. To test the expression of Sex hormone binding globulin (SHBG) and its clinical consequences
3. To investigate the clinical importance of cancer stem cell marker CD117 in ovarian cancer patients.
4. To analyse the expression pattern of cancer stem cell marker ALDH1 and its clinical role in ovarian cancer patient prognostication.

3 MATERIALS AND METHODS

3.1 Cell lines

Four ovarian cancer cell lines ES-2, SKOV-3, OVCAR-3 and OV-90 were purchased from American Type Culture Collection (ATCC, USA) and maintained in our laboratory. The ES-2 line was derived from a patient with ovarian clear cell carcinoma (CCC), and other three cell lines were derived from malignant ascites of ovarian adenocarcinoma patients.

3.1.1 Cell culture and cell treatment

All cells were cultivated in appropriate medium with supplementing 10% fetal bovine serum (FBS), 100units/ml penicillin and 100μg/ml streptomycin at 37℃ in a humidified 5% CO_2 incubator. In paper I, ES-2 line and SKOV-3 line were cultivated in McCoy's 5A medium (Life technologies) as ATCC recommends, with 10% FBS, 100units/ml penicillin and 100μg/ml streptomycin. In paper II, all four cell lines were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium.

In paper I, to block mtDNA replication, both cell lines were treated with EtBr at 50ng/ml and or 100ng/ml, 500ng/ml, 1000ng/ml. To provide intermediate nutrient for EtBr treated cells [187], 50μg/ml Uridine and 100μg/ml pyruvate were supplied together with EtBr in the above medium for a certain time.

3.1.2 Cell counting

Cells were harvested by 0.25% trypsin and EDTA (Invitrogen), resuspended in phosphate-buffered saline (PBS). A cell suspension was gently pipette up and down for several times to avoid cell aggregation before counting. 10μl of single cell suspension was mixed well with 10 μl of 0.4% trypan blue dye to distinguish dead cells and living cells. 10μl of the mixture was loaded onto a cell counting chamber and the chamber was applied into Countess® Automated Cell Counter (Life Technologies) for cell counting.

3.1.3 Cell growth curves

Counted single cells were resuspended at 200 cells/well for ES-2 line and 400 cells/well for SKOV-3 line in McCoy's 5A medium (Life technologies) with different concentrations of EtBr in six-well plates and cells were harvested every 24 hours for cell counting. This was replicated three times, and the mean values of cell numbers were obtained to make cell growth curve.

3.2 Clinical samples

3.2.1 Clinical Information

Two-hundred and forty-eight surgically removed ovarian carcinoma samples were randomly enrolled in this study. All patients were diagnosed and operated at The Norwegian Radium Hospitalduring 1983 to 2000. The patient age at diagnosis ranged from 19 to 89 years, with a median of 58 years. The patients were followed up for more than 12 years, ending on January 1st 2012. All patients were clinically staged by the criteria of International Federation of Gynecology and Obstetrics (FIGO) stage [192]. The primary tumours were histologically reclassified and graded as well, moderately and poorly differentiated according to WHO recommendations by two of the authors (J.M. and Z.S.) [6]. Disease progression was determined based on the definitions outlined by the Gynecologic Cancer Intergroup [193]. In the patients enrolled in this thesis, no Brenner tumour was involved, maybe due to its low incidence.

3.2.2 Ethics Statement

The Regional Committee for Medical Research Ethics South of Norway (S-06277a), The Social- and Health Directorate (06/3280) and The Data Inspectorate (06/5345) approved the study.

3.3 Immunocytochemistry (ICC) and Immunohistochemistry (IHC)

3.3.1 Cytoblocks

For each cell line, the 80% confluent cells were harvested by mechanical scraping, and cells in suspension were spun down at 2000 rpm for 5 minutes before the supernatant was discarded. The cells were rinsed twice with PBS to further delete the dead cells or cell organelles. Four drops of plasma and 2 drops of thrombin were added to the sedimentation after the supernatant was discarded, and the contents were carefully mixed by rotating tube for one minute before coagulation was formed. 4% buffered formaldehyde was added to the coagulation for cell fixation. The coagulated mass was then wrapped in a linen paper, put in a labeled cassette, and placed in 4% buffered formaldehyde. The material was paraffin-embedded to make cytoblocks. The cytoblocks were cut into 3µm paraffin sections for ICC and IHC.

3.3.2 IHC/ICC

Dako Envision™ FLEX+ system (K8012; Dako, Glostrup, Denmark) and the Dako Autostainer were used according to the manual instructions for ICC/IHC. Paraffin sections were deparaffinized and epitopes unmasked in PT link with appropriate target retrieval solution (Dako, Table 3), and then blocked with peroxidase blocking (Dako,) for 5 minutes. The slides were incubated with primary antibodies for an appropriate period time at optimized concentrations (Table 3). The slides were incubated with corresponding secondary antibody for 30 minutes, following up with mouse linker for 15 minutes and HRP for 30 minutes at room temperature. Slides were then stained with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes and counter-stained with hematoxylin for 20 seconds, dehydrated, and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Scientific, Waltham, MA, USA) before microscopy evaluation.

Table 3. Antibody information for IHC/ICC used in this thesis

Ab name	Company	Catalog No.	Resource	Dilution	Retrieval	Incubation condition
VEGFA	SANTA CRUZ	Sc-507	Rabbit	1/200	LPH	30 minutes at RT
WEE1	SANTA CRUZ	Sc-5285	Mouse	1/300	HPH	30 minutes at RT
HES1	Abcam	Ab87395	Mouse	1/100	HPH	30 minutes at RT

SHBG	R&D	AF2656	Goat	1/1200	LPH	Overnight at 4°C
CD117	Dako	A4502	Rabbit	1/400	HPH	30 minutes at RT
FAP	Abcam	Ab53066	Rabbit	1/300	HPH	30 minutes at RT
αSMA	BioGenex	MU128-UC	Mouse	1/750	HPH	30 minutes at RT
CD73	LSBio	LS-C138754	Rabbit	1/1600	LPH	30 minutes at RT
ALDH1	BD	611194	Mouse	1/3000	LPH	Overnight at 4°C

3.3.3 Scoring system of IHC/ICC

The Allred scoring system [194, 195] was used for evaluating each protein expression levels in ovarian carcinoma cells and tissues. The intensity of the immunohistochemical staining was scaled by 0 to 3 and the percentage of immunostaining cells was scaled by 0 to 5 (Table 4). The sum of intensity score and percentage score was seen as total score, which ranged from 0 to 8. In paper I the slide was regarded as positive if it showed immunostaining and negative if it did not. In paper II and paper IV, the slide was regarded as negative, low/weak expression and high/strong expression when the total score is 0, 1 to 6 and 7 to 8, respectively. In paper III, the slide was divided into negative and positive groups when the total score is 0 to 6 and 7 to 8. The ovarian carcinoma cells and the stromal cells were scored separately.

Table 4. The criteria of Allred scoring system used for evaluating ALDH1 expression in the ovarian carcinoma cells and the stromal cells in our study.

Table 4.1 The criteria of intensity scoring system

Intensity Score	0	1	2	3
	Negative	Weak	Moderate	Strikingly positive at low magnitude

Table 4.2 The criteria of percentage scoring system

Percentage Score	0	1	2	3	4	5
	0	<1%	1%-10%	11-33%	34-66%	67-100%

Table4.3

Total Score*	0	1-6	7-8
	Negative	Low	High

(Note: *The total score was obtained by adding the percentage score to intensity score. It ranges from 0 to 8.)

3.4 Western blotting

Cells were harvested by 0.25% trypsin and EDTA (Invitrogen) and rinsed twice with ice-cold PBS. Cell bullets were homogenized in lyses buffer, which contains RIPA buffer (Thermo scientific), 1% PMSF, 1% aprotinin, 1% leupeptin, 1% pepstatin and 0.5% vanadate, immediately before use. Samples were left on ice for 30 minutes before they were spun down at 14000rpm for 15 minutes at

4℃ to get total proteins in the supernatant. Total proteins were measured by the Bio-Rad protein assay (Hercules, CA, USA). Equal amount of proteins from each sample in SDS loading buffer was boiled for 5 minutes and subjected to 10% SDS-PAGE electrophoresis and then electro-transferred to high-quality polyvinylidene difluoride (PVDF) membrane in a Trans-Blot apparatus (Bio-rad, Hercules, CA). The membrane was blocked with 5% fat-free milk for 1 hour at room temperature and incubated overnight at 4℃ with primary antibodies with optimized concentrations (Table 5). After washing with PBS-tween 0.05% (PBST), the blot was incubated with the corresponding secondary antibodies conjugated with HRP (Table 5). After several washes with PBST, the blot was visualized using an enhanced chemiluminescence detection kit (ECL, Amersham) by following the manual guide. The experiments were performed three times.

Table 5. Antibody information for Western blotting used in this thesis

Ab name	Company	Catalog No.	Resource	Dilution	Incubation condition
VEGFA	SANTA CRUZ	Sc-507	Rabbit	1/500	Overnight at 4°C
WEE1	SANTA CRUZ	Sc-5285	Mouse	1/500	Overnight at 4°C
HES1	Abcam	Ab87395	Mouse	1/1000	Overnight at 4°C
SHBG	R&D	AF2656	Goat	1/500	Overnight at 4°C
GAPDH	R&D	AF5718	Goat	1/1000	Overnight at 4°C
Anti-mouse IgG-HRP	R&D	HAF007	goat	1/1000	45 minutes at RT
Anti-rabbit IgG-HRP	R&D	HAF008	goat	1/1000	45 minutes at RT
Anti-goat IgG-HRP	R&D	HAF017	Rabbit	1/1000	45 minutes at RT

3.5 Colony formation assay

Counted single cells were resuspended at 200 cells/well for ES-2 line and 400 cells/well for SKOV-3 line in McCoy's 5A medium (Life technologies) with different concentrations of EtBr in six-well plates for 14 days. The cells were gently washed with PBS and fixed with 4% buffered formalin for 15 minutes before stained with 1% crystal violet for 30 minutes. The plates were then gently washed with PBS and dried in the air. Colonies were evaluated under microscopy and those with more than 30 cells were considered valid. Colony formation efficiency was defined as valid colonies/input cells ×100%. Data are representative of three independent experiments.

3.6 DNA and RNA preparation

Total genomic DNA (gDNA), which includes nDNA and mt DNA, was extracted from approximately 10^6 cells using PureLinkTM Genomic DNA Mini Kit (Invitrogen), and total RNA was extracted from the same amount of cells using RNeasy Micro kit (Qiagen), according to the manuals. DNA and RNA quality and quantity were assessed by NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was considered to be good when OD 260/280 ratio was 1.8 to 2 and OD 260/230 was 1.8 or more.

3.7 MtDNA quantification

Total gDNA was amplified by polymerase chain reaction (PCR) to obtain the relative ratios of mtDNA to nDNA. The primers for mitochondrial gene ND1 were: forward 5′ACTACAACCCTTCGCTGACG 3′ and reverse 5′GCCTAGGTTGAGGTTGACCA 3′, with product length of 169bp. The primers for nuclear gene GAPDH were: forward 5′CCTCAAGATCATCAGCAATGC3′ and reverse 5′ TGGTCATGAGTCCTTCCACG3′, with product length of 101bp. Primers were optimized to avoid across interactions. 1ng gDNA was added to the PCR system for ND1 and GAPDH amplification simultaneously under the following PCR programme: initial denaturation at 95ºC for 10 minutes; followed by 35 cycles of 95ºC for 15 seconds, 57ºC for 30 seconds and 72ºC for 30 seconds; then 75ºC for 10 minutes and held at 4ºC. 4µl of PCR product was then well mixed with 5µl DEPC water and 1µl 10× Blue JuiceTM gel loading buffer (Invitrogen) and subsequently applied to 7.5% polyacrylamide gel electrophoresis. 50bp DNA ladder was used to confirm the correct band. The gel was incubated in EtBr Buffer for 10 minutes before exposed to G:Box imaging system. Quantity One software (version 4.3, Bio-rad Laboratories) was used to analyze the quality and the quantity of the bands detected.

3.8 Flow cytometry (FCM)

Approximately $1x10^6$ cells for each sample were collected and resuspended in 3ml ice cold PBS in Falcon® tubes for cell surface markers CD90, CD117 and ABCG2 investigation. Anti-CD90 and anti-

CD117 monoclonal antibodies directly conjugated with hycoerythrin (PE) and anti-ABCG2 monoclonal antibody directly conjugated with allophycocyanin (APC) were obtained from BD Pharmingen Company. The cells were twice washed with ice-cold PBS and incubated in optimized dilutions of above antibodies in dark for 30 minutes. The cells were then filtered in a 35 μm nylon mesh cell strainer cap (BD) right before applied on flow cytometer (BDTM LSRII yellow laser). PE mouse IgG2b and APC mouse IgG2b isotype controls, both obtained from BD Pharmingen Company, were used for negative controls. For each cell sample, variable and single cells were gated before fluorescence was analyzed. FlowJo (version 10.0.6) was used to analyze the data. The experiments were repeated at least three times, and statistical analyses were performed based on the fluorescence intensity values.

3.9 RNA sequencing (RNA-seq)

3.9.1 RNA-seq experiments

After the total RNA extraction and DNase treatment, magnetic beads with Oligo (dT) were used to isolate mRNA. The mRNA was fragmented into short fragments by fragmentation buffer. And then the mRNA fragments were used as templates to synthesize cDNA. Short fragments were purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments were connected with adapters. After agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification as templates. During the QC steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample library. At last, the library was sequenced using Illumina HiSeq^{TM}2000.

3.9.2 RNA-seq data analysis

RNA-seq experiments were performed in Beijing Genomics Institute (BGI), HongKong. Data filtering and quality control on raw sequencing data, alignment of raw sequence reads to human reference genome HG19 and genome annotations were done in BGI according to previous publication [196]. In total genome reads, there are 39693774 (77.31%), 41280763 (77.25%), 43254963 (78.26%) and

39800036 (77.39%) unique matches in sample E0 (ES-2 line control), E500 (ES-2 line treated with 500ng/ml of EtBr), S0 (SKOV-3 line control) and S500 (SKOV-3 line treated with 500ng/ml of EtBr) respectively. Gene expression level was calculated by the RPKM method (Reads per kilobase transcriptome per million mapped reads) [196]. Subsequently, differential gene expression analysis between the samples was carried out. The distributions were assumed to be normal when the difference between the log transformed RPKM levels of S0/E0 and S500/E500 samples was assessed. Genes with P-value <0.01 was chosen as significantly differential expressed between the two groups [197]. Based on those selected significantly differentially expressed genes, GO functional annotation was performed by DAVID tool [198]. Finally, selected genes highly enriched in certain functional annotation category were shown in color-coded heat maps where red and green represented up-regulation and down-regulation, respectively.

3.10 Statistical analyses

SPSS software (version 18.0) was used for data analysis. Laboratory experiments were performed at least three times. Statistical analyses of fluorescence intensity were performed by student's t-test. For clinical data analysis, associations between categorical variables were assessed by Chi-square tests (Pearson and linear-by-linear as appropriate). Survival analysis was estimated using the Kaplan-Meier method, and groups were compared with log-rank tests. For all the analyses, associations were considered to be significant if the p value was < 0.05.

4 SUMMARY OF RESULTS

I. Mitochondrial DNA deficiency in ovarian cancer cells and cancer stem-like probabilities

MtDNA deficiency has been long regarded as a risk factor in a variety of tumours. MtDNA-deficient ovarian cancer cell models were established by EtBr treatment with additive combination of pyruvate and uridine, to further investigate the biological characters. Cellular mtNDA quantity in both ovarian cancer cell lines ES-2 and SKOV-3 was decreased dose-dependently with increasing EtBr concentrations. The mtDNA deficient cells grew slowly and had a low efficiency of colony formation compared to the control cells. RNA sequencing revealed downregulation of mitochondrion-related genes and upregulation of cell proliferation and anti-apoptosis related genes. The expression of genes involved in cancer metastasis, proliferation, angiogenesis, drug resistance and cancer cell stemness were upregulated as well. Among these genes, the upregulations of WEE1, the angiogenesis factor VEGFA and the transcription factor HES1 were verified at the protein expression level as well. Intriguingly, cancer stem cell markers CD90 and CD117 were both upregulated with EtBr treatment and the associations were dose-dependent in both cell lines. To conclude, mtDNA deficiency may induce ovarian cancer cell stem-like properties through variable ways *in vitro*, and the clinical significances of stemness related markers are of great value for further studies.

II. Sex hormone-binding globulin (SHBG) expression in ovarian carcinomas and its clinicopathological associations

SHBG, known as a carrier protein, is classically thought to be mainly synthesized in the liver and then secreted into the circulating system and functions there. However, the local expressed SHBG may play an important role in tumour development. SHBG expression status and its clinicopathological significance in ovarian cancer cells were studied in this paper. Variable SHBG expression was detected in four ovarian cancer cell lines (OV-90, OVCAR-3, SKOV-3 and ES-2) by immunocytochemistry and confirmed by Western blotting. A series of 248 ovarian carcinoma samples were immunohistochamically studied then, and we did find SHBG to be variably expressed in these ovarian carcinomas. Higher level of SHBG expression was significantly associated with more

aggressive histological subtype (p=0.022), higher FIGO stage (p=0.018) and higher histological grade (grade of differentiation, p=0.020), although association between SHBG expression and OS/PFS was not observed. Our results demonstrate that ovarian cancer cells produce SHBG and higher SHBG expression in ovarian carcinoma is associated with unfavourable clinicopathological features.

III. CD117 expression in fibroblasts-like stromal cells indicates unfavourble clinical outcome in ovarian carcinoma patients

The stem cell factor (SCF) receptor CD117 (c-kit), is widely used for identification of hematopoietic stem cells and cancer stem cells, and its expression in carcinoma cells usually indicates a poor prognosis in a variety of cancers. We examined CD117 expression in tumour microenvironment and the potential predictive role in 242 EOC patients. Thirty-eight out of 242 cases were CD117 positive in fibroblast-like stromal cells and 22 cases were positive in EOC cells. The CD117 expression in fibroblast-like stromal cells in ovarian carcinoma was closely linked to advanced FIGO stage, poor differentiation grade and histological subtype (p<0.05), and it was significantly associated with poor overall survival (OS) and progression free survival (PFS) (Kaplan-Meier analysis; p<0.05, log-rank test). CD117 expression in ovarian carcinoma cells was not associated with these clinicopathological variables. The CD117 positive fibroblast-like stromal cells were all positive for mesenchymal stem/stromal cell (MSC) marker CD73 but negative for fibroblast markers fibroblast activation protein (FAP) and α smooth muscle actin (α-SMA), indicating that the CD117+/CD73+ fibroblast-like stromal cells are a subtype of mesenchymal stem cells in tumour stroma, although further characterization of these cells is needed. It was concluded that the presence of CD117+/CD73+ fibroblast-like stromal cells in ovarian carcinoma may predict an unfavourable clinical outcome.

IV. The expression of aldehyde dehydrogenase 1 (ALDH1) in ovarian carcinomas and its clinicopathological associations: a retrospective study

Aldehyde dehydrogenase 1 (ALDH1) is widely used as a specific cancer stem cell marker in a variety of cancers, and may become a promising target for cancer therapy. However, the role of its expression in tumour cells and the microenvironment in different cancers is still controversial. To clarify the

clinicopathological effect of ALDH1 expression in ovarian carcinoma, a series of 248 cases of paraffin-embedded formalin fixed ovarian carcinoma tissues with long term patient follow-up information were studied. The immunostaining of ALDH1was variably detected in both tumour cells and the stromal cells, although the staining in tumour cells was not as strong as that in stromal cells. High levels of ALDH1 expression were observed in the tumour cells in 15.7% of total 248 well-characterized EOC cases. Statistical analyses showed that high ALDH1 expression in tumour cells was significantly associated with histological subtypes, early FIGO stage, well differentiation grade and better survival probability ($p<0.05$), although ALDH1 was not an independent risk factor in multivatiate analysis. The expression of ALDH1 in the stromal cells had no clinicopathological associations in the present study ($p>0.05$). To conclude, high expression of cancer stem cell marker ALDH1 in ovarian carcinoma cells may be associated with a favourable prognosis. ALDH1 expression in tumour microenvironment may have no role in clinical behavior in ovarian carcinomas. More studies are warranted to explore the mechanisms.

5 GENERAL DISCUSSION

5.1 MtDNA quantity and CSC

Mitochondrion is the site for oxidative phosphorylation (OXPHOS), a process by which most of the cell's energy supply of adenosine triphosphate (ATP) is generated by aerobic respiration through tricarboxylic acid cycle (TCA cycle) in the presence of oxygen. The mtDNA-encoded proteins are all subunits of respiratory complexes I, III, IV and V, while the subunits of complex II are entirely nuclear DNA (nDNA)-encoded [181, 182]. Reduction of mtDNA quantity may lead to insufficient synthesis of respiratory chain complexes [181]. The cancer cells may be forced to the metabolic adaptation toward anaerobic respiration for the supply of energy and other intermediate nutrients. Compelling evidence shows that mtDNA reduction or mtDNA mutation, which may lead to oxidative DNA damage, abnormal expression and mitochondrial dysfunction, is a genetic risk factor for different cancers [199-204].

Decreased mtDNA quantity in ovarian cancer cells may force the cells to go through anaerobic respiration and produce lactate instead of TCA cycle using oxygen. This may have similar mechanisms with hypoxia environment treatment which has been proved in our group to induce cell stemness [174]. Hypoxia is one of the key ways to induce cancer stem cells or upregulate cancer cell stemness [175, 205, 206]. Ovarian cancer cells treated with hypoxia expressed high levels of the cancer cell stemness markers and showed low proliferation and colony formation efficiency, but significantly high proliferative and aggressive properties were generated after these cells were put back to normal oxygen condition [174].

In paper I, ovarian cancer cells generated relatively quiescent and low-proliferating characters compared to control cells, and this is coincidence with the dormancy probability of cancer stem cells [100, 101]. Meanwhile, plenty of stem cell markers were highly expressed in the mtDNA deficient cells, and the anti-apoptosis and the regulation of cell proliferation related genes were upregulated,

indicating the stem-like properties were induced. It may be an ideal method to induce the stem-like properties of ovarian cancer cells by blocking mtDNA.

5.2 Tumour microenvironment (TME) and CSC

Evidence of transcriptional and epigenetic programs confirms that cancer is a heterogeneous disease, and the intratumoural heterogeneity may contribute to therapy failure and disease progression [207]. However, a tumour does not mean a bulk of homogeneous malignant cells. Rather, a tumour is a complex ecosystem containing not only tumour cells, but also various cell types like infiltrating endothelial cells, hematopoietic cells, fibroblast cells, immune cells, the extracellular matrix (ECM) and others which compose the living environment and may impact the characteristics and the behavior of tumour cells as a whole, and it is therefore called tumour microenvironment (TME). They may influence tumour cells directly or through changing the metabolic approaches such as oxygen, pH and nutrient fluctuations, which contribute to heterogeneity in the function of malignant cells [110, 208]. TME, which is created by complicated interactions between tumour cells and stromal cells, yields cancer promoting effects, especially induction of a hypoxic environment. The importance of the tumour microenvironment for tumour initiation and progression is well established [209-212]. Anticancer treatment may show different responses on tumour cells *in vitro* and *in vivo*, reinforcing the significance of the tumour microenvironment. The role of hypoxia environment to induce stemness properties of cancer cells is well identified, and it was also found to play a critical role in the formation of the putative CSC niches [213]. The stromal cells also promote the engraftment of metastatic nodules which forms a partial CSC niche [214].

Cancer-associated fibroblasts (CAFs), as a predominant component in the microenvironment, are being used in carcinomas to prepare the microenvironment according to the “seed and soil” theory [212, 215]. CAFs produce and modulate the collagen [216] and ECM [217], and thus may facilitate carcinoma cell invasion. In addition, CAFs promote angiogenesis, and lymphangiogenesis in EOC [218]. Fibroblast activation protein (FAP) is widely used among other markers to mark CAFs in

tumours. Zhang and coworkers has proved by immunohistochemistry that over 90% fibroblasts in EOC specimens are positive for FAP [218].

The relationship between CSCs and TME is never a one-way road. Most of the CSC functions including self-renewal, differentiation and tumourigenesis are made with the help of environmental stromal cells, which form the CSC niche. Yet, CSCs are able to facilitate stromal cells to their needs, not only in the primary tumor, but also in distant organs where CSCs may manipulate the foreign soil by inducing a pre-metastatic niche for their arrivals [219].

5.3 Epithelial Mesenchymal Transition (EMT) and CSC

Epithelial Mesenchymal Transition (EMT) is a fundamental biologic process during which epithelial cells lose their polarity and acquire a mesenchymal phenotype responsible for invasion and metastasis [220-222]. Here, detachment and migration of small clusters of tumour cells from the neoplastic epithelium take place, both of importance for the local invasion and distant metastases [223, 224]. The mesenchymal state facilitates cells with the capacity of migration to distant organs and maintain stemness, allowing the initiation of metastasis [221]. Some CAFs may originate from cancer cells after EMT [225], and in the other hand, they may drive the EMT process through paracrine TGF-beta1 or HIF-1alpha/beta-catenin-dependent pathway and other pathways [226-228].

Initially, Groups of Weinberg [229] and Puisieux [230] found that EMT in transformed mammary epithelial cells may drive the generation of cells with properties of CSCs and express CSC marker expression by ectopic expression of the transcription factors Twist or Snail, or the oncogenic GTPase Ras. Similar results were reported for forced expression of the transcription factor Zeb1 [231] and TGF-beta [232], WNT5A [233], and some other factors [234]. These findings verified the possibility that, cancer stemness can be acquired by changing gene expression programs such like EMT. In paper II, SHBG is expressed significantly higher at the invasive/infiltration front (IF), known for EMT processes [235]. This demonstrated that the local produced SHBG in ovarian cancer cells might probably influence the proliferative activity, invasiveness and distant metastasis of human ovarian carcinoma cells.

Ricci and colleagues reported that ovarian CSCs may have a mesenchymal phenotype and a potential of EMT. In their study, ovarian stem-like cells which propagate as non-adherent spheres were isolated from primary ovarian carcinoma samples, and cultured in medium suitable for tumour stem cells. These cells were tumourigenic *in vitro*, able to self-renew, and acquired an epithelial morphology when grown in FBS-supplemented medium, but tended to lose their invasive potential and express mesenchymal and EMT markers when cultured in differentiating conditions [236].

The similarity of EMT cells and migrating CSCs may exist indeed, but we do know that many CSCs identified by tumourigenesis assay do not express EMT markers. It is still unclear whether they are regulated according to similar mechanisms and how do they communicate.

5.4 Mesenchymal stem cell (MSC) and CSC

Mesenchymal stem cells (MSCs) were initially isolated from the bone marrow and demonstrated the multipotency to differentiate into a variety of cell types [237, 238]. When a tumour initiates, they are able to recruit into the tumours as the progenitors of stromal cells from a distant organ such as bone marrow malignancies [214, 239, 240]. In one way, MSCs may act as a defender of cancer cells by infiltrating into the tumours, but they may also have unique contributions to tumour progression[214]. Moreover, CSC niche can be generated through paracrine signaling between carcinoma cells and mesenchymal stem cell (MSC) via EMT [241].

It is still an open question about how to define MSCs, because no single marker is specific to identify MSCs to date [242]. Cultured MSCs are uniformly and strongly positive for CD105, CD90, and CD73, regardless of their passage or time in culture [243], and it has been one of the minimal criteria to identify MSC [242, 244]. Moreover, the morphology of MSCs was reported to be fibroblast-like [245, 246]. Bone marrow-derived MSCs selectively express FAP but not other resources [247], and slightly express α-SMA [248]. But MSCs in tumour stroma are currently not reported to express these two markers. Therefore, in Paper III, the CD117+/CD73+ fibroblast-like stromal cells were possible to be MSC-derived, although currently we are not able to confirm this suppose.

5.5 The role of stem cell markers in the CSC study

The markers for CSCs are not universal depending on the tumour site. The candidates raised up to characterize ovarian CSCs include CD44, epithelial cell adhesion molecule (EpCAM), CD133, CD117, CD90 (Thy-1), CD24, ABCG2, LY6A, AGR5 and ALDH1, etc [249, 250]. Each marker or combination markers may generate a specific population with stem-like features; nevertheless, multiple populations from one single primary tumour cell population or cancer cell line can be established with different markers or combinations (Figure 2). It remains challenging to identify one single marker or several combined markers to specifically identify all the CSCs in an ovarian tumour. The exact roles of these stemness related markers are still poorly understood due to either a current lack of understanding of the biological functions of the markers, or frequently the lack of information correlating the varied isoforms, splicing variants or substrates to stem cell function [249, 250].

Moreover, although enriched cancer stem-like cells with CSC marker expression may indicate more aggressive features *in vitro*, but the roles of CSC markers would become more complicated and even controversial when it comes to the *in vivo* study. This may be due to multiple factors including the mutual influence of tumour cells and TME, the effect of immune system and others. For example, while most of the studies showed an overexpression of stem cell marker was a poor prognostic factor, it was reported that the loss expression of membranous CD44, CD166, and EpCAM was associated with tumour progression [251]. The role of stem cell marker ALDH1 in ovarian cancer patients was rather controversial as well. While some studies supported that cancer patients with ALDH1 positive tumor cells displayed treatment-resistant and poor survivals [161, 170, 252, 253], some other studies indicated that no predictive role was identified for ALDH1 in the clinical outcomes of ovarian cancer patients [254]. Moreover, the potential role of ALDH1 in tumour stromal cells was thought to be interesting, but still the results were quite debatable. In paper IV, the expression of ALDH1 in stromal cells had no associations with the clinical parameters, which was agreed by Woodward et, al. [255] and Ohi et, al [256].

5.6 Sex hormone, SHBG and CSC

The effects of sex hormone stimulation in sex hormone-related cancers including prostate, breast and ovarian cancers are well understood. The promoting roles of androgen and estrogen to EOC are discussed in the above [257-259]. However, more investigations are called for the mechanical study. Androgen and estrogen are traditionally known to play roles by binding with each receptor. It is found recently that both female and male sex hormone may bind to a complex of SHBG-SHBG receptor, which is formed by SHBG and its specific membrane receptor R_{SHBG} [260, 261]. And the intracellular cAMP may get increased when an appropriate steroid binds to the SHBG-R_{SHBG} complex, and it may thus play crucial roles in prostate and breast cancers [262-264]. Plasma SHBG can also participate in multiple signaling pathways for certain steroid hormones and therefore contribute to ovarian cancers. An *in vitro* study from our group showed that androgen treatment may upregulate the stemness of prostate cancer cells by SHBG, indicating the potential significance in CSC studies. In paper II, SHBG expression in ovarian cancer cells in tumour tissue was significantly associated with poor differentiation grade, advanced clinical stage and aggressive histological subtypes, indicating SHBG may contribute to ovarian cancer progression and aggressiveness. Moreover, it was observed that SHBG expression was higher in the IF, where the tumour initiates, showing that SHBG may contribute to the tumour initiating characters. The potential associations of SHBG and tumour initiation are in line with our previous findings. More molecular studies on the relationships of SHBG and cancer cell stemness are merited.

6 CONCLUSIONS

MtDNA deficient cells could be obtained by 500ng/ml of EtBr treatment for 4 days in ovarian cancer cell line ES-2 and SKOV-3. They grew slowly with lower capability in colony formation, a similar finding with the tumour cells in hypoxia condition. Furthermore, these mtDNA deficient cells highly expressed a series of genes related to stem cells, anti-apoptosis and regulation of proliferation. In short, mtDNA deficient ovarian cancer cells may be ideal models for CSC studies.

SHBG is found to be expressed in ovarian cancer cells, verified both in cell lines and clinical samples, and its expression is associated with unfavourable clinicopathological features. Further investigations on the SHBG variants and their potential molecular and biological functions in human ovarian carcinoma are warranted.

CD117+/CD73+ fibroblast-like stromal cells are significantly associated with poor clinical manifestations and poor survival probability in ovarian carcinomas, while CD117 expression in tumour cells does not show any clinical significance. Thus, it is worthy of further study for CD117 positive and CD73 positive stromal cells in EOCs in order to explore their potential application in prognostic prediction and targeting therapy.

Our long-term follow-up retrospective study reveals that high ALDH1 expression in tumour cells portends favourable prognosis and better survivals in patients with ovarian carcinoma, but the expression of ALDH1 in stromal cells has no associations with clinical outcomes. More studies are warranted to verify the potential role of ALDH1 in ovarian carcinoma progression and the original mechanisms involved.

7 FUTURE ASPECTS

Transplantation experiments, in addition to the colony formation assays, spheroid formation and molecular analyses are the more important methods currently existing to identify CSCs. EtBr induced ovarian cancer stem-like cells needs more verification, such as animal xenotransplantation examination. To date, xenotransplantion in immune deficient mice is the most accepted method to demonstrate cell´s ability to form tumour *in vivo*.

The clinical significances of some stem cell markers are of great interest, like for ALDH1.Therefore more clinical samples are needed to further investigate the associations. Research of related signaling pathways and molecular regulation of these markers are to be explored. In addition, new technology applying Nanostring is going to be used in retrospective studies of multiple genes, in addition to the application of the traditional technology IHC used in the current thesis.

Polyploidy giant cells (PGCC) have been observed over a century. Recently they were purified and investigated from ovarian cancer cell lines and ovarian primary tumours by Zhang and coworkers [265]. They express normal and cancer stem cell markers, divide asymmetrically and cycle slowly. They have the capability of self-renewal, spheroid formation *in vitro*, tumourigenesis in immunodeficient mice and multilineage differentiation. These PGCC-derived tumours gained a mesenchymal phenotype with increased expression of cancer stem cell markers CD44 and CD133 and become more resistant to cisplatin [265]. Similar PGCC were also observed during the ovarian cancer cell culture, and the proportion seems to be increased in EtBr treated cells, which intrigue us to purify these cells for the next genetic and epigenetic studies.

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9 ERRATA

In the main text of the thesis, on page 12 line 5, “among the EOCs [11][12]” should be “among the EOCs [11,12]”. On page 16 line 16, “computed tomography (CT) scans” should be “computed tomography scans”.

In the title part of Paper III, “Fibroblasts-Like Stromal Cells” should be “Fibroblast-Like Stromal Cells”. In the results part of Paper III, on page 4 line 10, “and others showed more” should be “and others showed less”. On the page 7 of Paper III, in Figure 5A and 5B, “CD117 in CAFs” should be “CD117 in fibroblast-like stromal cells”.