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Ovarian cancer stem cells and their role in drug resistance

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ABSTRACT

Ovarian cancer is typically diagnosed at advanced stages (III or IV), with metastasis ensuing at stage III. Complete remission is infrequent and is not achieved in almost half of the women diagnosed with ovarian cancer. Consequently, management and treatment of this disease is challenging as many patients are faced with tumour recurrence disseminating to surrounding organs further complicated with acquired chemo-resistance. The cancer stem cell theory proposes the idea that a drug resistant subset of tumour cells drive tumour progression, metastasis and ultimately, recurrent disease. In the ovarian cancer field, cancer stem cells remain elusive with significant gaps in our knowledge. The characteristics and specific role of ovarian cancer stem cells in recurrence still requires further research since different studies often arrive at contradictory conclusions. Here we present a review and critical analysis of current research conducted in the field of ovarian cancer stem cells and their potential role in drug resistance including several signalling pathways within these cells that affect the viability of targeted therapies.

1. Ovarian Cancer: background

Ovarian cancer (OC) is the deadliest gynaecological cancer with 90% of OCs being epithelial in origin (Deng et al., 2016; Stewart et al., 2018). The mortality rate of the disease is high, where the overall 5-year survival rate for patients with advanced epithelial OC is less than 25% (Roy and Cowden Dahl, 2018). This is mainly due to the late diagnosis of the disease, with 70% of patients usually diagnosed at advanced stages (III and IV) (Ottevanger, 2017). Early diagnosis of the disease is challenging as OC presents with nonspecific symptoms, such as abdominal discomfort and bloating (Dong et al., 2014). Epithelial OC is heterogenous, and can be characterised into various histological subtypes, such as clear cell, endometrioid, mucinous, low-grade serous and high-grade serous (Mitra, 2016).

Advanced OC is a highly metastatic disease, with dissemination often occurring within the peritoneal cavity, spreading to other areas of the abdomen (Lengyel, 2010). Patients receive either initial surgery or interval debulking surgery, with the aim of achieving complete removal of macroscopic tumours. The majority of patients with advanced stage disease respond well to treatment, however a major problem is the high relapse rate often associated with chemo-resistance (Luo et al., 2011). Tumour recurrence depends on the duration of platinum-free interval (PFI), which is often reflective of the patient's secondary response to chemotherapy. Thus, a short PFI is associated with a higher possibility of chemotherapy resistance (Markman et al., 1991). Patients with more than 6 months PFI are considered platinum sensitive or partially platinum sensitive (Luvero et al., 2014; Stuart et al., 2011). These patients often receive retreatment with a platinum-based chemotherapy and are likely to be still responsive initially, although the tumours become resistant after multiple rounds of chemotherapy (Pfisterer et al., 2006). Patients with less than 6 months PFI are 'platinum resistant'. Hence, chemo-resistance is one of the main contributing factors to the low survival rate in OC patients. Research into the underlying mechanisms behind OC chemo-resistance is required in order to develop improved treatment options for women affected by the disease. Advances in cancer stem cells (CSC) have widened our understanding of metastasis and chemo-resistance in OC (Ip et al., 2016).

2. Ovarian Cancer stem cells

Recent research has focused on the characterisation of CSCs within OC (Lupia and Cavallaro, 2017), given that tumours consist of heterogeneous cancer cells linked to cancer progression (Reya et al., 2001; Vlashi and Pajonk, 2015). The CSC theory suggests that a small population of drug resistant tumour cells (CSCs) are the driving force behind tumour initiation, dissemination, metastasis and recurrence (Albini

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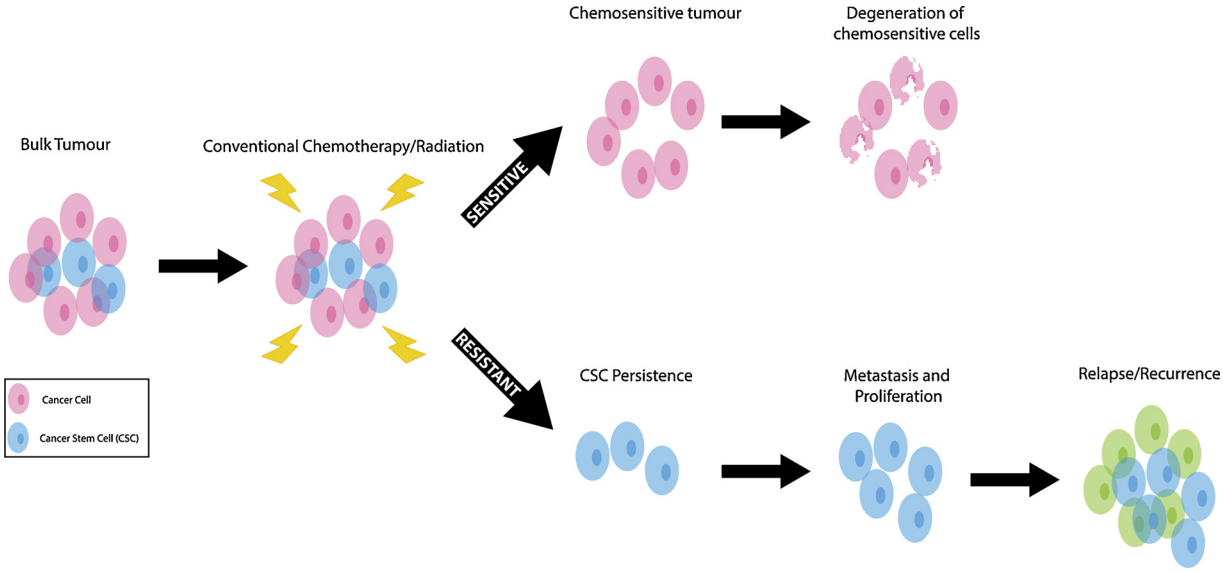

Fig. 1. Diagrammatic representation of CSC progression after therapeutic intervention. Cancer cells are sensitive to treatment ultimately leading to reduced tumour size and degradation of these cells. CSCs are resistant to treatment, further persisting enabling proliferation and metastasis, causing patient relapse and recurrent tumour formation (Albini et al., 2015).

et al., 2015). Conventional treatment can significantly reduce the size of the tumour and temporarily improve the patient's signs and symptoms, without specifically targeting this highly potent subpopulation. CSCs have a high capacity for self-renewal therefore play an important role in tumour formation, assist tumour dissemination and ultimately advance the progression of the disease (Fig. 1) (Zhang et al., 2018; Parte et al., 2018; Yasuda et al., 2014). Thus, CSCs can be left dormant with the possibility to repopulate again, leading to a more aggressive, drug-resistant disease (Clevers, 2011). Although we know little about the location of ovarian CSCs and their effects on disease progression, recent studies have aided in understanding their role (Parte et al., 2018). Interestingly, OCSCs are thought to be resistant to chemotherapy, and are the cells involved in metastatic spread of the cancer (Burgos-Ojeda et al., 2012). These chemo-resistant CSCs could contribute to the high recurrence rate of the disease (Zhang et al., 2018). Understanding the link between OCSCs and the progression of the disease could lead to developing better diagnosis and treatment options for patients, which could ultimately improve survival outcome. In this review article, we will discuss the current research on CSCs in OC.

3. Tumour-microenvironment influencing stemness

The exact mechanisms underlying the transformation of normal cells to aggressive cancer cells, particularly in OC, remains elusive. The tumour microenvironment consists of non-cancerous cells and secreted proteins surrounding the tumour. These non-cancer cells are collectively defined as the stroma, composed of endothelial cells, cancer-associated fibroblasts, adipocytes, mesenchymal cells, mesenchymal stem cells and immune cells. Some evidence points towards the microenvironment playing an important role in activation of CSCs such as maintaining their stemness, and enhanced chemo-resistance contributing to treatment failure (Varas-Godoy et al., 2017). For example, IL-17 produced by $CD4^+$ T cells and $CD68^+$ macrophages in the OC tumour microenvironment, promotes self-renewal of $CD133^+$ CSCs (Xiang et al., 2015). OC–associated mesenchymal stem cells (thought to be derived from the bone-marrow) identified in human ovarian tumour samples were shown to regulate tumourigenesis and CSCs *via* BMP production (McLean et al., 2011). In particular, these ovarian tumour-associated mesenchymal stem cells led to an increased percentage of $CD133^+$ and $CD133^+ALDH^+$ CSCs. Hence, the stromal cells and conditions within the ovarian tumour microenvironment can promote stem cell-like phenotype.

The microenvironment of a tumour can have different oxygen pressure depending on its angiogenic status. As the tumour mass expands, its blood supply may not develop adequately to support its growth. Initially, CSCs are located perivascularly within the tumour mass. However recent studies show that CSCs can be found at a distance from vessels, vacating the relatively hypoxic area of the tissue (Liang et al., 2012). The stronger survival capacity of CSCs associated with stemness is stimulated by the induction of Hypoxia-inducible factorα (HIFα) through activation of Oct-4, Sox2, Notch, VEGF and c-Myc gene expression in hypoxic conditions (Keith and Simon, 2007).

One significant characteristic of advanced OC is development of malignant ascites. In contrast to other types of cancers which mostly disseminate through the vasculature, ovarian tumour cells disseminate locally in the pelvic area, throughout the peritoneal and abdominal cavity. Therefore, OC metastasises from the primary tumour through a non-haematogenously-based process, and forms peritoneal carcinomatosis through ascitic fluid as single cells or spheroids (Lengyel, 2010). When OC cells expressing stem cell-related molecules such as Oct4, Nestin, and c-kit/CD117 were isolated from ascites, they had the ability to grow in an anchorage-independent manner *in vitro* as spheroids and form tumours *in vivo* that were able to cause peritoneal metastases (Bapat et al., 2005). Thus, OCSCs can be isolated from ascites and may have high metastatic ability, leading to disease progression.

4. OCSCs mediate chemo-resistance and recurrence

Chemo-resistance or drug resistance remains one of the major challenges to successful OC treatment. Although most OC patients respond well to initial combined treatment of debulking surgery and chemotherapy, many patients exhibit recurrent tumours most of which are resistant to subsequent chemotherapy (Luo et al., 2016). Recent research into CSCs and their effects on OC progression has aided our understanding of chemo-resistance (Ip et al., 2016). Indeed, it has been shown that multiple chemotherapy treatment rounds can enrich the CSC population (Kurtova et al., 2015).

In most OC cases, relapse is associated with a shorter progression free interval, and it is therefore important to improve prognosis of OC to mitigate the risk of mortality. A study by Tucker et al. (2014) showed that high expression of FABP4 and ADH1B in high grade serous OC (HGSOC) tumours was associated with disease relapse and poor clinical

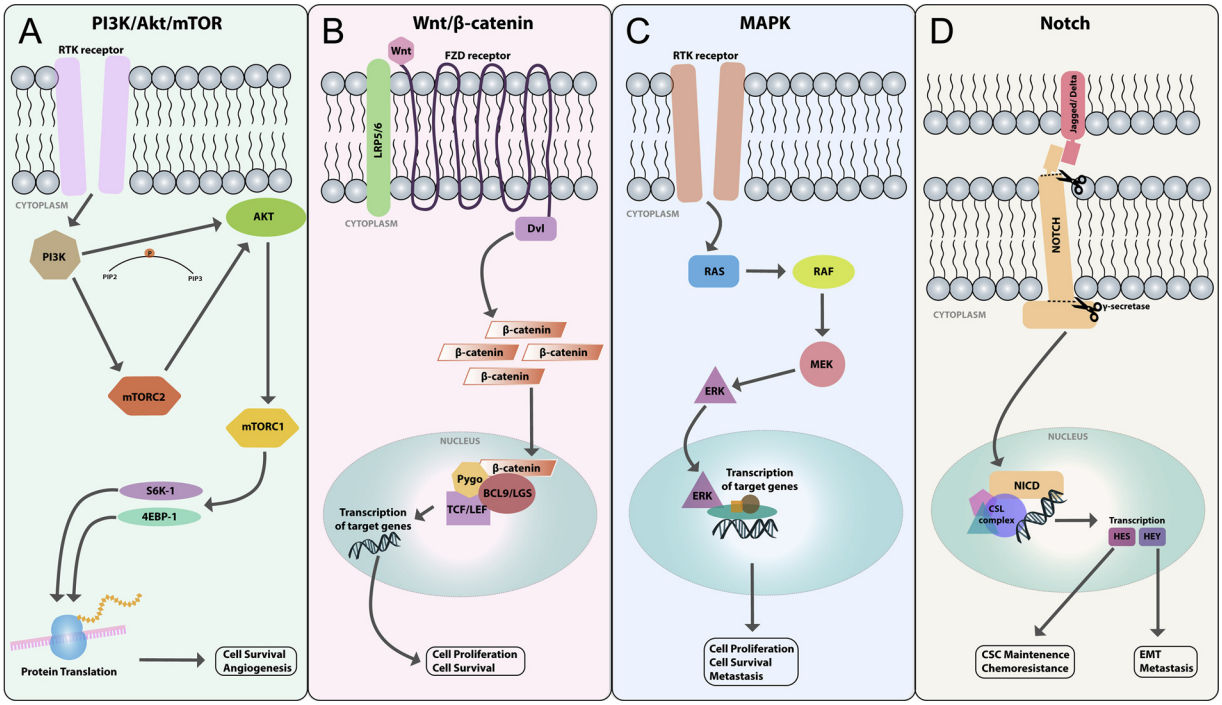

Fig. 2. A) The PI3K/AKT/mTOR pathway is widely implicated in cancer development. Upon activation after ligand binding, phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) which produces phosphatidylinositol 3, 4,5-triphosphate (PIP3). PIP3 recruits AKT to the plasma membrane triggering a cascade of phosphorylation processes. PI3K activates mTORC2, also leading to recruitment of AKT. Activated AKT phosphorylates mTORC1 leading to translation through S6K-1 and 4EBP-1 (translation regulating factors), ultimately leading to processes involved in cancer development (Mabuchi et al., 2015). B) The Wnt/β-catenin pathway is important for CSC maintenance. In the canonical pathway, Wnt ligands bind to transmembrane Frizzled (Fzd) receptor, leading to the recruitment of Dishevelled (Dsh) protein. Dsh triggers the cytoplasmic accumulation of β-catenin, which translocates into the cell nucleus, where it forms a complex with transcription factors lymphoid enhancer factor–T-cell factor (TCF/LEF), B-cell lymphoma 9/Legles (BCL9/LGS) and Pygopus (Pygo), promoting protein transcription of target genes for cellular processes (Arend et al., 2013). C) ECLs (extracellular ligands) bindto RTK receptor, activating cytoplasmic cascade proteins Ras, Raf and MEK through phosphorylation. This triggers ERK to translocate to the nucleus activating transcription of target genes implicating cell proliferation, survival and metastasis of cancer cells (McCain, 2013). D) Notch signalling pathway is activated through cell-to-cell contact. Delta/Jagged ligands bind to Notch receptors on the target cell which triggers cleavage of the extracellular domain through ADAM10/17. This allows intracellular cleavage through γ-secretase, producing Notch intracellular domain (NICD). NICD translocates to the nucleus binding with CBF-1/Su(H)/Lag-1 protein (CSL) complex, triggering transcription and leading to CSC maintenance, metastasis and chemo-resistance (Groeneweg et al., 2014).

outcome, suggesting they could be used as potential prognostic biomarkers (Tucker et al., 2014). FABP4 contributes to OC progression by stimulating the c-Kit/SCF pathway in cancer cells, leading to increased angiogenesis (Ottevanger, 2017; Elmasri et al., 2012). Moreover, Nieman et al. (2011) showed that the ablation of FABP4 in OC mouse models, significantly decreased the metastatic potential of OC cells, suggesting that it may be a therapeutic target to halt metastasis and ultimately prevent OC relapse (Nieman et al., 2011). There are more factors which contribute to chemo-resistance of OC such as alterations to signalling pathways and cellular mutations. Here we discuss the alterations in the OCSC signalling pathways that contribute to OC progression and chemo-resistance shown in Fig. 2. However, an important consideration in interpreting the literature, is the distinction between studies in animal models with cell lines *versus* clinical studies in patients correlating gene expression with patient outcome.

4.1. PI3K/Akt/mTOR pathway

The phosphoinositide 3-kinase (PI3K) pathway is responsible for cell proliferation. However, in OC cells, mutation or alteration of this pathway plays a critical role in tumourigenesis, progression and chemotherapy resistance (Dobbin and Landen, 2013). The PI3K/Akt/mTOR pathway is activated upon recruitment of growth factor to receptor tyrosine-kinases (RTKs) present in the extracellular membrane of cells, which causes phosphorylation of RTKs (Fig. 2).

Cai et al. (2014) examined changes in the PI3K pathway in chemotherapy resistant cells as compared to chemo-sensitive OC cells using the SKOV3 and SKOV3/DDP cell lines, and found that SKOV3/DDP attained a drug resistant phenotype, based on IC_{50} value of 13.96 mg/L compared to 3.31 mg/L (Cai et al., 2014). Furthermore, dual treatment with the PI3K inhibitor, PI-103 and cisplatin showed a commendable response, causing apoptosis in the SKOV3/DDP cells, suggesting that PI-103 increased the sensitivity of these cells to cisplatin. PI-103 inhibits activated Akt and rpS6, signalling molecules which cannot be solely targeted by cisplatin, suggesting that the PI3K/Akt/mTOR pathway plays an important role in apoptosis of OC cells (Cai et al., 2014). Similarly, Ip et al. (2016) investigated the role of the PI3K/Akt/mTOR pathway in maintaining stemness and drug resistance in OCSCs. Treatment of SKOV3 OCSCs with the PI3K/Akt inhibitor, LY294002 caused suppression of Oct 4, ABCG2 and P-gp expression, which are usually induced by cellular stress (Ip et al., 2016). The suppression of these signalling molecules caused increased sensitivity to chemotherapeutic drugs, indicating a crucial role of PI3K/Akt/mTOR in maintaining the drug resistance properties of OCSCs (Ip et al., 2016).

4.2. MAPK pathway

The mitogen activated protein kinase (MAPK) pathway is involved in cell proliferation, survival and apoptosis under normal circumstances. In this pathway, ERK1/ERK2 are activated *via* phosphorylation through the Ras/RAF pathway, initiating cell proliferation (Fig. 2). In OC, mutations in the Ras gene cause continuous activation of the MAPK pathway, leading to cancer progression (Roberts and Der, 2007). In contrast, the JNK and p38 MAPK cascades play a crucial role in cell

growth arrest and apoptosis during chemotherapy treatment. Studies have shown that in OC, decreased MAPK activity by JNK and p-38 is associated with chemo-resistance through cell growth arrest (Roberts and Der, 2007). Alvero et al. (2011) examined the role of ERK1/ERK2 in chemo-resistant OC cells by targeting Akt (Alvero et al., 2011). They showed that when primary ovarian $CD44^+/MyD88^+$ CSCs were treated with Akt inhibitor, NV-128, ROS-dependent activation of ERK was able to initiate mitochondrial-mediated apoptosis in the cells, suggesting a role for ERK in the inhibition of drug-induced apoptosis in OCSCs.

4.3. Wnt/β-catenin pathway

The Wnt/β-catenin pathway regulates cell proliferation, apoptosis and epithelial-to-mesenchymal transition (EMT), usually through the Frizzled (Fzd) receptor (Fig. 2) (Boyer et al., 2010). Cancer cells undergoing EMT acquire stem-like properties that enable them to become drug-resistant, mediating dissemination and metastasis (Deng et al., 2016). Alterations in Wnt pathway proteins lead to tumour initiation and progression of OC. Mutations in the β-catenin gene were observed in endometroid ovarian carcinoma, but are rare in other types of ovarian carcinoma *i.e.* serous, clear cell, and mucinous (Dubeau, 2008). Despite the absence of mutations, the Wnt/β-catenin pathway is still believed to be crucial in OC tumourigenesis (Boyer et al., 2010; Rask et al., 2003; Gatcliffe et al., 2008).

Fzd receptor levels are elevated in OC cells, leading to upregulation of the Wnt pathway, which has been associated with poor survival of patients (Badiglian Filho et al., 2009). CD117 (c-kit) which can also regulate the pI3K-Akt and JAK/STAT pathways, has gained much interest due to its involvement in Wnt pathway alterations leading to the acquisition of stem like properties in cancer cells. The role of c-kit in relation to chemo-resistance in OC tumour initiating cells (TICs) was investigated by Chau et al. (2013), who demonstrated that TICs were resistant to paclitaxel and carboplatin treatment. These chemo-resistant cells expressed CSC markers such as Bmi-1, Nanog, and Oct 4 and also had elevated SCF and c-kit levels. c-kit was targeted using short hairpin RNA (shRNA) or imatinib resulting in a significant reduction of the TIC population, inducing a chemotherapy-sensitive phenotype (Chau et al., 2013). Further, treatment with a combination of chemotherapy and shRNA/imatinib ablated the CSC marker positive subpopulation, leading to the conclusion that targeting c-kit causes TICs to be more sensitive to chemotherapy. In another experiment, the same study showed that under hypoxic conditions, the levels of c-kit were elevated, promoting chemo-resistance in OC cells. (Chau et al., 2013).

Members of the leucine-rich repeat-containing G protein-coupled receptors, specifically LGR5 and LGR6 are also important components of the Wnt pathway that are upregulated in ovarian tumours. (McClanahan et al., 2006). These proteins regulate cell proliferation, cell-cell adhesion and self-renewal in normal stem cells and cancer stem cells in a number of tissues such as skin and intestine. LGR5 is a marker for ovarian and fallopian tube epithelial cells in genetically engineered mouse models of OC (Flesken-Nikitin et al., 2013; Kessler et al., 2015). In OC patients, elevated levels of LGR5 have been reported in primary tumours compared to normal ovaries or benign tumours (n = 140) by immunohistochemistry. Notably, higher expression of LGR5 was observed in high-grade tumours and correlated with poor overall patient survival (Sun et al., 2015) Similar overexpression of LGR5 was also reported in another study comparing patient OC samples (n = 93) *versus* normal ovarian tissue (n = 5); moreover, siRNA knockdown of LGR5 in OC cell lines leads to decreased tumour growth in mice and decreased invasion *in vitro* (Liu et al., 2018).

4.4. Notch signalling pathway

The Notch signalling pathway is responsible for cell survival, proliferation, and maintenance of somatic stem cells. This pathway is also altered in cancer cells contributing to stem-like properties in CSCs. In OC, Notch3 is over-expressed in more than 20% of serous adenocarcinoma and is related to highly aggressive subtypes with poor prognosis (Choi et al., 2008; Park et al., 2006; Shih Ie and Wang, 2007). The Notch pathway is initiated after Notch ligands (Jagged and Delta) bind to Notch receptors. Additionally, Notch signalling cascades are activated *via* ϒ-secretase through a cascade of proteolytic cleavages (Fig. 2) (Choi et al., 2008).

McAuliffe et al. (2012) examined Notch signalling in the side population (SP) enriched in CSCs in murine models, human cancer cell lines and patient derived cells (McAuliffe et al., 2012). OC SPs exhibit CSC characteristics such as recognised cell surface markers including CD133 and ALDH1, greater tumour-initiation capacity and chemo-resistance, and are more invasive than the non-side population (NSP) in murine models, some OC cell lines and clinical samples (Szotek et al., 2006; Moserle et al., 2008). McAuliffe et al demonstrated significant upregulation of regulatory genes involved in pluripotency and maintenance of ovarian CSCs such as c-Kit, Nanog and Notch and multidrug resistance genes including ABCG2, ABCG5 and MDR1 (McAuliffe et al., 2012). SPs have also been isolated from ascites of OC patients and their CSC signature validated by demonstrating the expression of stem cell related genes such as Nanog, Oct4 and other Notch target genes *via* microarray and qRT-PCR (Vathipadiekal et al., 2012). Further, a key role for Notch signalling in SP maintenance was uncovered using Notch inhibitors or transduction with constitutively activated Notch (McAuliffe et al., 2012), coupled with increased resistance to platinum. Importantly Park et al. (2010) have shown that Notch3 expression is higher in recurrent tumors than in primary samples, further implicating Notch signalling not only in chemoresistance, but also in disease relapse.

5. Cancer stem cell markers in ovarian Cancer: chemo-resistance and therapeutic targets

Over the years, identification of CSCs has relied on various cell surface markers. For example, in breast cancer, CSCs are identified based on CD44 antigen (Al-Hajj et al., 2003) and in colorectal cancer, they are identified based on CD24 and CD133, among other markers (Kozovska et al., 2014). OC presents biological features and evolutionary trends characteristic of diseases driven by CSCs. It is speculated that OCSCs contribute to primary tumour growth, metastasis, relapse and acquired chemo-resistance as discussed previously (Lupia and Cavallaro, 2017). CSCs maintain a state of quiescence remaining in G_0 for a prolonged period of time (Giornelli and Mandó, 2017). This presents issues as most treatments administer therapeutics that target actively dividing cells in the S or M phases. Thus, CSC quiescence contributes to the acquisition of chemo-resistance in OC. As the proportion of OCSCs increases at relapse, a challenge for novel therapeutics is determining the efficacy of the drug in both adult stem cells and CSCs in the quiescent state (Giornelli and Mandó, 2017; Zhan et al., 2013). The association between CSCs and OC progression reveals the potential to improve targeted therapies for OC patients. However, further biological characterisation of CSCs, particularly the underlying mechanisms regulating their function is required. The development of novel CSC treatments requires a thorough understanding of the complex genomic profile of OCs, since their heterogeneity dictates the differential responses to treatment, particularly the specificity and efficacy of drugs (Zhan et al., 2013). In an effort to combat heterogeneity and optimise patient treatment response, a combined therapeutic approach is often utilised. Thus, the identification of reliable OCSC biomarkers is a critical prerequisite to improving patient prognosis, progression free survival (PFS) and overall survival (OS) (Burgos-Ojeda et al., 2012). A variety of OCSC markers have been reported (summarised in Table 1), but their validity remains controversial (Burgos-Ojeda et al., 2012). Their relationship to OC chemo-resistance and use as potential targets for therapies is discussed below.

Table 1
Summary of OCSC markers.

Marker	Author	Relevance as OCSC marker
CD24	Choi et al. (2005)	$CD24^+$ cytoplasmic localisation can be predictive of poor prognosis and recurrence.
	Surowiak et al. (2006)	$CD24^+$ correlates with metastasis and poor prognosis in OC patients.
	Su et al. (2009)	CD24 gene expression silencing with shRNA decreases cell viability *in vitro* and suppresses murine tumour growth *in vivo*.
	Gao et al. (2010b)	$CD24^+$ OCSCs have increased tumour initiating potential and expression of stemness-associated genes.
	Meirelles et al. (2012)	SKOV3 and OVCAR5 patient derived cell lines with $CD24^+CD44^+EpCam^+$ tumour cells show increased colony formation *in vitro* and shorter tumour-free survival.
	Jaggupilli and Elkord (2012)	CD24 expression increases metastasis and is a prognostic marker for poor clinical outcome.
	Burgos-Ojeda et al. (2012)	$Apc^-/Pten^-/Trp53^-$ murine OC model demonstrates a $CD24^+$ subset of cancer cells have increased tumour forming potential.
	Nakamura et al. (2017)	Overexpression of CD24 in ovarian tumour tissue correlates with poor prognosis. High expression of CD24 corrrelates with metastasis. Caov-3 cell line with $CD24^+$ cells are resistant to cisplatin treatment increasing metastasis and chemo-resistance.
CD44	Zhang et al. (2008)	$CD44^+CD117^+$ cells isolated from ovarian serous adenocarcinomas demonstrate cisplatin and paclitaxel resistance. $CD44^-$CD117- cells were non-tumourigenic.
	Casagrande et al. (2011)	siRNA knockdown of claudin-3/4 which has increased expression in $CD44^+$ OCSCs inhibits tumour progression in xenograft mouse models.
	Meng et al. (2012)	$CD44^+/CD24^-$ cells from patient tumours correlate with OC recurrence ($p = 0.003$) and PFS ($p = 0.01$).
	Zhang et al. (2013)	Questioned viability of CD44 as biomarker as expression was only found in 38% of patients with primary OC ($n = 483$). $CD44^+$ expression correlates with HGSOC ($P < 0.013$) and advanced stage (III – IV) OC ($P < 0.001$) $CD44^+$ expression does not correlate with OS ($P = 0.529$) or disease-free survival ($P = 0.218$).
CD117	Raspollini et al. (2004)	$CD117^+$/c-Kit^+ expression in OC is correlated with chemo-resistance.
	Schilder et al. (2008)	Treatment of cells with Imantinib Mesylate (CD117 inhibitor), reduces spheroid formation *in vitro*.
	Helland et al. (2011)	$CD117^+$ cells have increased tumourigenicity compared to $CD117^-$ cells, and are able to recapitulate the heterogeneity demonstrated by the patient.
	Luo et al. (2011)	$CD117^+$ cells injected into immunodeficient mice cause an increase in tumorigenicity. $CD117^-$ cells have increased sensitivity to chemotherapy when compared to $CD117^+$ populations.
	Conic et al. (2015)	CD117 overexpression or mutation correlated with poor clinical outcomes. CD117 expression is different for each OC subtype. Increased CD117 expression observed in HGSOC.
	Stemberger-Papic et al. (2015)	CD117 expressed in 81% of HGSOC, and overexpression is associated with shorter OS and PFS periods. CD117/SCF promotes tumour growth and the evasion of tumour cell death by proliferation.
CD133	Baba et al. (2009)	$CD133^+$ cells have significantly higher cisplatin IC_{50} value compared to $CD133^-$ cells, suggesting these cells are drug resistant.
	Curley et al. (2009)	$CD133^+$ cells from OC tumours exhibit CSC properties such as increased tumourigenicity compared to $CD133^-$ population.
	Zhang et al. (2012)	CD133 expressed in 31% of OC tumours and was associated HGSOC, advanced stage of the disease, ascites level and drug resistance. CD133 expression is associated with short PFS and OS periods.
	Skubitz et al. (2013)	Deimmunized pseudomonas exotoxin fused to anti-CD133 single-chain variable fragment with a KDEL terminus inhibits tumour progression in *in vivo* OC model.
	Rüdiger et al. (2017)	Chimeric antigen receptor treatment in conjunction with platinum based chemotherapy successfully used to kill $CD133^+$ cells.
ALDH	Landen and Jr (2010)	ALDH1A1 expression in OCSCs related to poor survival and drug resistance.
	Januchowski et al. (2016)	Knockdown studies of ALDH1A1 show increased drug sensitivity.
	Silva et al. (2011)	Expressed in all 13 human ovarian tumour and 5 ascites specimens from OC patients compared to other OCSC markers. ALDH levels are shown to increase during each round of cisplatin. Co-expression of ALDH and CD133 in OC cells improves tumour regeneration.
	Kryczek et al. (2012)	ALDH expressed in majority of OC patients compared to other CSC markers.
	Ma and Zhao (2016)	Expression of ALDH1A2 associated with poor overall survival.
Thymosin β4	Ji et al. (2013)	Overexpressed in primary OC tumours, with correlation shown to CD133 expression in these tumours.
Nestin	He et al. (2013)	Overexpressed in OC tumours. Associated with HGSOC, advanced disease and poor progression free survival and overall survival.
	Onisim et al. (2016)	Significantly higher correlation with poor OS and PFS compared to CD133 marker, from 85 serous OC tumours.
ROR1	Zhang et al. (2014a)	Prognostic factor associated with short term survival of OC patients
	Zhang et al. (2014b)	ROR1 expressed in OC tumours, with gene expression profile consistent with CSCs. ROR1 co-expressed with $ALDH1^+$ CSCs. ROR1+ cells have increased engraftment of OC tumours in immunodeficient mice.
Hoechst 33342 SP	Vathipadiekal et al. (2012)	SP cells from ascites of OC patients express stem cell related genes.
	Yasuda et al. (2013)	SP cells identified through Hoechst 33342 dye coexpressing $ALDH^{bright}$ show increased tumourigenic ability both *in vitro* and *in vivo*.

Abbreviations: OC, ovarian cancer; CSC, cancer stem cell; OCSC, ovarian cancer stem cell; OS, overall survival; PFS, progression free survival; HGSOC, high grade serous ovarian carcinoma; SP side population.

5.1. CD24

CD24 (Cluster of Differentiation 24) is a 27–30 amino acid cell surface protein anchored to the cell membrane by glycosyl-phosphotidyl-inositol, linked to a wide variety of cancers, such as breast, bladder, colorectal and OC (Kristiansen et al., 2004; Nakamura et al., 2017; Davidson, 2016; Jaggupilli and Elkord, 2012). CD24 is thought to increase metastasis by promoting the adhesion of tumour cells to P-selectin, an adhesion receptor found on platelets and endothelial cells. It has also been used as a prognostic marker for poor clinical outcome in cancer patients (Jaggupilli and Elkord, 2012). $CD24^+$ OCSCs have been isolated from tumours and reported to be enriched in tumour initiating potential and expression of stemness-associated genes (Gao et al., 2010a). Similarly, the association of CD24 expression with increased metastatic potential validates it as a CSC marker. Moreover, CD24 expression has been associated with metastasis and poor prognosis in OC patients (Surowiak et al., 2006), and its localisation in the cytoplasm has been used as a predictive marker for recurrence and poor prognosis (Choi et al., 2005).

Using an $Apc^-/Pten^-/Trp53^-$ murine OC model, Burgos-Ojeda

et al. (2015) reported that the $CD24^+$ subset of cancer cells had increased tumour forming potential, suggesting that $CD24^+$ cells were cancer initiating cells, or CSC; however CD24 alone has low specificity as a CSC marker for identifying a specific type or sub-type of OC as it is also expressed in other epithelial cancers (Burgos-Ojeda et al., 2015). Nakamura et al. (2017) also characterised the expression of CD24 in OC progression, demonstrating that 70.1% of ovarian tumour tissues overexpressed CD24, which correlated with poor prognosis. Furthermore, high expression of CD24 was correlated with advanced stages of the disease, supporting the association of CD24 with metastasis. Finally, their study found that $CD24^+$ cells from a Caov-3 cell line were resistant to cisplatin treatment, in contrast to $CD24^-$ cells. Hence, the association of CD24 with increased metastasis and chemo-resistance supported its validity as an OCSC marker (Nakamura et al., 2017). Meirelles et al. (2012) further refined the identity of OCSCs using patient OC cell lines such as SKOV3 and OVCAR5 showing that $CD24^+CD44^+EpCam^+$ tumour cells exhibited greater colony formation *in vitro* but also shorter tumour-free survival. Notably, $CD24^+CD44^+EpCam^+$ OCSCs could be further enriched by the absence of E-cadherin, an indicator of cells that had undergone EMT; moreover these triple marker positive E-cadherin negative cells also exhibited greater resistance against chemotherapeutic drugs compared to $CD24^-$ cells (Meirelles et al., 2012). There is limited research on CD24 as a therapeutic target in OC. Interestingly, treatment of OC using an shRNA to reduce CD24 expression resulted in decreased cell viability by activation of apoptosis *in vitro* and the suppression of tumour growth in mice *in vivo* (Su et al., 2009). The potential of CD24 OCSC marker as a therapeutic target still requires further research.

5.2. CD44

CD44 (Cluster of Differentiation 44), a transmembrane 85–90 kD glycoprotein, and the main surface receptor for hyaluronic acid facilitating cell-cell interactions and cell migration, is found in many solid tumours including OCs (Bartakova et al., 2018). This receptor has the potential to regulate metastasis, motility and invasion in cancer progression (Senbanjo and Chellaiah, 2017). CD44 is a proposed CSC marker due to its role in cell interactions, cancer cell metastasis and recurrence, although its use as an OCSC marker is controversial (Zhang et al., 2013).

Thus, despite reports of CD44 as a biomarker for OCSC (Alvero et al., 2009), several other papers have contradicted these findings. Zhang et al. (2013) demonstrated that CD44 was expressed in only 38% of patients (n = 483) with primary OC by immunohistochemistry. Although a correlation between CD44 expression and high-grade carcinoma ($P < 0.013$) and advanced stage (III–IV) of OC ($P < 0.001$) was evident, there was no association in OS ($P = 0.529$) or disease-free survival ($P = 0.218$). Furthermore, there was no correlation of CD44 expression between the primary and recurrent OC, leading to the conclusion that CD44 is not a prognostic or CSC marker for OC (Zhang et al., 2013). However, the combination of CD44 with other CSC markers may be prognostic of disease progression, a notion supported by several studies demonstrating resistance to chemotherapy, differentiation, invasion, migration and aggressiveness in different OC cell lines. For instance, Meng et al. (2012) demonstrated a correlation between $CD44^+/CD24^-$ cells from patient tumours and OC recurrence ($p = 0.003$) and PFS ($p = 0.01$) (Meng et al., 2012). $CD44^+$ OCSCs have also been linked to chemo-resistance. Zhang et al. (2008) isolated $CD44^+CD117^+$ cells from ovarian serous adenocarcinomas patients and showed that they had tumour initiating properties and exhibited cisplatin and paclitaxel resistance whereas $CD44^-CD117^-$ were non-tumourigenic (Zhang et al., 2008). CD44 has also been used to target OCSCs for ablation and decrease their chemo-resistance. Casagrande et al. (2011) found that $CD44^+$ OCSCs had increased expression of claudin-4, a tight junction protein with a naturally high affinity to *Clostridium perfringens* enterotoxin (CPE). siRNA knockdown of claudin-3/4 in $CD44^+$ OCSCs inhibited tumour progression in xenograft mouse models, after multiple treatments with CPE resulting in increased OS. This study supports the combination therapy of CPE with $CD44^+$ cells as a means to ablate chemo-resistant OCSCs (Casagrande et al., 2011). In other cancers such as breast cancer, targeted approaches to CD44 functions have been investigated. For example, development of interference peptides with a binding affinity for hyaluronic acid of CD44 CSCs, led to apoptosis in mammary carcinomas (Orian-Rousseau, 2010).

5.3. CD117

The proto-oncogene c-KIT encodes a type III receptor tyrosine kinase termed CD117 - a 145 kD transmembrane receptor which contributes to cell signalling, apoptosis, differentiation and proliferation (Lupia and Cavallaro, 2017). CD117 binds to stem cell factor (SCF), an important regulatory factor in cell growth (Stemberger-Papic et al., 2015; Conic et al., 2015; Klemba et al., 2018). In normal cells, CD117/SCF interaction is essential for stem cell development and survival, whereas in tumour cells, the CD117/SCF interaction promotes tumour growth by proliferation and also promotes the evasion of tumour cell death (Stemberger-Papic et al., 2015). CD117 expression has been found in different mesenchymal-like and epithelial-like tumours, and patients with CD117 overexpression or mutation have poor clinical outcomes (Conic et al., 2015).

Luo et al. (2011), showed that $CD117^+$ cells injected into immunodeficient mice had increased tumourigenic ability, where only 1000 $CD117^+Lineage^-$ cells gave rise to ovarian tumours in these mice. Furthermore, this cell population gave rise to more $CD117^+Lineage^-$ cells, suggesting self-renewal ability. Moreover, $CD117^+$ cells were less sensitive to chemotherapy compared to the $CD117^-$ population (Luo et al., 2011). Similarly, Raspollini et al. (2004) also demonstrated that $CD117^+$ expression in OC was significantly correlated with chemotherapy resistance (Raspollini et al., 2004). Other studies show the potential use of CD117 as a predictive marker for survival and OC subtype. Stemberger-Papic et al. (2015) reported CD117 expression in 81% of 64 primary HGSOCs by immunohistochemistry, and that CD117 overexpression was associated with shorter OS ($p = 0.014$) and PFS ($p = 0.025$), also noting that CD117 may be used as a predictive marker for disease progression (Stemberger-Papic et al., 2015). Similarly, Conic et al. (2015) showed that the expression of CD117 is different for each OC subtype. They reported increased CD117 expression in HGSOC (32.6%), compared to patients with clear cell OC (0%; $p < 0.05$) and mucinous OC (9.1%; $p < 0.05$). Furthermore, the patient prognosis for each OC subtype was different, *i.e.* patients with mucinous and endometrioid OC had poorer clinical outcome (20 and 26.8 months OS respectively) compared to 52.1 months OS in HGSOC with CD117 positive tumours (Stemberger-Papic et al., 2015).

Investigations into CD117 and its effect on drug sensitivity in OC, revealed that knockdown of CD117/c-kit decreased tumour progression and recurrence in mouse xenograft models (Chau et al., 2013). Other targeted treatment approaches specific to CD117 OCSCs have been investigated. In a study conducted by Helland et al. (2011), $CD117^+$ OC cells isolated from patient xenografts had a 100-fold higher tumourigenic potential than $CD117^-$ cells. Moreover, the $CD117^+$ population was able to recapitulate the heterogeneity demonstrated in the patient and could be serially transplanted (Helland et al., 2011). This confirmed the speculated differentiation, renewal and other CSC characteristics of $CD117^+$ cells. Imatinib Mesylate, a CD117 inhibitor has been tested for a variety of cancers including recurrent OC, demonstrating a significant reduction in sphere-forming potential (Schilder et al., 2008). There are several trials of pharmacological tyrosine kinase inhibitors as therapeutics for OC, however to optimise therapeutic action, the biological role of CD117 requires further mechanistic insight.

5.4. CD133

CD133, known also as prominin-1, is a 120 kD penta-membrane glycoprotein initially discovered in murine neuroepithelial stem cells. CD133 has also been found on different adult stem cells and believed to suppress cell differentiation (Klemba et al., 2018). The role of CD133 in CSC has not been fully established, but may be associated with maintaining the cells' lipid membrane composition due to its interaction with cholesterol (Stemberger-Papic et al., 2015). Cell surface CD133 expression has been used as a CSC marker for many different types of cancers. In terms of OCSCs, it is one of the most widely investigated and better understood markers. CD133 has distinct epitopes identified as CD133-1 and CD133-2 which are only overexpressed in ovarian tumours but not in benign tumours or normal ovary tissues (Stemberger-Papic et al., 2015; Klemba et al., 2018). A study conducted by Curley et al. (2009) demonstrated that $CD133^+$ cells have CSC capabilities in OC as these cells isolated from primary human ovarian tumours had increased tumour initiating abilities compared to the $CD133^-$ population when tested in NOD/SCID mice (Curley et al., 2009). Similar to CD117, CD133 expression has also been correlated with survival outcome of OC patients. Zhang et al. (2012) demonstrated that CD133 was expressed in 31% of OC samples (n = 400) using tissue microarray analysis (Zhang et al., 2012). Furthermore, CD133 expression positively correlated with HGSOCs ($p = 0.035$), late stage ($p < 0.001$), ascites level ($p = 0.010$), and non-response to treatment with chemotherapy ($p = 0.023$) by Fisher's exact test and one-way variance analysis. Furthermore, they showed that CD133 was also associated with shorter PFS ($p < 0.001$) and shorter OS ($p = 0.007$) using log-rank tests. These findings suggest that CD133 expression may be used as a CSC marker in OC to monitor and predict clinical outcomes following chemotherapy (Zhang et al., 2012).

CD133 has been significantly associated with chemo-resistance in OC cells. Previous reports show that $CD133^+$ cells are more tumourigenic, highly active and possess chemo-resistance as compared to $CD133^-$ OC cells (Curley et al., 2009; Ferrandina et al., 2008). Baba et al. (2009) also showed that the cisplatin IC_{50} value for $CD133^+$ cells was significantly higher than $CD133^-$ cells, confirming drug resistance in $CD133^+$ cells (Baba et al., 2009).

There are many examples of therapies that have been established through exploiting our knowledge of CD133 and its associated CSC properties. A combination of a murine derived anti-human CD133 antibody and monomethyl auristatin F (a cytotoxic drug) has been used to inhibit cell growth in hepatocellular and gastric cancers (Smith et al., 2008), and could potentially be applied to OCs. An alternate approach utilised a deimmunized Pseudomonas exotoxin fused to anti-CD133 single-chain variable fragment with a KDEL terminus (dCD133KDEL) to inhibit tumour progression in an *in vivo* OC model. dCD133KDEL was specifically designed to target drug resistant cells, providing a novel treatment approach to counter chemo-resistance in OC (Skubitz et al., 2013). A CSC specific chimeric antigen receptor approach in conjunction with platinum-based treatment has also been successfully used against OC cells, focussing on treating recurrence through the activation of natural killer cells with an improved affinity for killing $CD133^+$ cells following cisplatin treatment (Rüdiger et al., 2017).

5.5. ALDH

In humans, Aldehyde dehydrogenase (ALDH) currently consists of 19 genes divided into 11 families and 4 subfamilies, found in different tissues and organs at varying levels depending on the type of enzyme family and subfamily. The ALDH superfamily of NAD (P)+ dependent enzymes catalyse oxidation of aldehydes into their corresponding carboxylic acids (Ma and Allan, 2011; Liebscher et al., 2013). Of the ALDH superfamily of enzymes, ALDH1 and its isoenzymes, mainly ALDH1A1 and ALDH3A1, have been proposed as candidate CSC markers due to their association with poor survival in many cancers including OC (Liebscher et al., 2013).

Five ALDH1 isoenzymes (ALDH1A1, ALDH1A2, ALDH1A3, ALDH1B1, and ALDH1L1) were assessed for their prognostic value in patients with OC by Ma et al. (2016) using an online "Kaplan-Meier plotter" database, to assess their prognostic ability in OC. mRNA overexpression of five ALDH1 isoenzymes was not associated with OS for all the OC patients assessed. However, expression of ALDH1A2 was associated with poor OS, and ALDH1A3 was associated with better OS in wild-type TP53 patients. These results suggest that ALDH1A2 and ALDH1A3 may be used as prognostic markers for certain types of OC patients (Ma and Zhao, 2016).

ALDH has been studied with other CSC markers for OC, including CD24, CD44, CD117, and CD133 to determine the expression level of each marker in OC patients. A study by Silva et al. (2011) involving 13 human ovarian tumours and 5 ascites specimens showed that ALDH was expressed in all ovarian tumours and ascites specimens compared to other CSC markers (Silva et al., 2011). Consistent with this, OC cells co-expressing ALDH and CD133 show improved tumour regeneration in mice compared to cells expressing either marker individually (Silva et al., 2011). Another study by Kryczek et al. (2012) demonstrated that levels of ALDH and CD133 were detected in the majority of OC patients compared to other epithelial CSC markers (Kryczek et al., 2012). Notably, significant variability and overlap of these CSC markers was observed between different OC patients. Although these results suggest that ALDH is highly expressed in OC patients compared to other OCSC markers, the specific ALDH family and subfamily members that were expressed was not investigated.

Several reports have emerged stating that ALDH1A1 plays a critical role in chemo-resistance of OCSCs (Silva et al., 2011; Landen and Jr, 2010). Landen et al. (2010) showed that ALDH1A1 expression was related to poor survival and drug resistance in OCSCs and that ALDH1A1 knockdown resulted in increased sensitivity of CSCs against taxane and platinum drugs (Landen and Jr, 2010). Similarly, Silva et al. (2011) demonstrated that the expression levels of ALDH in SKOV3 cells lines was increased during each dose of cisplatin. However, the same cells with high ALDH expression showed better viability against drug treatment as compared to $ALDH^-$ cells indicating correlation between ALDH expression and chemo-resistance in OCSCs (Silva et al., 2011).

The inhibition of ALDH could potentially be used as a therapeutic to achieve the elimination of drug-tolerant populations, ultimately delaying or preventing cancer relapse (Mele et al., 2018; Raha et al., 2014). Drug-tolerant subpopulations encourage CSCs to acquire chemo-resistance. These subpopulations have elevated ALDH expression which provides CSCs with a survival advantage given that ALDH protects drug-tolerant subpopulations from elevated levels of reactive oxygen species (ROS). When ALDH activity is impaired through the administration of therapeutics, ROS accumulates to toxic levels causing DNA damage and apoptosis of the drug-tolerant subpopulation (Raha et al., 2014).

The ALDH1A1 isoform is considered to be the predominant driver of ALDH activity in CSCs (Mele et al., 2018), given that ALDH1A1 subpopulations have been associated with chemo-resistance and poor prognosis in OC specifically (Landen and Jr, 2010; Liu et al., 2013). Elevated ALDH1 expression has also been correlated with a significant reduction in PFS of OC patients (Mizuno et al., 2015). However, controversy and debate continue in the field as contradictory results have also found ALDH to be indicative of favourable prognoses (Chang et al., 2009; Ricci et al., 2013). Since $ALDH1A1^+$ populations display CSC characteristics associated with both taxane and platinum resistance, and knockdown of ALDH1A1 can re-sensitise OC cells to chemotherapy, ALDH1A1 is an attractive therapeutic target for OCs (Landen and Jr, 2010; Januchowski et al., 2016), with the potential to prevent relapse and improve patient prognosis.

5.6. Thymosin β4 (Tβ4), Nestin and ROR1

Thymosin β4 (Tβ4) is a polypeptide consisting of 43 amino acids found in all cell types. Tβ4 is the major G-actin sequestering protein, which is involved in regulating cell growth, migration and cell structure maintenance. Tβ4 involvement in promoting stem cell activity has made it a potential CSC marker in various cancers (Lever et al., 2017). Tβ4 overexpression has been reported in breast, ovarian and uterine cancers, playing a role in metastasis in colorectal, renal and lung cancers (Ji et al., 2013). These studies suggest that Tβ4 expression is increased in cancer and may be used as a CSC marker. A study by Ji et al. (2013) demonstrated that Tβ4 may be used as an OCSC marker due to its overexpression in primary OC compared to primary stomach cancer using western blot and immunohistochemistry. Furthermore, a positive correlation between Tβ4 and CD133 expression has been reported in immunofluorescence and confocal microscopy experiments (Ji et al., 2013).

Nestin belongs to the class VI intermediate filament proteins, comprised of more than 1600 amino acids, with a molecular weight of about 240 kD, and is involved in cellular activities such as migration and cell adhesion by cytoskeletal regulation (Onisim et al., 2016; Ishiwata et al., 2011). Nestin has gained recognition as a CSC marker in various cancers including bladder, prostate, cervical, brain, testicular, pancreatic, and OC. It plays an active role in tumour angiogenesis and its expression is associated with poor prognosis (Ishiwata et al., 2011). Several studies have investigated the correlation of Nestin expression with OS and PFS in OC patients. A study by He et al. (2013) found that Nestin was overexpressed in OC compared to benign and borderline ovarian tumours. Furthermore, Nestin expression was associated with advanced stage and HGSOC, as well as poor PFS and OS (He et al., 2013). The predictive value of Nestin was further confirmed by Onisim et al. (2016) in 85 serous OC samples, showing an association of Nestin expression with poor OS ($p = 0.025$) and PFS ($p = 0.05$) compared to CD133, which had no correlation (Onisim et al., 2016).

The tyrosine kinase-like orphan-receptor type 1 (ROR1), is a transmembrane protein typically expressed during embryogenesis and in various cancer types, including OC. Interestingly, higher ROR1 protein expression is a predictive factor for short term OC survival (Zhang et al., 2014a, b), and moreover is associated with OCSCs (Zhang et al., 2014b). Thus, patient derived $ROR1^+$ OC cells displayed a CSC gene enriched signature with greater ALDEFLUOR activity and had greater tumour-initiating capacity compared to $ROR1^-$ OC cells (Zhang et al., 2014b). Notably, treatment with an anti-ROR1 mAb or ROR1 shRNA knockdown resulted in inhibition of *in vitro* spheroid formation and migration, as well as decreased tumourigenicity in mice, suggesting that $ROR1^+$ is required for CSC renewal (Zhang et al., 2014b).

5.7. Hoechst 33342 exclusion assay

Recent studies have identified CSCs through the Hoechst 33342 exclusion assay. This assay is based on the principle that like normal stem cells, CSCs have a greater capacity to efflux the Hoechst dye allowing them to be detected by FACS as a distinct sub-population of Hoechst dim cells known as the side population (SP) (Vathipadiekal et al., 2012). As described above (Notch signalling section), several studies have shown that the SP in OC cells and tumours is enriched for CSCs (McAuliffe et al., 2012; Szotek et al., 2006; Moserle et al., 2008), co-expressing known CSC markers. Enrichment for tumourigenic cells in the SP (*versus* non-SP or main population) in OC cell lines has been reported (Vathipadiekal et al., 2012; Yasuda et al., 2013). In addition, Yasuda et al. (2013) showed that SPs expressing high levels of ALDH1A1 ($ALDH^{bright}$) were significantly more tumourigenic than either $ALDH^{bright}$ or SP cells alone and when compared to non-SP/main population or $ALDH^{low}$ cells in OC cell lines (Yasuda et al., 2013). Importantly, SPs with a demonstrable CSC signature have also been found in ascites of OC patients supporting the clinical relevance of this sub-population of cancer cells (Vathipadiekal et al., 2012).

6. Conclusion

Defining stem cell markers that identify OC stem cells unequivocally is still in its infancy and complicated by the heterogeneous nature of OC. It may be unrealistic to expect that a single marker could identify OC stem cells as evidenced by experimental studies to date which reveal that continued enrichment of candidate markers is possible for instance by the combination of $CD133^+$ and $ALDH^+$; or the inclusion of markers that are not expressed such as E-cadherin, alongside positive markers. Ultimately, the suitability of any specific combination of markers over another needs to be compared directly in the same experiments to ascertain if one is better than the other and by how much. The assays used to ascertain the veracity of OCSC markers are also critical – thus while using OC cell lines in tumourigenicity assays is attractive due to ease of access, it remains just the first step. Extending candidate OCSC marker analysis to real patient tumours using tumour microarrays is particularly important, combined with linking expression with clinical patient outcome; ultimately, testing candidate OCSC subsets within patient tumour tissue for greater tumour initiating ability and demonstrating that these cells are more chemo-resistant is required to unequivocally validate OCSC identity. The latter is of course difficult not only due to limited patient tumour tissue availability, but also the need for a clearer classification of patient OC subtypes. In that context, classifying available OC cell lines into subtypes based on genomic profiling into distinct subtypes has been very helpful (Domcke et al., 2013). The classification of actual patient OC samples by transcriptional profiling correlated with clinical prognosis (Tothill et al., 2008) has been critical to advance our understanding of the inherent heterogeneity among ovarian tumours. Given that HGSOC is the most aggressive OC, it is highly desirable that markers that clearly identify CSCs within this subtype of OC, are rigorously defined.

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