International Journal of Biochemistry and Cell Biology 107 (2019) 92–103

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Ovarian cancer stem cells: What progress have we made?

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ARTICLE INFO

Keywords:
Cancer stem cell
Ovarian cancer
Stem cell markers
Drug resistance
Pathways
Clinical trials

ABSTRACT

Ovarian cancer (OvCa) is the most lethal gynecological malignancy in the United States primarily due to lack of a reliable early diagnostic, high incidence of chemo-resistant recurrent disease as well as profuse tumor heterogeneity. Cancer stem cells (CSCs) continue to gain attention, as they are known to resist chemotherapy, self-renew and re-populate the bulk tumor with undifferentiated and differentiated cells. Moreover, CSCs appear to readily adapt to environmental, immunologic and pharmacologic cues. The plasticity and ability to inactivate or activate signaling pathways promoting their longevity has been, and continues to be, the challenge faced in developing successful CSC targeted therapies. Identifying and understanding unique ovarian CSC markers and the pathways they utilize could reveal new therapeutic opportunities that may offer alternative adjuvant treatment options. Herein, we will discuss the current state of ovarian CSC characterization, their contribution to disease resistance, recurrence and shed light on clinical trials that may target the CSC population.

1. Introduction

Ovarian Cancer (OvCa) is the most lethal gynecologic malignancy in the United States (Torre et al., 2018). In 2018, it is estimated that 22,240 new cases of OvCa will be diagnosed and 14,070 women will succumb to their disease (National Cancer Institute, 2018). The high mortality rate is primarily due to our inability to detect early onset of OvCa resulting in most women presenting with advanced stage disease at the time of diagnosis. The standard of care for OvCa includes cytoreductive surgery followed by adjuvant platinum-based chemotherapy (Ozols et al., 2003; Armstrong et al., 2006). Some studies suggest that neoadjuvant chemotherapy prior to interval debulking surgery could further reduce the rates of recurrence (Kehoe et al., 2015; Wright et al., 2016). Regardless of the treatment regimen, most patients will develop recurrent platinum resistant disease. Recurrence is attributed to the inability to completely eradicate all the tumor by surgical and/or pharmacological strategies. It is also believed that among the residual cancer cells, some have inherent or acquired stem like properties serving as seeds for the development of recurrent disease. In some reports, cells with distinguishable stem like biological

Abbreviations: Akt, Protein kinase B; ALDH, aldehyde dehydrogenase; ALDEFLUOR, aldehyde dehydrogenase detection reagent; ATP, adenosine triphosphate; Cadherin, calcium dependent adhesion (for E-cadherin); CD, cluster of differentiation; CSC, cancer stem cell; CXCR4, C-X-C motif chemokine receptor 4; CXCL12, C-X-C motif chemokine ligand 12; DNA, deoxyribonucleic acid; Dsh, dishevelled; EMT, epithelial-mesenchymal transition; EZH2, enhancer of zeste homologue 2; FDA, food and drug administration; Fz, frizzled; GLI1, GLI family zinc finger 1; GLI2, GLI family zinc finger 2; H3K27me3, trimethlylation of lysine residue 27 on the amino (N) terminal tail of histone H3; H3K4me3, trimethlylation of lysine residue 4 on the amino (N) terminal tail of histone H3; IκB, inhibitor of kappa B (1 and in IKKa); IKKα, IκB Kinase alpha; IPI-926, drug name; JAK, Janus kinase; MAPK, mitogen activated protein kinase; MDR1, multidrug resistance 1; MEK, mitogen activated protein kinase kinase; miR, microRNA; mTOR, mammalian target of rapamycin; MyD88, myeloid differentiation primary response gene 88; NANOG, nanog homeobox; NCT, National clinical trial; NFκB, nuclear factor kappa light chain enhancer of activated B cells; NICD1, Notch1 intra-cellular domain; NSC, Normal Stem Cell; OCT4, octamere binding transcription factor 4; OS, overall survival; OvCa, ovarian cancer; PARP, poly (ADP-ribose) polymerase; PDX, patient derived xenograft; PFS, progression free survival; PI3K, phosphatidylinositol-4, 5-bisphosphate 3-kinase; PKC, protein kinase C; PRC2, polycomb repressive complex 2; PTEN, phosphatase and tensin homolog deleted on chromosome TEN; ROR1, receptor tyrosine kinase like orphan receptor 1; SC, stem cell; SMO, smoothened; SOX2, sex determining region Y (SRY)-box 2; SP, side population; ST6GalNAc1, alpha2,6-sialyltransferase gene; STAT3, signal transducer and activator of transcription 3; STn, Sialyl-Tn; TCGA, the cancer genome atlas; TEAD, TEA domain family member; TGF-β, transforming growth factor beta; TLR, toll like receptor; TNFα, tumor necrosis factor alpha; TP53, gene that transcribes p53; Wnt, wingless/integrated signaling pathway; YAP, Yes-associated protein; ZEB1, zinc finger E-box binding homeobox 1

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https://doi.org/10.1016/j.biocel.2018.12.010
Received 16 November 2018; Received in revised form 14 December 2018; Accepted 16 December 2018
Available online 17 December 2018
1357-2725/

characteristics were found to efflux the Hoechst33342 dye by cell transporters the way cells would efflux toxic drugs. These unique cells were found in a side population (SP) fraction as determined by flow cytometric analysis (Christgen et al., 2010; Luo et al., 2012; Meirelles et al., 2012; Oates et al., 2009). Thorough investigation revealed the SP fraction was enriched for cancer stem cells (CSCs), also known as tumor or cancer initiating cells, due to their shared characteristics with stem cells (SCs).

OvCa is a highly heterogenous disease which is most evident by the different histopathologies and diverse genomic signatures (Cancer Genome Atlas Research, 2018b). Epithelial OvCa, the most common (~90%), can be further sub-classified into the serous, endometrioid, mucinous, or clear cell histology. The high-grade serous carcinomas (HGSC) make up the majority of the epithelial ovarian cases and portend poor 5-year survival rate of 35–40% (Berns and Bowtell, 2012; Ledermann et al., 2011; Conklin and Gilks, 2013, Torre et al., 2018). The remaining ~10% of OvCa subtypes are of germ cell or sex cord-stromal origin (Torre et al., 2018). While the name suggests OvCa is derived from the ovary itself, the exact origin of OvCa remains somewhat controversial. Mounting evidence supports the concept that OvCa can be derived from multiple sites including the surface epithelium of the ovary, surface epithelial cells trapped in inclusion cysts within the ovarian stroma, the distal portion of the fallopian tube, or from (or near) an endometriotic lesion (Nik et al., 2014; Seidman, 2015; Abubaker et al., 2013; Merritt and Cramer, 2010; Dubeau, 2008). Due to the lack of a reliable diagnostic method and the vague symptoms with which patients present, more than 70% of OvCa is diagnosed at a late stage at which point the survival rates are dismal (National Cancer Institute, 2018). While the histology, grade and stage at which the disease was diagnosed can influence the clinical outcome of OvCa patients, it is the overall heterogeneity of OvCa that often proves to be the most challenging variable in successfully managing the disease. The aim of this review is to discuss recent developments in the ovarian CSC field, the role of CSC plasticity, and briefly review CSC-based therapies, some of which are already in clinical trials.

2. Cancer stem cells

The genesis of cancer has long been thought to be a product of the clonal evolution whereby a cell that withstands multiple mutational hits or mutations eventually undergoes malignant transformation as shown in Fig. 1**A**. Alternatively, there are those that argue cancer can be derived from an aberrant stem cell (SC) or somatic cell that has undergone key genetic hits allowing the cell to gain stem like properties, including but not limited to asymmetric division. The first indication of the presence of tumor cells with SC properties was in 1858, when Virchow proposed that cancers might originate from immature cells. In 1875, Cohnheim refined this model and suggested that embryonic-like cells remain in the adult tissue and develop into cancer upon activation later in life (Sell, 2004; R., V., Editoral Archiv fuer pathologische, 1855; J, C., 1867). There are roughly 37.2 trillion cells that make up the human body (Bianconi et al., 2013). This massive network of cells requires careful organization, maintenance, and regeneration, which is primarily achieved by SCs. Stem cells are best known for their ability to regenerate skin, blood, and intestinal cells that constantly need to be replenished (Jiang et al., 2002). Both normal stem cells (NSCs) and CSCs are regulated by similar signaling pathways (Wnt, Hedgehog, Notch…etc.) and share many other similarities as shown in Fig. 2. CSCs, like their NSCs counterparts, are believed to have the enhanced detoxification ability (Moitra, 2015) by altering drug transporters (Dean, 2009; Hedditch et al., 2014), and repair their DNA (Wang, 2015). In contrast, there are differences that distinguish CSCs from NSCs. For example, NSCs are characterized by the highly regulated homeostatic balance of self-renewal, while CSCs have lost this ability (Shackleton, 2010). Unlike the bulk tumor population, CSCs maintain their capacity to generate new tumor. Their slow turnover rate renders them unresponsive to current cytotoxic strategies designed to target the more rapidly replicating bulk tumor cells (Moitra, 2015). It is believed the differentiation of CSCs is hierarchal, allowing subsets of cells to have varying levels of replicative ability, often termed transient progenitor cells. Collectively, these properties serve to differentiate CSC from non-CSC. In response to stress initiated by surgery, therapy and/or the tumor microenvironment, some CSCs, progenitors or differentiated tumor cells can undergo further selection or accumulation of genetic mutations leading to more genetically diverse and heterogeneous tumor groups. It remains a point of debate in the scientific community as to which model is primarily responsible for tumorigenesis, metastasis and/or recurrent disease.

In OvCa, Bapat and colleagues first characterized the presence of OvCa stem-like or progenitor cell properties using cells isolated from patient ascites. Using a combination of clonal isolation, anchorage-independent growth and spheroid formation techniques they identified cells that possessed SC like properties. While not all tumor cells have the capacity to form new tumors, these ovarian stem-like cells were tumorigenic (Bapat et al., 2005). Clinically, Steg and colleagues analyzed matched primary/recurrent OvCa samples for expression of CSC markers and showed an enrichment of CSCs and SC pathway mediators after primary therapy (Steg et al., 2012a). Limited functional evidence suggests that treatment of OvCa cell lines or ovarian tumors in vivo with cisplatin or other cytotoxics may push cells to take one or more

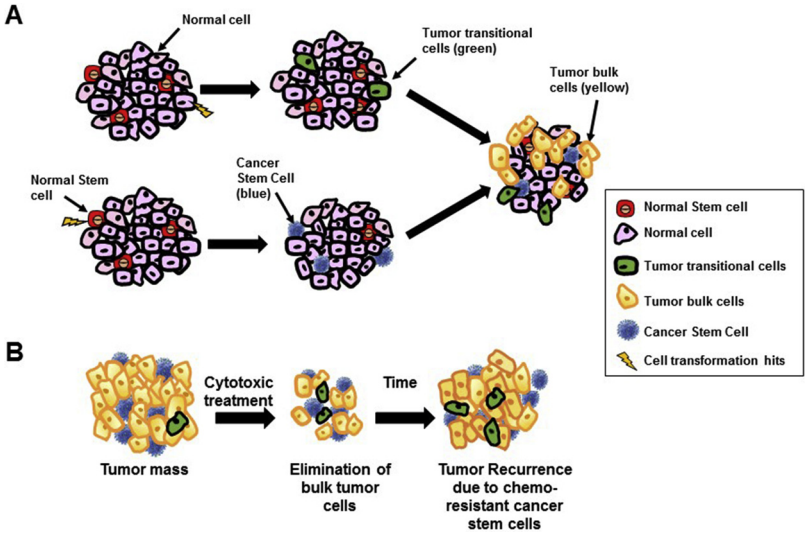

Fig. 1. The CSC model proposes that NSCs or somatic cells (red and pink respectively) undergo transformations that render them CSCs (blue and green cells). These CSCs are then capable of giving rise to more differentiated daughter tumor cells (yellow cells) **(A)**. The CSCs have been shown to be chemo-resistant. Although most of the bulk tumor cells are eliminated, the CSCs remain. The remaining CSCs have been hypothesized as one of the primary drivers for disease recurrence since they can generate additional tumor daughter cells **(B)**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

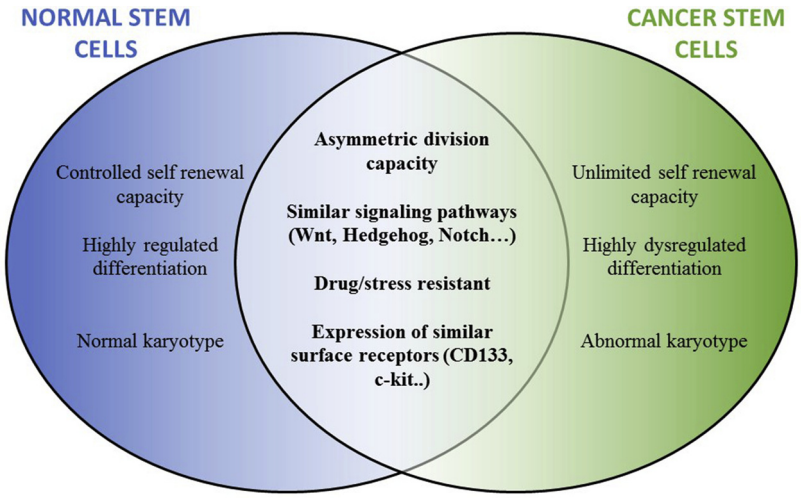

Fig. 2. NSCs and CSCs can have independent as well as shared phenotypes and utilize similar pathways.

CSC-like properties (i.e. chemo-resistance) or cause the enrichment of CSCs as shown in pre-clinical data (Chaffer et al., 2011; Chaffer and Weinberg, 2011) and depicted in Fig. 1**B**.

Recently, a relatively new concept in the CSC research field described as CSC plasticity has brought additional controversy to an already turbulent field. The plasticity of cells refers to the ability of a specific population of cells to switch between different phenotypical states; a more differentiated state to a CSC phenotype. Bidirectional interconversion between stem and non-stem like is attributed to genetic, epigenetic, pharmacologic and/or microenvironmental changes (van Neerven et al., 2016). This process reportedly combines elements of the clonal evolution model and the hierarchal CSC model (Kreso and Dick, 2014; Rich, 2016).

The integration of CSCs, clonal evolution and potential for bidirectional interconversion ensures the durability and longevity of the tumor. More than likely, OvCa is a byproduct of all these models contributing to the heterogeneity of the disease, which is further modified by clinical treatment regimens. Although the interpretation of these models will be further refined, presently, we believe the combination of both clonal evolution and hierarchal CSC models are culprits in the development and resurgence of cancer.

3. CSC markers and challenges in their identification

Identifying and functionally characterizing ovarian CSCs is crucial for developing effective therapies. Several surface antigens, molecular markers and enzymatic activity have been used to identify CSC populations. Herein, we will discuss the more established ovarian CSC markers including CD44, CD117, CD133, CD24 and assessment of ALDH activity and/or expression and the challenges associated with CSC identification. While these are described individually, it is important to note that these markers/enzymatic activities are also used in combination in different cancer types and subtypes. The co-expression as well as changes in marker expression/activity with disease progression are only a few factors to consider when determining the best strategy to use while identifying CSCs.

3.1. CD44

CD44 is a cell-surface receptor with a role in cell-cell interaction, adhesion and migration (Karan Krizanac et al., 2018). CD44 levels are enriched in various cancers and can contribute to tumor metastasis through interaction with different extracellular matrix ligands such as hyaluronic acid (Senbanjo and Chellaiah, 2017). CSCs isolated from solid tumors and ascites of ovarian serous adenocarcinoma patients as well as from established OvCa cell lines (SKOV-3, OV90 and 3AO) were enriched with CD44+ cell surface expression (Alvero et al., 2009; Meng et al., 2012; Shi et al., 2010; Zhang et al., 2008). The CD44+ cells had high sphere-forming ability, possessed asymmetric replicative properties and were resistant to carboplatin and paclitaxel treatment (Alvero et al., 2009). In addition, the CD44+ population had enriched capacity for tumorigenesis (Alvero et al., 2009; Shi et al., 2010; Zhang et al., 2008). Comparing matched ovarian primary, metastatic and recurrent samples showed that CD44 levels were elevated in metastatic and recurrent samples compared to the primary tumors (Gao et al., 2015). Moreover, elevated CD44 levels were also associated with shorter progression free survival (PFS) and reduced overall survival (OS) (Gao et al., 2015). Additional studies revealed an association between CD44+ cells by IHC with poor OS but not with PFS (Karan Krizanac et al., 2018; Lin and Ding, 2017).

3.2. CD117

CD117, also known as c-Kit, is a transmembrane protein tyrosine kinase receptor involved in the embryonic development of hematopoietic SC, primordial germ cells, and melanocytes. As evidenced by IHC, CD117 was highly expressed in various solid tumors including OvCa and its elevation was correlated with cancer grade (Schmandt et al., 2003). Spheres derived from SKOV-3, HEYA8 and HO8910 OvCa cell lines were enriched for CD117+ cells (Chau et al., 2013; Yan et al., 2014). Inhibition of CD117 expression by shRNA or CD117 kinase activity by imatinib in SKOV-3 and HEYA8 reduced the number and size of spheres and sensitized them to cisplatin or paclitaxel treatment (Chau et al., 2013). Independently, inhibition of CD117 gene expression or CD117 activity reduced the tumorigenic potential, while tumors that did form were smaller in size (Chau et al., 2013). CD117+ cells isolated from a serous OvCa PDX formed new tumors at a higher rate and with the original heterogeneity compared to CD117- cells when re-injected into BALB/c-nu mice (Chau et al., 2013). Clinically, elevated CD117 levels as determined by IHC were positively correlated with patient chemo-resistance (Raspollini et al., 2004) and responsiveness to chemotherapy(Chau et al., 2013). Collectively, these studies support that CD117+ enriched cells, like other CSC markers, have many of the required properties of CSCs and can be used as a CSC marker.

3.3. CD133

CD133, also known as Prominin-1, is a trans-membrane glycoprotein with a potential role in organizing plasma membrane topology (Ferrandina et al., 2008). The CD133 marker is widely used for

identifying and isolating CSCs in several solid tumor types. Ferrandina et al. showed that CD133+ cells isolated from OvCa samples had increased colony-forming capacity compared to CD133- populations (Ferrandina et al., 2008). Elevated CD133+ expression was detected in human OvCa samples, epithelial cells derived from ascites, and in OvCa cell lines including IGROV1, OVCAR8, A2780, PEO-1, OVCAR3, OVCAR4, and OVCAR5. These CD133+ cells were highly tumorigenic which recapitulated the original tumor heterogeneity, were drug resistant and/or had vasculogenic potential (Baba et al., 2009; Ferrandina et al., 2009; Curley et al., 2009; Kusumbe et al., 2009; Stemberger-Papic et al., 2015; Cioffi et al., 2015). CD133+ cells were enriched in samples collected from platinum resistant patients and post-chemotherapy treated mouse OvCa cells, supporting the concept that CD133+ cells were chemo-resistant (Steg et al., 2012a; Kulkarni-Datar et al., 2013). However, there is discrepancy within the field as at least two studies show no difference between the tumorigenic potential of CD133+ and CD133- cells in OvCa (Stewart et al., 2011; Ishiguro et al., 2016). Such inconsistencies could be attributed to different analytical tools used and various methodological limitations. Some studies have shed light on regulators of CD133 expression hinting to their possible involvement in CSCs. One such regulator is DNA binding protein ARID3B which is overexpressed in serous OvCa (Cowden Dahl et al., 2009). Overexpression of ARID3B in SKOV-3IP cells was shown to increase mRNA expression of CD133 by binding to its upstream transcription start site. SKOV-3 cells overexpressing ARID3B when treated with cisplatin formed chemo-resistant spheres that displayed elevated levels of CD133+ cells. Mice injected with cells overexpressing ARID3B and CD133 ShRNA had increased survival and less ascites formation compared to mice injected with cells overexpressing ARID3B alone, confirming that ARID3B mediated CD133 regulation is involved in tumor growth and metastasis (Roy et al., 2014, 2018). Another player known to indirectly regulate levels of CD133 is miR-200a. CD133+ cells isolated from OVCAR3 cells had decreased expression of miR-200 compared to CD133- cells as determined by qPCR. Overexpressing a miR-200a mimic in CD133+ cells led to inhibition of their migratory and invasion potential indicating a possible role of miRNA in regulation of CSC marker expression (Wu et al., 2011).

It is worthy of note that both CD117 and CD133 were investigated using an FDA approved OvCa treatment strategy: Poly (ADP-ribose) polymerase inhibitors (PARPi). The treatment of OvCa cell lines with the PARPi olaparib and rucaparib induced an enrichment of cells expressing the CSC markers CD133 and CD117 (Bellio et al., 2018). These CSCs displayed a more efficient DNA repair pathway, compared to their non-CSC counterpart. This enhanced DNA repair observed in the ovarian CSCs is believed to be due to activation of a DNA meiotic recombinase 1 (DMC1), a DNA recombinase, which is normally associated with meiosis, provides CSCs with an advantage to survive the synthetic lethality of PARPi (Bellio et al., 2018). These findings may provide some insight as to why patients treated with PARPi develop resistance despite a promising initial clinical response.

3.4. CD24

CD24 is a glycoprotein attached to the cell surface via a glycosylphosphatidylinositol link and is expressed in a variety of solid tumors. Immunohistochemistry analysis of 69 epithelial ovarian tumors showed membrane and cytoplasmic expression of CD24 in 84% and 59% of low and high-grade cases respectively (Kristiansen et al., 2002). A subset of CD24+ cells that were isolated from human OvCa samples were shown to be more quiescent, chemo-resistant, tumorigenic in nude mice, and possessed the ability to self-renew and differentiate (Gao et al., 2010). CD24 was found to be a specific diagnostic marker in differentiating malignant mesothelioma from OvCa with its expression being uniformly absent in malignant mesothelioma (Davidson et al., 2016). While being present in a majority of OvCa samples, as analyzed by IHC, CD24 expression was elevated in OvCa effusions compared to solid tumors and metastatic lesions suggesting that its expression might be linked with acquisition of a CSC-like phenotype (Davidson et al., 2016). CD44+, CD24+, EPCAM+, and E-cadherin$^-$ cells isolated from established OvCa cell lines SKOV-3 and OVCAR-5 showed increased colony formation, resistance to doxorubicin treatment in vitro and displayed shorter tumor-free intervals in vivo (Meirelles et al., 2012). Combined, these studies indicate that CD24+ enriched cells possess CSC like phenotypes. However, like other makers, use of CD24 alone may not be sufficient to detect CSCs but may gain efficiency when combined with other CSC markers (Jaggupilli and Elkord, 2012).

3.5. Aldehyde dehydrogenase (ALDH)

Aldehyde dehydrogenase is a family of enzymes that catalyzes the oxidation of aldehydes to their carboxylic acid forms (Chang et al., 2009). ALDH enzymatic activity detected by ALDEFLUOR assay has been used to identify stem-like cells in several solid cancers (Chang et al., 2009). Expression analysis of a member of the ALDH family, ALDH1, comprising of multiple isoforms such as ALDH1A1, ALDH1A2…etc has also been utilized to characterize the CSC population in several malignancies (Landen et al., 2010). Deng and colleagues showed that expression levels of ALDH1 by IHC correlated with ALDH activity in human epithelial cancers (Deng et al., 2010). Hence, studies using the ALDEFLUOR assay to determine ALDH activity as well as those using IHC to detect ALDH1 expression in CSCs should be considered. Briefly, elevated ALDH1 expression and ALDH activity were directly related to cells possessing high tumorigenic potential and chemo-resistance in OvCa cell lines and patient samples (Landen et al., 2010; Ayub et al., 2015; Liao et al., 2014; Meng et al., 2014; Sharrow et al., 2016; Wang et al., 2014). Knockdown of ALDH1 or inhibiting ALDH activity using a DNA methyltransferase inhibitor sensitized OvCa cells to chemotherapy and reduced their stem-like properties (Ishiguro et al., 2016; Landen et al., 2010; Meng et al., 2014; Wang et al., 2014). Treatment of drug-resistant OvCa cell lines with all-trans-retinoic acid (all-trans-RA) to downregulate ALDH1, or with di-ethyl-amino-benzaldehyde (DEAB) to inhibit ALDH activity led to re-sensitization of these cells to paclitaxel and topotecan (Januchowski et al., 2016). These results suggest that ALDH may be involved in drug resistance and is therefore an interesting therapeutic molecular target (Januchowski et al., 2016). Several studies have shown a negative correlation between ALDH1 expression and poor clinical outcome including reduced OS and PFS in OvCa patients (Landen et al., 2010; Deng et al., 2010; Ayub et al., 2015; Meng et al., 2014; Liebscher et al., 2013). On the contrary, other studies present positive correlation between patient outcome and ALDH1 expression (Chang et al., 2009; Huang et al., 2015). Collectively, there remains some controversy, which is likely based on analysis, technique, methodology and group composition.

The utility of ALDH has been realized by multiple researchers and was used as a read-out to determine whether there are drugs that can be re-purposed to enhance the ability of chemotherapeutic measures by targeting CSCs. Metformin hydrochloride, a biguanide, was developed as an anti-diabetic. Alternatively, metformin was able to suppress tumor growth and relapse in vivo in breast cancer xenografts when combined with standard chemotherapy, implying its ability to target a different cell population (Iliopoulos et al., 2011). In OvCa, Metformin reduced the percentage of ALDH+ cells below control levels. Metformin also decreased the number of tumor spheres formed from both ALDH+ and unsorted primary human ovarian tumor or ascites cells, further supporting its involvement in targeting a CSC population (Iliopoulos et al., 2011; Shank et al., 2012; Wu et al., 2012). Several potential mechanisms of action for metformin have been proposed and are currently being studied in a Phase II clinical trial focused on OvCa (NCT02122185). It will evaluate PFS along with radiological and biochemical progression. In addition to determining whether metformin thwarts recurrence, it would be of interest to confirm if metformin targets the ALDH+ population clinically. Similarly, its impact on other

cells that share CSC like phenotype but were ALDH- would be of value. Metformin's unique repurposing opens the door to consider the potential of already approved drugs that could also target CSCs.

3.6. Altered glycosylation and its impact on CSCs

Tumor associated carbohydrate antigens (TACAs) have emerged as contributors to the CSC phenotype. It has been shown that sialyl-Tn (STn) expression in cells can overlap with SC markers such as CD133 (Starbuck et al., 2018). Specifically, cells isolated via STn share several properties that are normally associated with CSCs including colony and sphere formation, enhanced tumorigenic capacity and chemo-resistance. Additionally, targeting STn positive cells with anti-STn-ADC led to decreased tumor volume in OvCa PDX models (Eavarone et al., 2018).

From another perspective, the ST6Gal-I glycosyltransferase (ST6GalNac-I) adds α2-6-linked sialic acids to substrate glycoproteins and has been implicated in carcinogenesis (Schultz et al., 2016). ST6Gal-I was shown to be upregulated in OvCa, enriched in metastatic tumors and associated with reduced patient survival (Christie et al., 2008). More importantly, ST6Gal-I upregulation in cancer cells directly altered spheroid growth, chemo-resistance and augmented tumor initiating potential (Schultz et al., 2016). Tumor associated ST6GalNac-I has been previously shown to regulate SC transcription factors such as Sox9 and Slug (Schultz et al., 2016). The tumor initiating capacity was reversed following knockdown of ST6Gal-I. Collectively, these studies support a role for altered glycosylation promoting a CSC phenotype.

3.7. Challenges associated with identifying and sorting CSCs

Due to their low frequency and hierarchal nature, accurately identifying a pure CSC population from bulk tumor cells can be difficult. In addition to technical complexities, the most common CSC markers are recognized for their ability to enrich for CSC populations, but not all cells displaying CSC markers have CSC properties. The purification of CSCs is commonly done using an immunomagnetic approach or fluorescent activated cell sorting. Using a variety of positive and negative markers for CSC inclusion and exclusion criteria aids in the isolation of CSC populations with high fidelity. However, establishing parameters for CSC frequency of surface markers is further complicated by the inter- and intra-tumor heterogeneity of OvCa. Additionally, it is important to take into consideration that tumor cells change in response to fluctuations in the local microenvironment typically caused by diagnostic modalities such as positron emission tomography (PET) scans, surgical intervention and/or pharmacological treatments which can further complicate CSC isolation (Predina et al., 2013).

Relying on marker expression alone to determine CSC frequency is risky in studying NSC as well as CSCs. Similarly, the use of only a single marker may be risky and not be sensitive enough to detect CSCs. However, the efficiency of CSC identification may be increased by using a combination of CSC markers (Jaggupilli and Elkord, 2012). More recently, Gonzalez et al have used single cell mass cytometry to identify the co-expression of various proteins with CSC markers/antigens that may augment the identification and their functional characterization (Gonzalez et al., 2018). In addition to CSC marker identification, functional assays should be employed to verify SC like properties of CSCs, which can vary widely. While the xenograft in vivo model is considered a 'gold standard' for determining whether cells truly display stem like properties it is costly and time consuming. Moreover, not all primary tumor cells or established cell lines grow in the more commonly used immunocompromised mouse models or grow slower than the original tumor in vivo (Gomez-Cuadrado et al., 2017). Further, these in vivo models have limitations, perhaps the most relevant to the current emerging immune-oncology field is their immunocompromised state. Thus, multiple methods should be employed for higher confidence in proper CSC isolation.

4. Signaling pathways directly or indirectly involved in CSC maintenance, self-renewal, differentiation and/or drug resistance

Understanding the signaling pathways involved in the maintenance, self-replication, differentiation and/or drug resistance properties of CSCs would provide a platform to identify novel therapeutic targets. Here, we will highlight the major pathways that are known to be active in ovarian CSCs. It is important to note that these pathways are not necessarily linear and like many other cancer subtypes, there is cross-talk between the various pathways. It is equally important to note that these pathways are also essential in the normal homeostatic control needed in cells, which renders studying and targeting them a challenge.

4.1. Notch

The Notch pathway is highly conserved and extensively studied in many areas including embryonic development, cell fate and CSC maintenance, replication and differentiation (Venkatesh et al., 2018; Patel et al., 2005). The contribution of Notch to the pathology of OvCa has been extensively reviewed in 2014 (Groeneweg et al., 2014a). Briefly, the Notch pathway is activated when one of its receptors (Notch-1, 2, 3, 4) is cleaved by coupling with a Notch Ligand (Jagged-1, 2, Delta-like 1, 4) expressed on neighboring cells (Karamboulas and Ailles, 2013). Notch cleavage is mediated by Gamma-secretase and A-disintegrin and metalloproteinase 10 (ADAM-10) allowing nuclear translocation of the Notch intra-cellular Domain (NICD). Inside the nucleus, NICD binds to Core binding factor-1 (CBF-1) which recruits transcription factors to regulate gene transcription (Karamboulas and Ailles, 2013; Takebe et al., 2015). Gamma secretase inhibitors (GSI) were used in in vitro and in vivo experiments to determine the functional role of Notch signaling in OvCa. GSI inhibited the proliferative ability of the OVCAR3 and SKOV-3 OvCa cell lines. Additionally, GSI mediated inhibition of the Notch pathway negatively impacted tumor growth in OvCa PDX models (Groeneweg et al., 2014b). Use of GSI in combination with paclitaxel had a synergistic effect in platinum-resistant ovarian tumors compared to single agent treatment. Interestingly, the use of GSI did not have a synergistic effect in platinum-sensitive tumors which suggests a role for the Notch pathway, particularly Notch 1 and Notch 3 in the ability of the tumor to become chemo-resistant indicating a link with CSCs (Groeneweg et al., 2014b). In a separate study, blocking gamma-secretase cleavage activity by GSI, and silencing Notch-3 using siRNA in vivo resulted in increased tumor sensitivity to cisplatin and reduced tumor burden (McAuliffe et al., 2012). The significance of Notch in OvCa was also determined through epigenetic analysis using cancer genome atlas (TCGA) data. Analysis of DNA methylation, miRNA and gene expression for Notch showed that modifications of Notch regulate multiple downstream cancer related genes including *PPARG*, *CCND1*, and *RUNX1* (Ivan et al., 2013). With relation to CSCs, gene expression levels of *Notch 1, 2, 3* and *ALDH1* were measured in OvCa tumors, and the results showed that *ALDH1* was positively correlated with *Notch-3* (Kim et al., 2017). Additionally, via clinicopathological analysis of patient outcomes, overexpression of Notch-3 was shown to be an independent poor prognostic indicator for patient survival (Kim et al., 2017). Notch-3 was specifically implicated in promoting chemo-resistance in OvCa (McAuliffe et al., 2012). Overexpression of the Notch-1, 2 or 3 intracellular domains (NICD1, 2, 3) in the 4306 murine OvCa cell line showed a positive correlation between *NICD3* and levels of *CD44* via gene expression suggesting a possible connection between the Notch and CD44 signaling pathways (McAuliffe et al., 2012).

Overexpression of Galectin-3 (a lectin with affinity to β-galactose-containing glycoconjugates) resulted in an increase in nuclear translocation of NICD1 suggesting that Galectin-3 could support ovarian CSC maintenance via Notch-1 activation. Galectin-3 has been shown to increase drug resistance and is associated with poor survival rates in OvCa by multiple studies utilizing primary OvCa samples and OvCa cell

lines (Kim et al., 2011; Mirandola et al., 2014; Oishi et al., 2007). Silencing Galectin-3 in SKOV-3, SNU-840, DOV13 and RMUG-1cell lines led to a decrease in the cleaved form of the NICD1 as well as Notch signaling pathway target genes *HEY1* and *HES* (Kang et al., 2016). Conversely, the overexpression of Galectin-3 in A2780 and OVCAR3 cell lines led to an increase in NICD1 and downstream Notch regulated genes (Kang et al., 2016).

4.2. Hedgehog

There are three main ligands that activate the Hedgehog pathway, namely Sonic, Desert, and Indian. These ligands convey their downstream actions via Patched, Smoothened (SMO) and Gli transcription factors (Merchant and Matsui, 2010). The Gli transcription factors are the targets of TGFβ/SMAD and can activate the Hedgehog pathway independently, rendering it as a positive feedback loop (Dennler et al., 2007). The Hedgehog pathway cascade is involved in the development of the nervous system, skeleton, major body organs and regulation of SCs (Merchant and Matsui, 2010). The expression of the Hedgehog ligand, Sonic, has been shown to be present in ~47% of the malignant primary ovarian epithelial tumors, yet absent in benign tumors (McCann et al., 2011). The TGF-β co-receptor endoglin (CD105) and the Hedgehog mediator Gli 1/2 were reported to be overexpressed in recurrent OvCa and contribute to cisplatin resistance (Ivan et al., 2013). In a separate study, it was shown that treatment of OvCa cell lines with a Sonic Hedgehog antibody decreased cell proliferation (Bhattacharya et al., 2008). Steg and colleagues demonstrated that in tumors collected from patients with recurrent platinum-resistant disease, the knockdown of CD105, Gli1 and Gli2 decreased cell viability. They speculated that the loss of resistance indicated the importance of this pathway in platinum-resistance (Steg et al., 2012a). The Hedgehog pathway inhibitor, saridegib (IPI-926), conveys its action by inhibiting SMO (Tremblay et al., 2009). Treatment of multiple ovarian PDX models with IPI-926 was shown to be effective as a single agent and effective as a maintenance therapy strategy post-chemotherapy (McCann et al., 2011). Similarly, Coffman and colleagues showed that using IPI-926 in vivo, resulted in the elimination of chemo-resistance and angiogenesis, alluding to the role of this pathway in CSCs (Coffman et al., 2016).

While many of the trials weren't designed to target CSCs specifically, their outcomes yield some interesting results that indirectly hint at their ability to influence CSCs. Sonidegib is an antagonist for the SMO protein. Sonidegib is FDA approved for basal cell carcinoma (Doan et al., 2016). A recent study using the SMO antagonists sonidegib and cyclopamine in A2780cp20 and SKOV-3TRip2 cells in xenograft models showed decreased tumor burden compared to the placebo. Additionally, both SMO antagonists significantly increased the sensitivity of the chemotherapy-resistant OvCa cell lines A2780cp20, HeyA8MDR, and SKOV-3TRip2 to paclitaxel. In A2780cp20 and SKOV-3TRip2 cell lines, sonidegib decreased multidrug resistance 1 (MDR1) expression compared to vehicle control, while paclitaxel increased MDR1 expression (Steg et al., 2012b). A Phase I clinical trial in combination with paclitaxel (NCT01954355) found two of eight OvCa patients showed a partial response and established a recommended Phase II dose (Stathis et al., 2017). A Phase IB clinical trial (NCT02195973) using lower doses assessed the development of adverse events and tumor response. However, no results have been released as of yet. Additional preclinical and clinical studies are warranted to elucidate the specific cell populations that sonidegib targets. It would be of interest to know whether it reduces CSC number or only prevents their self-replicative or differentiative properties in the patients that responded. A second Hedgehog pathway inhibitor, vismodegib, also acts through SMO. A phase II trial tested vismodegib in a maintenance therapy regimen in patients diagnosed with OvCa after 2nd or 3rd complete remission (NCT00739661). Unfortunately, no marked increase in PFS was observed (5.8 months in the placebo compared to 7.5 months in the treatment group) (Kaye et al., 2012) suggesting that inhibition of Hedgehog pathway alone was insufficient to overcome recurrent disease. It is unclear if the CSC population was affected post treatment. Whether combining a Hedgehog inhibitor with a different cytotoxic treatment or an additional inhibitor targeting a different supportive CSC signaling pathway would extend the PFS remains to be seen.

4.3. Wnt/β-catenin

The wingless/integrated (Wnt) signaling pathway is critical during embryogenesis where it controls cell proliferation, differentiation, migration, organ development and body axis formation (Clevers, 2006). Canonically, the Wnt ligand binds to the frizzled (Fz) complex resulting in phosphorylation of the dishevelled (Dsh) and lipoprotein receptor-related protein (LRP) by Casein kinase 1-γ (CK1-γ) and Glycogen synthase kinase 3β (GSK3β). These phosphorylation events allow docking of the scaffold protein, Axin, to the Fz/Dsh complex resulting in stabilization of β-catenin. β-catenin accumulates in the nucleus where it forms complexes with transcriptional coactivators such as T cell factor (TCF) for transcription of Wnt-responsive genes (MacDonald et al., 2009). Wnt/β-catenin signaling is important in normal as well as CSCs. TCF3 is a downstream Wnt/β-catenin transcription cofactor that forms a complex with Nanog and octamer binding transcription factor 4 (OCT4) transcription factors to maintain the gene expression necessary for embryonic stem cell pluripotency in mice (Cole et al., 2008). Moreover, work by Chen and colleagues, demonstrated the importance of Wnt signaling in tissue regeneration as well as SC function and self-renewal in a zebrafish model (Chen et al., 2009). Wnt/β-catenin pathway activity has been linked to the CSC population in several cancer types including colorectal (Vermeulen et al., 2010), breast (Xu et al., 2015) and lung (Jiang et al., 2015). Elevated levels of Wnt signaling were detected in OvCa (Arend et al., 2013) and may contribute to chemo-resistance and poor patient OS (Nagaraj et al., 2015). Treatment of OvCa cell lines (SKOV-3 and HEYA8) with Compound K, the downstream metabolite of the Saponin ginesenoside-Rb1, led to reduced proliferation as determined by MTT assays, epithelial-mesenchymal transition (EMT) as determined by protein expression and phosphorylation as well as sphere forming ability (Deng et al., 2017). Additionally, Nagaraj and colleagues underlined the importance of Wnt/β-catenin signaling in ovarian CSCs as knock down of β-catenin or use of the Wnt/β-catenin inhibitor, iCG-001, reduced sphere formation, sensitized platinum-resistant cells to cisplatin, and reduced CSC frequency in chemo-resistant OvCa cell lines (Nagaraj et al., 2015). Treatment of CSCs isolated from prostate, breast, and OvCa cell lines with Secreted frizzled-related protein 4 (sFRP4), a Wnt antagonist, in combination with doxorubicin/cisplatin reduced sphere forming capacity, proliferation as measured by MTT assay, and downregulated stemness related genes (Deshmukh et al., 2017). Crosstalk between signaling pathways is common. Chen and colleagues showed the inhibition of the Signal transducer and activator of transcription 3 (STAT3) pathway epigenetically inactivated Wnt/ β-Catenin signaling resulting in reduced sphere formation and chemo-resistance in cells isolated from ascites from recurrent OvCa patients as well as established OvCa cell lines (Chen et al., 2017). Additionally, in a mouse model of intraperitoneal OvCa, paclitaxel treatment combined with STAT3 knock down synergistically reduced peritoneal tumor seeding and extended mouse survival (Chen et al., 2017).

4.4. NFκB and TLR2-MyD88-NFκB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is involved in multiple normal cellular functions; nonetheless, its constant activation has been implicated in invasion of several forms of cancer including OvCa (Prasad et al., 2010). The NFκB signaling pathway has been thoroughly studied in inflammation and several cancer subtypes (Hayden and Ghosh, 2008; Hoesel and Schmid, 2013). There are five proteins in the NFκB transcription factor family that

dimerize but are typically inactivated by IκB (Hayden and Ghosh, 2008). The NFκB pathway can be activated in a canonical or non-canonical manner. In the canonical pathway, NFκB activation occurs through the binding of TNFα or IL1β to specific receptors. In the non-canonical pathway, activation occurs via different receptors such as CD40 and signals through IKKα (Hayden and Ghosh, 2008; Hoesel and Schmid, 2013). House et al has shown that the non-canonical NFκB pathway regulates ALDH activity, spheroid formation and tumorigenesis in OvCa via the transcription factor RelB (House et al., 2017). However, they also show the canonical pathway is also involved in tumorigenesis but not ALDH activity (House et al., 2017). CD44+ CSCs isolated from OvCa patient ascites displayed constitutive activation of both canonical and non-canonical NFκB pathways (Alvero et al., 2009). Additionally, inhibition of NFκB activity in ovarian CSCs using eriocalyxin reduced cytokine production and increased sensitivity to FasL and TNFα-mediated cell death of ovarian CSCs in vitro (Leizer et al., 2011).

Myeloid differentiation primary response gene 88 (MyD88) is an adapter protein used by almost all Toll Like Receptors (TLRs) to activate NFκB and was shown to be present in neoplastic cells as assessed by IHC of OvCa tumors (d'Adhemar et al., 2014). The expression of MyD88 was also associated with decreased PFS and OS (d'Adhemar et al., 2014; Steffensen et al., 2011; Block et al., 2018). A recent study that analyzed 5263 OvCa tumor samples by IHC and microarray analysis concluded that MyD88 expression was modestly correlated with OS. However, MyD88 expression was strongly associated with advanced stage of OvCa. In contrast, low grade OvCa tumor expression of both MyD88 and TLR4 were associated with improved survival (Block et al., 2018). MyD88 protein levels of isolated tumor cells measured by western blot revealed that patients with low MyD88 expression responded better to carboplatin and paclitaxel treatment and had better OS compared to those with elevated MyD88 (Silasi et al., 2006). The presence of MyD88 and CD44 in epithelial OvCa cells was used to isolate and establish clonal cells that could re-capitulate an original tumor in mice (Chefetz et al., 2013). Chefetz and colleagues then used these CD44+/MyD88+ as well as CD44-/MyD88- cells to show that the CD44+/MyD88+ cells had increased wound healing capacity as determined by scratch assays (Chefetz et al., 2013). Alvero and colleagues demonstrated that one of the phenotypes of ovarian CSCs is the expression of CD44+/MyD88+ as well as the activation of the NFκB pathway (Alvero et al., 2009). The presence and activation of the TLR4/MyD88/NFκB signaling pathway also contributes to the inflammatory microenvironment which typically drives an aggressive OvCa phenotype (Li et al., 2016). In fact, Silasi and colleagues showed that MyD88 protein expression, as determined by IHC, was seen in immune cells infiltrating OvCa tumors as well as tumor cells (Silasi et al., 2006). Collectively, these data suggest the expression of MyD88 in addition to specific CSC markers confers some of the chemo-resistance properties seen in CSCs.

4.5. YAP/TEAD

The Hippo pathway is best known for its ability to regulate organ size and is associated with OvCa progression and drug resistance (Hall et al., 2010). TEA domain family member (TEAD) is a transcription factor targeted by the Hippo pathway effector Yes-associated protein (YAP) to transcribe downstream target genes. The importance of the YAP/TEAD complex in ovarian CSCs was demonstrated by knock-down of YAP/TEAD in A2780 spheres which reduced Oct4 and Notch1 protein levels as measured by western blot and RNA levels of *Sox2*, *Oct4*, *Nestin*, *Notch1*, and *Nanog* as measured by RT-PCR (Xia et al., 2014a). Moreover, knock-down of YAP alone reduced sphere forming ability of A2780 cells. In the same model system, the authors show that the YAP/TEAD complex was involved in chemo-resistance to cisplatin, paclitaxel, and bleomycin through crosstalk with the RAS-MAPK and PI3K pathways (Xia et al., 2014a, b). Collectively, this data hints that the YAP/TEAD pathway may influence CSC pluripotency, drug resistance, and self-renewal. YAP/TEAD targeting therapies are in development and promising results have been shown with the inhibitor of YAP/TEAD, verteporfin. This inhibitor disrupts the YAP-TEAD interaction which led to decreased proliferation, migration, and invasion of OVCAR3 and OVCAR8 cell lines in vitro and reduced tumor burden in OVCAR8 xenografts (Feng et al., 2016). While the results of verteporfin are encouraging, it would be interesting to see its effect specifically on ovarian CSC populations.

4.6. JAK/STAT3

The Janus kinases (JAKs), Signal Transducer and Activator of Transcription proteins (STATs) (JAK/STAT) pathway has been implicated as a promoter of OvCa pathology. Activation via phosphorylation was found in more than 85% of ovarian tumors and overall phospho-STAT3 nuclear expression has been correlated with poor OS in OvCa (Rosen et al., 2006; Shang et al., 2017). CD24, a marker for OvCa cells with stem like features, has been shown to correlate with increased phosphorylation of STAT3. The importance of the JAK/STAT pathway in metastasis was shown by using a JAK2 inhibitor TG101209 in an in vivo model in which intra-bursal injections of adenovirus Cre depleting Apc−; Pten−; Trp53− were used to induce ovarian tumors (Wu et al., 2007). Mice were treated with a JAK2 inhibitor/cisplatin combination or cisplatin alone. The mice treated with the combination JAK2 inhibitor/cisplatin had increased survival. Additionally, when mice were treated with TG101209 only 1/14 mice exhibited metastases compared to mice treated with vehicle (Burgos-Ojeda et al., 2015). When these cells were sorted to CD24+ and CD24- populations and treated with the JAK2 inhibitor, a decrease in STAT3 phosphorylation was observed and there was an induction of cytotoxicity in resistant CD24+ cells (Burgos-Ojeda et al., 2015). Similarly, HEY OvCa cell line and OvCa cells isolated from patient ascites treated with paclitaxel in combination with JAK2-specific small molecule inhibitor CYT387 were more sensitive to paclitaxel treatment than those treated with paclitaxel alone. This therapeutic combination additionally led to decreased tumor volume compared to paclitaxel treatment alone (Abubaker et al., 2014). While impactful, it is not clear whether JAK2 specific inhibitors affected other potential CSCs isolated by additional parameters (i.e., CD133 or CD117 or ALDH activity). These results and others render members of the JAK/STAT pathway as potential therapeutic targets in OvCa. The durability of the response may provide some insight as to whether it is truly impacting the CSC population.

4.7. PI3K/PTEN/AKT

The Phosphatidylinositol-4, 5-bisphosphate 3-kinae (PI3K)/Protein kinase B (also known as Akt)/mammalian Target of Rapamycin (mTOR) pathway is hyperactive in approximately 70% of OvCa. It is a major pathway that regulates cell survival, growth, metabolism, proliferation, and angiogenesis (Li et al., 2014). Activating mutations of Akt have been linked to poor PFS and OS rates (Cai et al., 2014). Seo and colleagues utilized the SP method to enrich and isolate CSCs from A2780 cells. The isolated SP A2780 cells demonstrated increase sphere forming ability, chemo-resistance against cisplatin and paclitaxel, and were enriched for ALDH1 relative to the bulk cell fraction (Seo et al., 2016). These findings were supported in vivo as evidenced by accelerated tumor growth by the SP fraction relative to the bulk population. Furthermore, knock down of Akt in A2780 SP cells resulted in decreased expression of ALDH1 (Seo et al., 2016). Moreover, Autotaxin, an enzyme that produces lysophosphatidic acid (LPA), was found to promote ovarian CSC properties through upregulation of Akt. Conversely, inhibition of Autotaxin attenuates ovarian CSC sphere formation and drug resistance (Seo et al., 2016).

4.8. PKCι

Protein kinase C (PKC), a serine/threonine kinase, is most

commonly associated with regulating diacylglycerol (DAG) and calcium ion (Ca^{2+}) concentrations in cells. An isoform of PKC, PKCι, was found to be an oncogene in OvCa (Zhang et al., 2006). Wang and colleagues found that PKCι knockdown negatively affects cells in colony and sphere formation assays. Similarly, inhibition of PKCι using the anti-rheumatic gold compound Auranofin (ANF) resulted in loss of clonal expansion, sphere formation, and anchorage-independent growth in vitro, as well as reduced tumor growth in vivo by ovarian CSC-like cells (Wang et al., 2013). PKC is known to convey its action through MAPK, which is also known to cross-talk with several cell death/survival pathways, including the Mammalian target of rapamycin (mTOR) inhibitor. The use of an mTOR inhibitor, NV-128, induced cell death in ovarian CSCs through two independent non-canonical pathways via the degradation of Cox-I and IV (Alvero et al., 2011). Collectively, these studies combined support a role for PKCι as a contributor to CSC pathology.

4.9. S100B

An interesting protein that has gained attention in multiple cancers is S100B which is a member of the S100 family (Heizmann, 2004). S100B RNA and protein expression was shown to be elevated in OvCa samples compared to normal tissues. Additionally, S100B expression was further elevated in advanced stage OvCa tumors compared to low stage and in cisplatin resistant OvCa compared to cisplatin sensitive tumors (Yang et al., 2018). A positive correlation between the expression of *S100B* and the CSC markers *CD133*, *Nanog* and *OCT4* was observed. Similarly, CD133 + A2780 cells had increased levels of S100B compared to CD133- cells as determined by RT-PCR, western blots and immunofluorescence (Yang et al., 2017). Knockdown of S100B in A2780-derived CSC-like cells induced loss of tumorigenicity and self-renewal ability as assessed by sphere formation assays (Yang et al., 2018, 2017). In contrast, overexpression of S100B in A2780 non-CSC-like cells led to an increase in sphere forming capacity and the percentage of CD133+ cells. Although other members of the S100 family have been investigated in various cancers, S100B was the only one to our knowledge thus far related to ovarian CSC.

4.10. ROR1

Another interesting CSC related marker is Type I Receptor tyrosine kinase-like orphan receptor (ROR1). It has been shown that while ROR1 expression is absent from adult tissues, it is expressed in many different types of cancers and is known to regulate EMT and metastasis of breast cancer cells (Cui et al., 2013a). However, a contrasting study showed that ROR1 was expressed on cell membranes of normal tissues such as esophagus and colon (Balakrishnan et al., 2017). In OvCa specifically, ROR1 was reported to be a predictor of poor clinical outcome and decreased disease-free survival (Zhang et al., 2014a). The protein expression of ROR1 overlapped with the activity of ALDH1 in OvCa PDX samples. Additionally, ROR1+ cells formed more spheres, exhibited increased protein EMT markers expression and higher tumorigenicity compared to ROR1$^-$ cells (Zhang et al., 2014b). Treatment of mice harboring OvCa PDX with an ROR1 monoclonal antibody inhibited the tumor growth compared to the vehicle control (Zhang et al., 2014b). In addition to ROR1, ROR2 was also shown to be elevated in OvCa tumors as evidenced by IHC (Henry et al., 2015). Knocking down ROR1 or ROR2 decreased the invasive and migratory capacity of OVCAR3 cells. Interestingly, knocking down both ROR1 and ROR2 had a more pronounced decrease in migration and invasion compared to knocking down one receptor alone in OVCAR3 cells (Henry et al., 2015). A similar result was seen when ROR1 and/or ROR2 was/were silenced in OVCAR4 cells in a 3D co-culture model. Knockdown of both ROR1 and ROR2 had a synergistic effect on the cell's adhesion and invasion abilities (Henry et al., 2017). To determine whether ROR1 was a valid target for therapy in OvCa, Gohil and colleagues developed a bi-specific T cell engager (BiTE) against ROR1 and tested it in solid tumor xenografts such as those derived from SKOV-3. ROR1-BiTE treatments prevented the engraftment of SKOV-3 cells and the animals had reduced tumor burden (Gohil et al., 2017). Based on multiple studies that provided supportive evidence that ROR1 was a viable target in multiple cancers, clinical trials were performed to determine cytotoxicity of an anti-ROR1 monoclonal antibody or ROR1-CAR T cells in breast cancer and chronic lymphocytic leukemia (CLL) (NCT02222688, NCT02776917, NCT02194374), two of which are still active (Gohil et al., 2017).

Another ROR1 monoclonal antibody was developed by Yin and colleagues (ROR1-cFab), which they tested in ROR1 expressing OvCa cell lines (A2780) and the ROR1- cells (Iose386). Treatments of cells with the ROR1-cFab antibody resulted in a decrease in viability and migratory ability of A2780 cells but not the Iose386 cells. These data demonstrate the specificity of ROR-cFab antibody and treatment with this antibody could inhibit the tumor cells migratory capacity (Yin et al., 2017). Although there are some studies suggesting ROR1's involvement in CSC survival, maintenance and overlap with CSC markers, it remains to be seen whether the results of the clinical trials confirm that it targets ovarian CSC.

5. Challenges in targeting drug resistant CSCs

One of the major challenges in successfully treating OvCa is the development of recurrent and chemo-resistant disease. The TCGA research network published a report on expression profiling, mutation and copy number analysis, methylation and whole genome sequencing from treatment-naïve tissue samples collected from the patient's initial OvCa debulking surgery (Cancer Genome Atlas Research, 2018b; Patch et al., 2015). Although this in-depth investigation of OvCa samples has allowed insight into alterations that contribute to the pathology of the disease, it does not capture the equally important molecular drivers of chemo-resistant and recurrent disease. The same can be said for investigations focused on ovarian CSCs whereby most studies that evaluate their prevalence or function rely on chemo-naïve tissue. The lack of in-depth molecular information derived from chemo-resistant and recurrent disease samples is due to the limited number of secondary debulking or biopsies that are performed after the initial surgery or post cytotoxic therapy. In those instances, that interim debulking occurs after neoadjuvant treatment and tissue is obtained, the amount of viable tissue is often rate limiting. Obtaining more fresh samples post treatment over time for in depth molecular investigations could help further delineate the dynamic changes that take place in the surviving cells. Similarly, focused biopsies could provide insight into the continuum of changes that may be brought about by the tumor microenvironment.

6. Immune cells and ovarian CSCs

It is of interest to better understand how CSCs evade the immune system. The term "immuno-editing" is often used to describe how tumors evade the immune system (Dunn et al., 2004). Immuno-editing is described as consisting of three phases: elimination, equilibrium, and escape (Dunn et al., 2004).

With a focus on ovarian CSCs, Lai and colleagues explored the effect of the γδ T cells on CSCs. γδ T cells differ from normal T cells as they use γ and δ glycoproteins, instead of the more common α and β glycoproteins, to form the T cell receptor (TCR) (Lai et al., 2012). γδ T cells were shown to have cytotoxic effects on ovarian CSCs as they reduced SKOV-3 sphere formation and increased SKOV-3 sphere derived cell sensitivity to cisplatin and paclitaxel (Lai et al., 2012). Additionally, γδ T cells added to SKOV-3 sphere derived cells reduced tumor burden compared to SKOV-3 sphere derived cells alone after being injected into nude mice. The effect of ovarian CSCs on macrophage differentiation has been addressed by Deng and colleagues who demonstrated that ovarian

CSCs use the NFκB and PPARγ pathways to promote anti-inflammatory/pro-tumorigenic M2 macrophage polarization over pro-inflammatory/anti-tumorigenic M1 macrophages (Deng et al., 2015). Myeloid-derived suppressor cells (MDSCs) are known to suppress T cell activity and Cui and colleagues, observed that MDSCs initiate miR101 expression in primary patient OvCa cells (Cui et al., 2013b).

Mir101 inhibits the transcriptional co-repressor C-terminal binding protein 2 (CtBP2) resulting in an increase in the genes responsible for promoting stemness in cancer cells such as *SOX2*, *OCT4/3*, and *Nanog* (Cui et al., 2013b). The chemokine/receptor complex: C-X-C motif chemokine receptor 4/C-X-C motif chemokine ligand 12 (CXCR4/CXCL12) are involved in dissemination, metastatic colonization, and maintenance of CSCs (Cojoc et al., 2013). Inhibition of CXCR4 reduced CSC sphere formation of modified syngeneic OvCa cells, ID8-T (Gil et al., 2014). Additionally, CXCR4 blockade stimulated anti-tumor immunity by reducing infiltration of T regulatory cells and increasing infiltration of T helper as well as cytotoxic T cells into the tumor microenvironment of mice bearing tumors and ascites fluid accumulation. Together these data suggest a role for ovarian CSCs in shifting the immune system away from attacking tumor cells and towards promoting tumor progression.

7. Epigenetic modulation of stem like markers and/or properties

Studies have shown that the CSC drug-tolerant subpopulations in other tumor types can revert to being drug sensitive in the absence of continued drug exposure (Sharma et al., 2010). However, it remains to be seen whether this occurs in ovarian CSCs. The rapidly re-acquired drug sensitivity described suggests that CSC drug-adaptability is not driven by a heritable change such as gene mutations, but by a poised epigenetic state (Brown et al., 2014; Easwaran et al., 2014). Two main players implicated in this poised-state are the histone modifications H3K27me3, with a chromatin repressive role, and H3K4me3, with a chromatin permissive role (Lesch and Page, 2014). Both are involved in the regulation of chromatin in the embryonic stem cells (ESCs) during cell differentiation and tissue development (Grandy et al., 2016; Sen et al., 2008). It has since been demonstrated that these two modifications are important in epigenetic regulation of CSCs and their acquired drug resistance (Munoz et al., 2012). When both modifications are located on the same gene promoter, the gene is in a poised state for transcriptional activation or silencing (Bernhart et al., 2016). This chromatin bivalent state was studied in OvCa cells by analyzing the status of H3K27me3 and H3K4me3 histone modifications in both benign and tumor samples. Results showed that tumor samples were characterized by increased H3K27me3 marking which in turn, is mediated by Enhancer of zeste homologue 2 (EZH2), and overlapped with Polycomb Repressive Complex 2 (PRC2) (Curry et al., 2018). Chapman-Rothe and colleagues demonstrated that transcriptional silencing mediated by H3K27me3 was more frequent in platinum-resistant OvCa samples than platinum-sensitive. Similarly, silencing of genes that are mediated by H3K27me3 in the OvCa cell line IGROV1 led to cancer recurrence and to a chemo-resistant phenotype (Chapman et al., 2012).

Specifically, EZH2 is involved in the trimethylation of the histone H3 (H3K27me3) and it is associated with a repressive chromatin state. Rizzo and colleagues used OvCa ascites samples as well as the IGROV1, PEO14 and PEO23 cell lines to elucidate the role of EZH2. They determined that knock down of EZH2 reduced H3K27me3 methylation, which reduced the SP fraction and spheroid formation in IGROV1 cells (Rizzo et al., 2011).

Another potential epigenetic regulation of CSCs involves the CSC marker CD133. Using OvCa samples, Baba and colleagues demonstrated that the transcription of CD133 was regulated by histone modifications and promoter methylation (Baba et al., 2009). Treatment with DNA methyltransferase and histone deacetylase inhibitors in CD133- cells enhanced CD133 expression, suggesting a transformation of non-CSCs to CSCs (Baba et al., 2009). However, whether enhancement of CD133 via DNA methyltransferase and histone deacetylase inhibitors is sufficient to elicit stem like properties remains to be shown functionally.

These epigenetic regulations of CSCs could be a possible target to eradicate this chemo-resistant population. Wang and colleagues shed light on the connection between DNA hypomethylation and chemo-resistance in ovarian CSCs via ALDH activity. By using a DNA methyltransferase inhibitor, SGI-110, they re-sensitized CSCs to platinum, decreased the number of ALDH+ cells and reduced sphere formation in A2780-CR5 chemo-resistant cell line (Wang et al., 2014). Moreover, cells pre-treated with SGI-110 and then injected into mice showed reduced tumorigenesis compared to those that were treated with vehicle control. Lastly, they also showed the utility of using SG-110 as a maintenance therapy as evidenced in A2780 cells in an in vivo experiment where mice were treated with carboplatin for 21 days and then randomized to either vehicle or SGI-110. Animals treated with SGI-110 showed a ~50% decrease in tumor volume compared to continued vehicle treatment to day 35 (Wang et al., 2014).

In lieu of the current findings, CSCs' adaptability to drug treatment and non-CSC-to-CSC plasticity are likely correlated, in part, to epigenetic mechanisms. Treatment strategies aimed at eradicating CSCs are insufficient as the CSC pool could be replenished by the non-CSC counterpart. Consequently, therapies targeting epigenetic modulations and chromatin bivalent state could improve cancer treatments, overcoming the reversible resistance of CSCs.

8. Summary and conclusions

Despite our increasing knowledge of the idiosyncratic attributes of ovarian CSCs, there remains much that we do not know. The inherent heterogeneity of ovarian tumor cells will continue to provide therapeutic challenges, whether they be stem or non-stem like cells, is further complicated by the acquired heterogeneity on top of the inherent heterogeneity. This heterogeneity can be driven by the culmination of microenvironmental, pharmacologic, epigenetic and/or immunologic induced selection pressures. Optimally, the use of complex therapeutic cocktails designed to have ample upfront cytotoxic effects on the non-stem populations allowing for the enrichment of targetable populations of CSCs followed by a maintenance strategy targeting the remaining resistant residual cells. To meet this goal, it will require we continue to identify a dynamic field of targetable signaling drivers which can influence CSC self-replication, hierarchical heterogeneity, differentiation, drug resistance, DNA damage repair and most importantly their plasticity.

Acknowledgements

Dr. Rueda is supported in part by the Advanced Medical Research Foundation and The Vincent Memorial Research Funds.

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