

Evidence for cancer stem cells contributing to the pathogenesis of ovarian cancer

Michael D. Curley¹, Leslie A. Garrett^{1,2,3}, John O. Schorge^{2,3}, Rosemary Foster^{1,3}, Bo R. Rueda^{1,2,3}

¹Vincent Center for Reproductive Biology, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, Boston, Massachusetts 02114, USA; ²Division of Gynecologic Oncology, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, Boston, Massachusetts 02114, USA; ³Department of Obstetrics, Gynecology and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02114, USA

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1. ABSTRACT

Ovarian cancer represents the most lethal gynecologic malignancy, primarily due to a lack of early detection, which results in most patients being diagnosed at an advanced stage of disease. Though the ovarian surface epithelium is thought to provide the primary site of tumorigenesis, the exact etiology of the various tumor types associated with this disease remain undefined. Recent evidence suggests that ovarian tumors, like other solid tumors, contain distinct populations of cells that are responsible for tumor initiation, maintenance and growth. These specialized cells, termed cancer stem cells, display some of the hallmarks of normal stem cells and are thought to evade current chemotherapeutic strategies, resulting in an increased risk of recurrence. Here we review evidence for the existence of cancer stem cells in ovarian malignancies and their contribution to the pathology of this

disease, critically evaluate the methods used for ovarian cancer stem cell definition and isolation, and discuss their clinical relevance.

2. INTRODUCTION

Ovarian cancer is the second most common, but most lethal, gynecologic malignancy in the United States and was estimated to affect over 21,000 women with more than 14,000 deaths in the USA in 2009 (1). Lifetime risk for sporadic ovarian cancer is 1.4% and is over 50% for women harboring mutations in BRCA 1 or 2 (2). Ovarian cancer encompasses a broad group of histologically distinct cancer subtypes, which can vary significantly in terms of incidence, malignant potential and clinical responsiveness. Epithelial ovarian cancers (EOC) comprise the majority of malignant ovarian tumors and may be classified into eight distinct histologic subtypes, namely serous, endometrioid,

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mucinous, clear cell, transitional cell, squamous cell, mixed epithelial and undifferentiated (3). The most common serous, endometrioid and mucinous subtypes are defined based on their histological similarity to normal oviduct, endometrium and cervix, respectively (4). The oviduct, endometrium and cervix are all derived from the embryonic Mullerian ducts suggesting there are plastic cells in the adult gonadal and reproductive tract tissues that serve as targets for malignant transformation (5).

The specific site (or cell) of origin of ovarian cancer is unclear. Until recently, the most accepted hypothesis was that epithelial ovarian cancer (EOC) was derived from the ovarian surface epithelium (OSE) and/or cortical inclusion cysts (3, 6). The OSE is composed of a simple mesothelial lining of cells on the surface of the ovary, which undergoes local disruption and trauma as a result of the ovulating follicle and requires continual repair during menstrual cycling (6). It is believed that the continued exposure of the OSE to damage, cytokines and wound repair renders these cells more susceptible to malignant transformation (7). Alternatively, the aging ovary has an increased number of inclusions cysts, which are believed to arise from OSE cells that become trapped within the ovarian stroma as an artifact of the wound and healing processes involved in ovulation and repair (8-10). These cells are potentially exposed to irregular levels of growth factors and/or cytokines, which can promote formation of epithelial tumors of low malignant potential (11-13). Recent evidence has indicated the existence of putative ovarian stem cells within the OSE layer of adult human ovaries (14, 15), though further functional analyses will be required for confirmation. Whether or not these cells serve as the precursor to the more common ovarian cancers is yet to be elucidated. More recently, it has been proposed that some cases of ovarian cancer may actually originate from the epithelial lining of the distal fallopian tube (16, 17), suggesting that if ovarian cancer is a stem cell based disease, the cells of origin are not limited to the ovary.

Currently, there is no effective screening strategy for early detection of epithelial ovarian cancer and over two-thirds of ovarian cancer patients are diagnosed with stage III or IV disease. These women will often undergo aggressive cytoreductive surgery in an attempt to remove all visible disease. One of the most important prognostic factors in patients with advanced disease is the volume of residual disease following primary surgery (18). Despite advances in therapy and delivery, recurrence and chemotherapy resistance are still challenging problems. Indeed, the majority of ovarian cancer patients who achieve a complete remission with first line platinum-based chemotherapy will ultimately develop recurrent disease, usually indicated by rising levels of the serum marker CA125. These clinical scenarios support the hypothesis that ovarian tumors contain a subpopulation of highly specialized cells, deemed cancer stem cells (CSCs), which escape cytoreductive procedures and have the capacity to sustain tumor progression (see Figure 1). Ineffective targeting of this cell population is responsible for the therapeutic failures and tumor recurrences currently

experienced in clinical settings (19, 20). Efforts to identify specific genetic and signaling pathway alterations in these cells have led to the discovery of novel biologic targets that can be used to design adjuvant therapies that could potentially overcome chemoresistance and lead to improved response rates and overall survival. The focus of this review will be to discuss both the implications of the CSC hypothesis in relation to ovarian cancer and the essential role putative ovarian CSCs could have in formulating new therapeutic strategies aimed at mitigating their role in recurrent disease.

3. CANCER STEM CELL HYPOTHESIS

Historically, cancer has been viewed as a disease of unregulated cell proliferation with tumor cells outcompeting surrounding cells for vital nutrients, blocking vital organ functioning and invading other tissues and/or body systems leading to organ failure. Such defective cellular homeostasis within tissues is brought about through the accumulation of mutations that result in constitutive proliferative signaling (21) and increased survivability. The originally perturbed tumor cells are often more susceptible to further genetic hits due to inherent genomic/chromosomal instability (21) and progressively lose their responsiveness to normal growth regulatory signals (22), resulting in (epi)genetic changes that drive tumor progression.

It is well established that tumors are heterogeneous in nature, based not only on the histopathology and function of the cells that comprise them, but also on their responsiveness to clinical therapy (for review, see (23)). Such heterogeneity has led investigators to renew their interest in a decades-old hypothesis (24, 25) that tumors, like certain normal tissues, are arranged in a cellular hierarchical order in which only certain populations of cells are responsible for generating the multiple cell types within the tumor. This 'cancer stem cell hypothesis' postulates that tumors contain phenotypically-distinct populations of stem-like cells with self-renewal capacity and the potential to reconstitute the entire cellular heterogeneity of a tumor (26). These CSCs are thought to be responsible for tumor initiation, progression and metastasis (23). Thus, based on the CSC hypothesis, a tumor may be regarded as an aberrant organ comprising a heterogeneous mix of tumorigenic CSC and their non-tumorigenic progeny. The resurgence of this hypothesis has stimulated fervent pursuit of CSC populations across most tumor types.

4. KEY CHARACTERISTICS OF CANCER STEM CELLS

A workshop convened by the American Association for Cancer Research in 2006 declared that, in its simplest form, a CSC may be defined as a cell that possesses the capacity for self-renewal and can generate heterogeneous lineages of cells that comprise a tumor (27). In essence, this hypothesis posits that CSCs sit at the pinnacle of a hierarchically organized tumor cell population and are solely capable of dividing asymmetrically to

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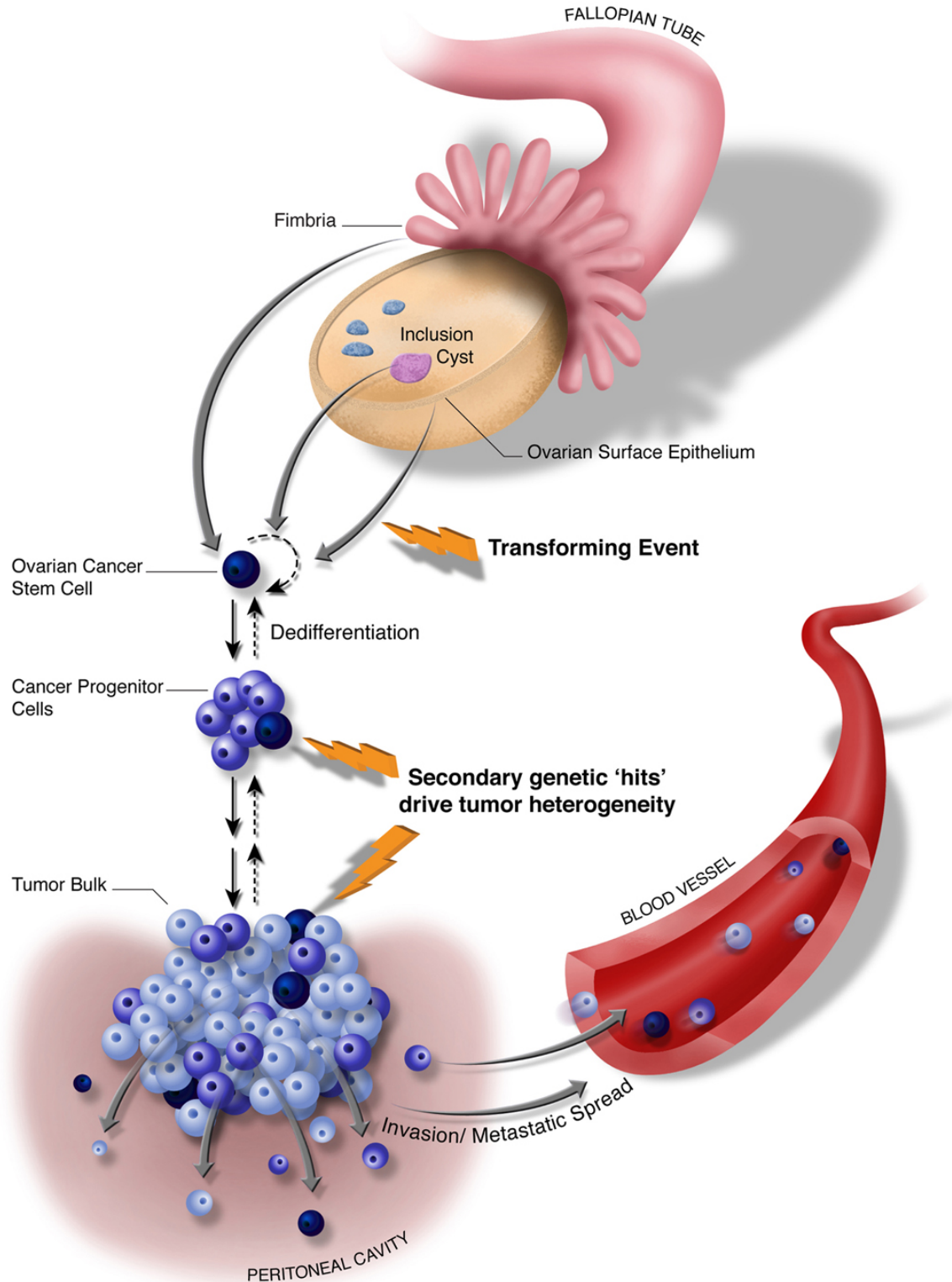


Figure 1. Schematic illustrating the proposed cancer stem cell hierarchy in human ovarian cancer. Cancer stem cells may derive from normal cells within the ovarian surface epithelium, inclusion cysts or the fimbriae located at the distal ends of the fallopian tubes. Though the initial transforming event(s) that derive CSCs remain undefined, secondary genetic hits will likely drive further tumor heterogeneity. Tumor cell dissemination into the peritoneal cavity or possibly into the blood and/or lymphatic systems may facilitate the development of secondary metastases.

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generate an exact copy of themselves (self-renewal capacity) and a more differentiated progenitor cell. These more differentiated progenitor cells divide rapidly to generate large numbers of daughter cells that will form the bulk of the tumor. Evidence supporting a shift from asymmetric to symmetric division of CSCs has also recently been reported (28), which may allow for continued expansion of CSC populations sufficient to maintain the tumor, while providing the necessary balance with asymmetric division to support tumor heterogeneity and growth.

CSCs are biologically distinct from their more differentiated progeny (29) and though both populations contain the same oncogenic mutations that result in tumorigenesis, the latter population lacks the capacity for continuous self-renewal (30). In addition to unlimited proliferation potential, CSCs also possess an increased longevity and are usually slow-cycling in nature, a feature that may endow these cells with inherent resistance to current chemotherapeutic strategies that target actively dividing cells. Though the precise mechanisms responsible for chemoresistance remain poorly understood, they likely include increased expression of ATP-binding cassette (ABC) transporter proteins (31-33) and/or detoxifying enzymes (e.g. aldehyde dehydrogenase (34, 35), reactive oxygen species (ROS) antioxidants (36)) given the potential for increased exposure to toxins throughout the extended CSC life cycle (37), as well as disruption to apoptotic pathway mechanisms ((38); for review, see (38-40)). CSCs have also been shown to be refractory to the effects of radiation, based on increased viability and decreased ROS production in breast cancer cells (36, 41) and gliomas (42).

The propensity of CSCs to evade current chemo- and radio-therapeutic strategies, due in part to their intrinsic stem-like properties, suggests that these cells are responsible for recurrent cancer growth. Often initial treatment strategies will positively select for chemoresistant CSC, thereby accelerating the pace of secondary disease progression (43). Additional (epi)genetic events may select for more aggressive cell populations with increased metastatic potential, possibly through an epithelial-mesenchymal transition (44) that enables these cells to become niche independent and migrate to other tissues. In light of these characteristics, it is essential that effective cancer therapies target both the tumor bulk and CSCs in order to prevent recurrent malignancies. This goal requires a better understanding of the origin of CSCs.

5. ORIGIN OF CANCER STEM CELLS

The cell of origin in cancer is the subject of longstanding debate and remains so in the context of the CSC hypothesis. It is unclear if the target cell of transformation is a normal adult stem cell, a transit-amplifying cell (also referred to as a committed precursor cell) or a more differentiated non-stem cell. It has been suggested that CSCs are derived from normal adult stem cells that undergo mutation and lose self-regulation

mechanisms while maintaining self-renewing capacity. Alternatively, CSCs may arise through mutation of daughter cells that undergo dedifferentiation and acquire self-renewal capacity and other key stem-like features (45, 46).

Normal adult stem cells reside in most somatic tissues and are responsible for maintaining tissue homeostasis. The essential characteristics of self-renewal and multi-lineage potential allow stem cells to replenish damaged or terminally differentiated cells, thereby maintaining their organs in a healthy state. Given their self-renewal capacity and proliferative potential, these cells are dependent on molecular signals from their surrounding microenvironmental niche to direct both their differentiation and proliferation when needed (47, 48). Though stem cells are believed to be few in number and slow-cycling within normal tissues, their increased life-span greatly increases the chances that they will persist long enough to accumulate the genetic hits necessary for malignant transformation (23, 47, 49). Such mutations, whether due to internal (DNA replication error) or external (toxins/UV irradiation) influences, could drive normal stem cells to escape the homeostatic control mechanisms provided by their niche environment and permit uncontrolled cell proliferation, thus making these cells attractive targets for complete oncogenic transformation (for review, see (47, 50, 51)).

Although there is accumulating data in some tumor types suggesting that normal adult stem cells are the initial precursor cells to malignant transformation (32, 33), definitive evidence implicating these cells as the putative cells of origin in all cancers is lacking and the possibility that dedifferentiation of lineage-committed cells or progenitor cells to stem-like cells cannot be ruled out. Indeed, Jamieson and colleagues (52) demonstrated that normal blood progenitor cells could give rise to leukemic stem cells in human chronic myelogenous leukemia (CML) through aberrant beta-catenin accumulation. Thus, any genetic changes in differentiated cells would require them to obtain both self-renewal capacity and increased longevity in order to effectively drive tumor progression (53). Though such transformation is possible, the molecular energy (i.e. number of essential mutations) required for conversion of these cells to CSCs would be substantial, given their limited life-span and the number of (epi)genetic mutations necessary to reacquire self renewal capacity and facilitate malignant transformation (54).

Ultimately, the term 'cancer stem cell' refers to a definitive set of cellular characteristics that contribute to tumor formation rather than any indication or implication as to the cell of origin (30, 55). The terms 'cancer-initiating cells' (CICs (41, 56-58)) or 'tumor-initiating cells' (TICs (43, 46)) have been used interchangeably with CSCs in the literature, though neither term literally implies that the cells it describes are those that *initiated* the tumor. Given the multiple mutational events necessary to drive malignant transformation, it is unlikely that the phenotype of the cell of origin will necessarily have a high degree of

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homology with that of the CSCs (54), or that this cell will even persist as the tumor evolves. It seems more likely that further mutations, generating better adapted, more tumorigenic and more drug-resistant CSCs, will be selected for as the cancer develops (59). Therefore, it may never be possible to clearly define the cell that suffers the initial genetic insult (60).

6. EVIDENCE OF CANCER STEM CELLS IN HUMAN CANCERS

Experimental evidence in support of the CSC model was first published in two seminal papers from the laboratory of John Dick (61, 62) in which the hierarchical organization of human acute myeloid leukemia (AML) was described. These reports stimulated further investigation to determine whether such hierarchical organization was a common trait across all cancer subtypes. Since the initial description of the cell surface marker signature of human breast CSCs by Clarke and colleagues (56), several laboratories have defined subpopulations of tumorigenic CSCs in solid tumors of various origins based on expression of specific cell surface markers (32, 46, 57, 58, 63-73).

The upsurge in CSC surface marker identification has provided researchers with the necessary tools to prospectively isolate defined CSC populations and investigate the molecular intricacies of the mechanisms involved in tumor pathogenesis. However, some words of caution are necessary when considering the significance of these methods to identify CSCs. Whether specific cell surface markers are sufficient to define CSC populations across all tumors of a common subtype remains to be determined and will have obvious repercussions from a clinical treatment perspective. Also, the models used to define these populations must be thoroughly assessed to minimize external factors that may influence marker expression. Thus, careful interpretation of both the markers presented and the methods used to define them will provide the best information to delineate the precise pathways that could be targeted therapeutically. Those markers that have been implicated in ovarian cancer are discussed in more detail below.

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Since the initial report by Clarke and colleagues identified a CSC phenotype in a solid tumor (breast cancer), several studies have sought to determine whether CSCs may also perpetuate ovarian tumor growth and metastasis. Table 1 summarizes the salient points of all studies to date describing CSC populations in ovarian malignancies. Initial work by Bapat and colleagues (74) identified clonogenic cells isolated from the ascites of a single patient with advanced ovarian cancer. These clones, propagated as multilayered spheroids in serum-containing media, possessed stem-like properties and expressed several markers of pluripotency. These clones also generated differentiated progeny *in vitro*, formed xenograft tumors *in vivo* and could be serially transplanted in nude mice.

In mouse ovarian cancer cell lines, a side-population (SP) of cells was isolated and reported to encompass the mouse ovarian CSC population (75). SP cells are distinguished based on their ability to extrude vital dyes and are hypothesized to be CSC-like cells that have increased expression of ABC proteins and drug transporters (20). SP cells isolated from MOVCAR cell lines exhibited enhanced chemoresistance, could reconstitute colonies *in vitro* and were shown to have an increased tumorigenic capacity *in vivo* when compared to their non-SP counterparts (75).

SP cells were also identified at low frequency in selected human ovarian cancer cell lines, including OvCAR3 (76), SK-OV3 (31), and IGROV-1 (75). For OvCAR3 cells, the identified SP was reported to have increased clonogenic capacity and expression of stemness genes relative to the non-SP (76). The presence of SP fractions in ascites cells derived from patients with epithelial ovarian cancer was identified by two independent groups (75, 77). In subsequent experiments, Moserle and colleagues identified both large and small SP fractions in distinct ascites-derived human ovarian tumor cell lines (78), with SP cells exhibiting an increased proliferative activity, decreased levels of apoptosis and increased tumorigenicity compared to the equivalent non-SP (77). These studies also indicated that interferon-alpha (IFN-alpha) treatment adversely affected the growth and survival of primary cultures containing large SP fractions, implying a possible treatment option for patients with such tumors. However, it was concluded that these SP cells might not encompass ovarian CSC populations, based on the fact there was no difference in expression of stem-like markers between SP and non-SP fractions.

Ferrandina and colleagues were the first to identify CD133 expression in primary human ovarian cancer (79). Previous reports had identified CD133 as a marker of CSC-like populations in other solid tumors (46, 57, 67, 70, 80). CD133 expression was much higher in primary human ovarian tumors as compared to its expression in normal ovary and benign ovarian lesions. The identified CD133⁺ cells were almost completely (<1%) non-endothelial in nature, based on the absence of vascular endothelial growth factor receptor 2 (VEGF-R2), CD105 (endoglin) and VE-cadherin (79). In further experiments performed on primary ovarian tumor cells propagated *in vitro*, CD133⁺ cells possessed increased clonogenic and proliferative capacities compared to their CD133⁻ counterparts.

More recently, propagation of dissociated primary human ovarian tumor cells as non-adherent spheroids in serum-free growth factor defined media has been utilized to enrich for self-renewing, stem-like populations from ovarian tumors. Using this methodology, Zhang and colleagues (81) reported that rare fractions of spheroids derived from dissociated primary human ovarian tumor cells and maintained under stem cell-selective conditions possessed self-renewal capacity, over-expressed stem cell marker genes and were resistant to current chemotherapeutic strategies. Furthermore, these sphere-

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Table 1. Overview of published scientific reports detailing putative CSC populations in ovarian cancer

Original Source	Cultured <i>in vitro</i> ?	Colony formation?	Chemo-sensitivity?	Cell surface marker positivity (method)?	Injected <i>in vivo</i> (site; mouse strain)?	Serial Transplantability	Tumor formation <i>in vivo</i> ?	Ref.
Human ov. ascites-derived clones	Yes, as attached spheroids	Yes, for specific clones in soft agar	Not tested	Not tested	Yes (sc/ip; nude mice)	Yes, for 2 unsorted isolated clones	Yes, 2 isolated clones formed tumors	74
Mouse/human ov. cancer cell lines, human ov. ascites	Yes, cell lines as adherent cultures	Yes, MOVCAR7 SP formed colonies	Yes, MOVCAR7 SP Dox. resistant, Pac. sensitive	Not tested	Yes, MOVCAR7 (sc dorsal fat pad; nude mice)	Not tested	MOVCAR7 SP more tumorigenic vs. non-SP	75
Human 1° ov. tumors	Yes, as adherent cultures	Yes, CD133 ⁺ cells had increased clonogenicity	Not tested	Yes, CD133 ⁺ (FC, IHC)	No	Not tested	Not tested	79
Human 1° ov. tumors	Yes, as spheroids	Not tested	Unselected spheroids more resistant to Pac./cisplatin	Yes, CD44 ⁺ and CD117 ⁺ (FC, IF)	Yes (sc/ip; BALB/c-nu/nu mice)	Yes, unsorted, CD44 ⁺ /CD117 ⁺ and CD44 ⁺ /CD117 ⁻ spheroids	CD44 ⁺ /CD117 ⁺ cells more tumorigenic vs. CD44 ⁻ /CD117 ⁻ cells	81
Human 1° ov. tumors, ascites-derived and cancer cell lines	Yes, ascites-derived cell lines as adherent cultures	Yes, SP isolated cells formed colonies	SP more sensitive to IFN-alpha treatment	Not tested	Yes (ip; SCID mice)	Reported previously for ascites-derived cell lines (78)	Yes, SP from ascites-derived cell line xenografts more tumorigenic	77
Human ov. cancer cell lines	Yes, as adherent cultures	Not tested	A2780/PEO1 CD133 ⁺ cells more resistant to cisplatin	Yes, CD133 ⁺ in cell lines (ICC, IHC)	Yes (sc; BALB/cAnNCr-nu/nu mice)	Not tested	Yes, A2780 CD133 ⁺ cells more tumorigenic vs. CD133 ⁻	84
Human 1° ov. tumors and ascites	Yes, as adherent cultures and spheroid suspensions	Not tested	CD44 ⁺ cells more resistant to Pac./carboplatin	Yes, CD44 ⁺ (IHC)	Yes (sc/ip; nude mice)	Yes, CD44 ⁺ cells only	CD44 ⁺ cells formed tumors (sc/ip)	82
Human 1° ov. tumors and ascites	Yes, as (un)attached spheroids	Yes, CD133 ⁻ cells clonogenic, CD133 ⁺ cells non-clonogenic	Not tested	Yes, CD133 (FC)	Yes (sc/ip; NOD/SCID mice)	Yes, for unsorted and CD133 ⁺ clones	CD133 ⁻ cells tumorigenic; CD133 ⁺ cells non-tumorigenic	85
Human 1° ov. tumors, ascites, and cell lines	Yes, CD44 ⁺ cells as spheroids, monolayer and Matrigel™	Not tested	Not tested	Yes, CD44 ⁺ (FC)	Yes (sc; NCr nude mice)	Not tested	Yes, CD44 ⁺ cells were tumorigenic	83
Human ov. ascites-derived clones, tumor cell lines	Yes, as (un)attached spheroids	Yes, PKH67 ^{hi} cells had increased clonogenicity	Yes, Pac.-resistant clones generated	Yes, CD44 ⁺ , CD117 ⁺ (FC)	Yes (sc/ip; NOD/SCID mice)	Yes, for PKH67-labeled sorted clone	Yes, PKH67 ^{hi} cells more tumorigenic vs. PKH67 ^{lo}	209
Human 1° ov. tumors and ascites	No	Not tested	Not tested	Yes, CD133 ⁺ , CD24 ⁺ , CD44 ⁺ , EpCAM ⁺ (FC, IHC)	Yes, (sc; NOD/SCID mice)	Yes, both unsorted and CD133 ⁺ cells	CD133 ⁺ cells more tumorigenic vs. CD133 ⁻ cells	86

Abbreviations: Ref: reference, ov: ovarian, sc: subcutaneous, ip: intraperitoneal, Dox.: Doxorubicin, Pac.: Paclitaxel, 1°: primary, ICC: immunocytochemistry, FC: flow cytometry, IF: immunofluorescence, IHC: immunohistochemistry

forming cells were tumorigenic and could be serially propagated in nude mice *in vivo* generating tumors histologically similar to their original primary tumors. Expression of CD44 and CD117, identified previously in ovarian CSC studies (74, 75), was shown to be enriched in these non-adherent spheroids. Prospective fluorescence-activated cell sorting (FACS) and injection assays of primary and spheroid-derived xenograft ovarian tumor cells

indicated that rare subpopulations of CD44⁺/CD117⁺ cells comprised a highly tumorigenic population in primary human ovarian cancer (81).

Subsequently, Alvero and colleagues (82) identified CD44⁺ cells in primary ovarian and metastatic tumors and malignant ovarian ascites. CD44⁺ populations isolated directly from malignant ascites formed self-

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renewing spheroids *in vitro* and were tumorigenic *in vivo*. Molecular analyses indicated these CD44⁺ cells were enriched for MyD88 (myeloid differentiation factor 88) protein, displayed constitutive NF-kappa B activation and cytokine production and were chemoresistant *in vitro*. Another report (83) from the same group indicated that CD44⁺ cells, which they termed Type I epithelial ovarian cancer cells, could also serve as progenitors for tumor vascularization. This report indicated that CD34⁺ cells lining blood vessels centrally located within human ovarian xenograft tumors were human in origin and solely derived from the Type I EOC cells. These data also confirmed that this neovascularization process was I kappa B kinase-beta (IKK-beta) dependent, but independent of VEGF and various other external and secreted factors.

Meanwhile, Baba and colleagues (84) confirmed CD133 as a marker of tumorigenic populations, albeit in human ovarian cancer cell lines. Having detected CD133 expression in ovarian cancer cell lines, primary human ovarian tumors and ascitic fluid, they showed that CD133⁺ cells derived from ovarian cancer cell lines divide asymmetrically *in vitro*, generating both CD133⁺ and CD133⁻ progeny. CD133⁺ cells also exhibited increased resistance to current chemotherapeutic strategies and were more tumorigenic *in vivo* compared to their CD133⁻ counterparts. Interestingly, they also determined that expression of CD133 was epigenetically regulated through histone modification and promoter methylation.

Several laboratories attempting to further elucidate the relevance of CD133 expression in primary human ovarian tumors and ascites have reported conflicting results. Kusumbe and colleagues (85) found that CD133⁺ cells from ascites-derived *in vitro*-selected clones (74) were non-tumorigenic *in vivo* and represented endothelial stem cell populations that function primarily to augment tumor vascularization, a role similar to that described by Alvero and colleagues (83), but through a VEGF-dependent process. Their co-injection experiments using tumorigenic clones and/or green fluorescent protein positive (GFP⁺) CD133⁺ populations resulted in formation of vascularized tumors from the co-injected populations alone with increased expression of a variety of human endothelial cell markers in tumors derived from the co-injected populations.

Our own report (86), however, indicated that CD133 delineates a tumorigenic population in human ovarian cancer that may encompass a component of the ovarian CSC population. Using primary human ovarian tumor cells derived from both serous and clear cell tumors that were propagated *in vivo* in the absence of *in vitro* culturing, we demonstrated that CD133⁺ cells have an increased tumorigenic capacity in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (86), confirming the report by Baba and colleagues (84) for ovarian cancer cell lines. We reported that primary human ovarian tumor cells could be propagated by xenograft transplantation in immunocompromised mice and that the tumors generated phenocopied the histology of the original parent tumors. Our serial dilution analyses indicated that not all cells within ovarian tumors have the

capacity to form tumors, which provides evidence in support of the existence of ovarian CSC populations. Serial transplantation of the xenografted tumors indicated that these tumors contained self-renewing cells that could recapitulate tumor growth (74, 86).

We also performed cell surface marker expression analyses using candidate markers previously implicated in other tumor types to test their potential relevance in distinguishing CSC populations in ovarian malignancies. Our results indicated that CD133 was consistently expressed across both serous and clear cell tumors, though at varying proportions, with clear cell tumors demonstrating higher expression of CD133 relative to serous tumors. Prospective FACS based on differential CD133 expression indicated that CD133 expression defines a tumor initiating cell population in both serous and clear cell primary human ovarian tumors.

The observed difference in the tumorigenic capacity of CD133⁺ populations reported in these independent studies may be indicative of differences in both the source material used (ascites-derived clones isolated from a single patient sample versus dissociated primary human ovarian tumors from multiple individual patients) and the propagation methodologies employed (*in vitro* expansion and clonal selection versus *in vivo* tumor growth) to generate sufficient tumor material for the described analyses. Both CD44⁺ and CD133⁺ ovarian cancer-derived subpopulations have been implicated as the CSC fraction in human ovarian cancer. The postulated neovasculogenic versus tumorigenic capacities of these subpopulations pose some interesting dilemmas. Although purely speculative, it may well be that these properties are not mutually exclusive and may functionally help define the cell populations that possess them as ovarian CSC. These dual functions have been defined for CD44⁺ cells within equivalent cell populations (81-83). The potential for such a role for CD133⁺ cells in human ovarian cancer is of significant interest and has already been described in some hematopoietic malignancies (for review, see (87)).

The potential overlap between CD44 and CD133 expressing populations remains unclear, though our initial results with flow analyses indicate that CD44⁺CD133⁺ populations exist in both serous and clear cell primary human ovarian tumors (86). Overlapping CD44⁺CD133⁺ populations have already been reported for prostate cancer (67) and co-expression of CD44 and CD133 has been shown to define tumorigenic populations in colon cancer (88). Similar double positive cells may represent a highly enriched CSC population in ovarian cancer. While the use of various methodologies has been successful in defining populations of cells with increased tumorigenic/clonogenic capacity, further analyses specifying the exact ovarian CSC surface marker signature are required. It is likely that this signature may vary between patients (or within tumor) and therefore careful consideration of the markers involved will be required. The striking variety of markers reported to define CSC populations across and even within different tumor types would suggest that single marker-defined populations are insufficient in defining the entire CSC

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population. It will be important to fully elucidate the relevance of the putative ovarian CSC markers and optimize the methodologies used to propagate ovarian tumor tissue in order to extract the most clinically applicable information. The merits and drawbacks of the principal ovarian CSC surface markers and methodologies utilized to date are dissected in detail below.

7.1. Side population (SP)

The isolation of a side population of cells based on differential dye exclusion was first described by Goodell and colleagues (89) and has been utilized for many years in stem cell biology. These original studies using murine whole bone marrow indicated that distinct cell populations that were more refractive to certain vital dyes (e.g. Hoechst 33342) expressed markers associated with multipotent hematopoietic stem cells (HSCs). Further analyses with verapamil, an inhibitor of ABC transporter activity, confirmed that this phenomenon was due to increased expression of drug-resistant membrane transporters and thus could be used as a basis for the isolation of stem cell populations from blood and various tissues.

CSCs are also postulated to be more resistant to current chemotherapeutic and environmental toxins, in part due to increased expression of plasma membrane drug transporters, including members of the ABC protein superfamily and breast cancer resistance protein 1 (Bcrp1) (for review, see (20)), which have the capacity to extrude certain chemotherapeutic drugs (31). Therefore, cell sorting methodologies based on differential dye exclusion have been exploited to isolate drug-resistant SP cells that may encompass the CSC population from various human solid tumors (31, 77) and cancer cell lines (90-95), including ovarian (75, 77).

Isolation of CSC populations from various cancer sources based on the definition of a drug resistant SP has significant limitations, however, due to the toxicity of the Hoechst dyes. Indeed, increased dye exclusion may allow SP cells to avoid the toxic effects of the dye and thus confer a growth advantage to these cells in *in vivo* models. Also, although the SP has helped define potential CSC populations in some tumors, other studies have provided evidence to suggest that this correlation does not apply to all tumor types (96). As suggested by Moserle and colleagues, the SP may not define the CSC population in ovarian ascites-derived cells based on the similarities in selected stem-like marker gene expression in the SP and non-SP (77). This study did report promising effects of IFN- α treatment on cells containing large SP fractions. Further analyses incorporating SP fractions derived from primary solid tumors may provide definitive evidence as to the relevance of the differential dye exclusion technique in defining ovarian CSC populations and the possible clinical application of IFN- α treatment for ovarian disease.

7.2. CD44

CD44 is a single chain transmembrane glycoprotein that is ubiquitously expressed. Multiple isoforms of CD44 exist due to extensive alternative splicing of the 19 exons comprising the gene that encodes it. The

standard CD44s isoform, generated from a mRNA in which the 10 central exons are spliced out, is an ~85kD glycoprotein that is a major receptor for hyaluronan (97, 98). Hyaluronan is one of the principal glycosaminoglycans present in mammalian extracellular matrix and plays important roles in both extracellular structure and cell signaling (for review, see (99)).

CD44 principally functions as an adhesion molecule, mediating cell-cell and cell-extracellular matrix (ECM) interactions by binding to hyaluronan. CD44 can also activate many intracellular signaling pathways and has been implicated in cell proliferation, cell differentiation, cell migration, cell motility, angiogenesis and metastasis (for review, see (100)). This molecule has been reported to be critical for the maintenance and survival of leukemic CSC by keeping these cells in contact with their supportive niche cells (101, 102). Indeed, both CD44 and hyaluronan have been reported to play significant roles in drug resistance and cell survival (103, 104).

In breast cancer cells, genes associated with cell motility and angiogenesis were more highly expressed in CD44⁺ populations, consistent with the notion that these cells have a more motile and less proliferative stem cell-like phenotype (19). Indeed, CD44 has been used either alone or in combination as a cell surface marker distinguishing putative CSC populations across a variety of tumor types (56, 64, 66, 69) including ovarian cancer (81, 82).

In analyses of clinical ovarian cancer samples, CD44 over-expression was correlated with disease progression (105). Interestingly, if CD44 positivity denotes a CSC population then this increase in expression would suggest that the ratio of stem to daughter cells has shifted. Auzenne and colleagues (106) indicated that CD44 could provide a putative therapeutic target for delivery of novel hyaluronan-paclitaxel copolymers aimed at reducing tumor burden in ovarian malignancies. This combination therapy may well target stem and daughter cells. Indeed the therapeutic potential of anti-CD44 agents has been highlighted by experiments in which targeting of CD44 using specific antibodies, antisense and CD44-soluble proteins significantly reduces the proliferative and malignant capabilities of various cancer subtypes (for review, see (100)). The use of specific CD44 antibodies to block hyaluronan binding has been shown to inhibit tumor growth and invasion in human melanoma xenograft tumors (107).

As with every candidate CSC marker, the potential role of CD44 is somewhat controversial. CD44 treatment has been reported to have anti-apoptotic effects in lung cancer cells (108). Also several reports investigating the possible use of CD44 as a prognostic marker in ovarian cancer have yielded conflicting results, with CD44 expression linked to both favorable and unfavorable outcomes (105, 109, 110). This discrepancy, however, may be dependent on the CD44 isoform analyzed. Despite the reported contrasting effects of CD44 treatment on various cancer cell populations, CD44 may still provide a useful

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target for novel chemotherapeutic strategies, though more in-depth analyses of the CD44 variant isoforms involved will likely be required for optimal treatment outcome.

7.3. CD117/c-KIT

The *c-kit* proto-oncogene encodes a type III receptor tyrosine kinase (CD117/c-KIT), which is involved in signal transduction across many normal and tumor cell types. The kinase activity of CD117 is stimulated by dimerization and autophosphorylation after binding of its ligand stem cell factor (SCF), which results in the activation of multiple transcription factors that control various cellular processes including cell proliferation, cell differentiation, apoptosis and cell adhesion (for review, see (111)).

CD117 and SCF expression in normal ovarian surface epithelial cells and ovarian cancer has previously been described (112, 113) and CD117 expression was reported to correlate with cancer progression after first line chemotherapy in advanced ovarian serous low grade carcinomas (114). Bapat and colleagues reported varying levels of c-KIT expression in clones generated from a multi-layered spheroid derived from the ascites of an ovarian cancer patient. Subsequently, Zhang and colleagues (81) reported that dual positive CD44⁺CD117⁺ cells comprised the ovarian CSC population in primary human ovarian tumors.

However, there have been conflicting reports regarding CD117 expression in ovarian cancer. Although Szotek and colleagues determined that SP cells derived from the mouse ovarian cancer cell line MOVCAR7 were enriched for c-KIT expression, their parallel analyses of human ovarian cancer cell lines and ascites-derived cells indicated no positive c-KIT expression (75). In our own screening of multiple human ovarian primary and ascites tumor cells and xenografts derived from human ovarian tumors, we detected no significant expression of CD117 in any source (86). The discrepancy in CD117 expression in ovarian tumors is puzzling and may be due to differences in the specific antibodies used or the methods of tumor propagation employed (*in vitro* spheroid culture versus direct *in vivo* propagation). Regardless, the finding that its expression is correlated with ovarian cancer progression after first line chemotherapy and that it defined one aspect of the dual positive CD44⁺CD117⁺ CSC cells (81) suggest that targeting CD117⁺ is a worthwhile venture.

7.4. MyD88

Myeloid differentiation factor 88 (MyD88) is an intracellular adaptor molecule associated with the Toll-like receptor (TLR) signaling pathway. TLRs play critical roles in the control of infection, tissue renewal and repair and have also been implicated in tumor formation. After stimulation, cell surface TLR recruits interleukin-1 (IL-1) receptor associated kinase via MyD88, thus inducing activation of the NF-kappa B and mitogen activated protein kinase signaling pathways (115).

Alvero and colleagues (82) characterized ovarian CSC based on a CD44⁺/MyD88⁺ phenotype. Although

MyD88 was expressed only in sorted CD44⁺ ovarian cell populations, assessment of the relative tumorigenicity of FACS isolated CD44⁺/MyD88⁺ cells when compared to CD44⁺ was not reported. Any such difference in tumorigenicity might provide some additional specificity for the development of targeted therapy.

7.5. CD133

CD133 is a cholesterol-binding cell surface glycoprotein comprising five transmembrane domains and two glycosylated extracellular loops with a molecular weight of 97-120kDa (116). Originally described in the mouse as prominin (117), the human homologue was identified as the target for the AC133 antibody which was generated to specify the CD34⁺ population of hematopoietic stem cells (118).

CD133 is distinctly expressed on plasma membrane protrusions of certain epithelial surfaces and interacts with membrane-cholesterol (119) to form microdomains that have been proposed to be carriers of important molecular factors necessary for the maintenance of the stem cell phenotype (48, 120). The tissue-specific regulation of CD133 expression is mediated by exon 1 which produces nine distinct 5' untranslated regions that can result in the formation of multiple splice variants of CD133 mRNA (121, 122). Epigenetic silencing of CD133 by hypermethylation of its promoter region has added further complexity to the regulation of CD133 expression in various cancers (e.g. glioblastoma (123)) including ovarian (84).

CD133 has been described as a marker of undifferentiated stem cells in several organs and appears to be a common CSC marker for many tumor types including brain (46, 80), pancreas (65, 124), colon (57, 58), liver (70, 125), prostate (67, 126), lung (68, 127), skin (73) and ovary (84, 86). Despite its reported utility as a marker of CSC populations, little is known about the exact function of the CD133 protein or the signaling pathways or molecules with which it interacts. It has been postulated to play a role in many processes including regulation of cell-cell interaction, ligand-receptor interaction, cell migration and plasma membrane topology as well as in the determination of cell polarity and the maintenance of homeostasis in normal adult organs (118, 128, 129). Given the fact that the most currently used anti-CD133 antibodies target glycosylated epitopes of the CD133 protein (AC133), it is possible that the glycosylation status of CD133, not actual expression of this protein, is more important for defining cells possessing CSC characteristics (for review, see (130, 131)). However, an antibody designed to target CD133 independent of glycosylation indicated no difference in CD133 expression or cellular location (132).

Interestingly, expression of CD133 has been correlated with expression of VEGF in primary pancreatic cancers (133), which may indicate a role for CD133 in promoting tumor neovascular- and/or angiogenesis. Additionally inhibition of the Notch signaling pathway in medulloblastoma cell lines and glioblastoma-derived neurospheres leads to loss of xenograft formation capacity

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(134, 135). The Hedgehog (Hh), Wnt and bone morphogenetic protein (BMP) signaling pathways have also been implicated in CD133⁺ human brain tumor CSC function (136-138).

CD133⁺ cells have been shown to be resistant to the effects of radiotherapy in medulloblastoma (139), which may be due to induction of DNA damage checkpoint kinases, as shown for gliomas (42). In glioblastoma, CD133⁺ cells have also been shown to be resistant to current chemotherapeutic agents and express higher levels of drug-resistant transporters and anti-apoptotic genes (140). It is still unclear whether CD133 is simply a marker of resistant cells or if its expression contributes to the mechanisms of resistance present in these cells (43).

In addition, the level of CD133 expression has been found to vary widely across a variety of tumor subtypes. In brain (80), pancreas (65), prostate (67) and lung (68, 127) cancer, CD133⁺ cells were reported to comprise a relatively rare subpopulation of the tumor bulk. However, Shmelkov and colleagues (141) reported that in primary colon cancer, CD133 expression is not limited to a small population of cells as previously reported (57, 58), but is more widely expressed. This phenomenon was also reported in the human liver cancer cell line Huh-7 (70) and we reported divergent expression of CD133 between serous and clear cell primary human ovarian tumors (86). Shmelkov and colleagues also indicated that the down-regulation (seen as low expression) of CD133⁺ cells might represent the transformation of these cells to more aggressive CD133⁻ cells that are responsible for metastatic growth. More recent evidence in colon cancer has suggested that the use of CD133 as a marker for putative CSCs should be interpreted with caution (142). The findings of Kemper and colleagues indicate that the epitope for AC133, one of the antibodies most commonly used to detect CD133, is masked upon differentiation of colon CSCs, possibly due to differential folding of the protein as a result of differential glycosylation. Therefore, though the AC133 epitope can define colon CSCs, its expression is likely regulated at the (post-) translational level.

Our results using the monoclonal antibody 293C/AC141 suggest that CD133 is an important marker for identifying tumor-initiating cell populations in ovarian cancer. Though CD133 positivity ranged from <12.5% in serous to >65% in clear cell tumors, respectively, we noted increased tumorigenic capacity consistently in CD133⁺ populations relative to their CD133⁻ counterparts in both subtypes (86). Moreover, although we observed tumor growth in both positive and negative cell populations, we ascribed tumor formation mediated by the negative cell fraction to post-sort contamination with a minority of CD133⁺ cells (i.e. <1%).

Somewhat surprisingly from a clinical perspective, Ferrandina and colleagues (143) reported that CD133 expression did not correlate with increased time to progression of disease or decreased overall survival in 160 primary ovarian cancer patients. This finding was in contrast to previous reports in hepatocellular carcinomas

(144), colorectal cancer (145) and in various brain tumors (for review, see (146)), which indicated that CD133 expression correlated with poor prognosis and advanced stage of disease. Whether this is due, in part, to the more heterogeneous nature of ovarian cancer is not yet known. Although previous studies (84, 86) suggest that CD133 expression does identify a tumor-initiating fraction in human ovarian cancer, future studies will require analysis of multiple marker-defined populations in ovarian tumors to delineate the most clinically relevant prognostic markers.

As is the case for CD44⁺ cells, CD133⁺ populations have been described as serving as either tumorigenic stem-like populations or tumor vascular progenitors in human ovarian cancer (81, 83, 85, 86). These findings are significant and suggest that the capacity of these marker-defined cells to form tumors may be inherently linked to their ability to sequester or generate a vascular blood supply to support tumor growth. It will be important to determine whether expression of these markers actually defines *bona fide* ovarian CSCs or merely distinguishes tumor sub-populations that include CSCs.

8. CURRENT METHODOLOGIES FOR HUMAN OVARIAN TUMOR CELL PROPAGATION

Current methodologies used to distinguish tumorigenic or CSC populations in ovarian cancer are varied across multiple laboratories. While each methodology has been successful in identifying important cell surface markers that delineate tumorigenic populations, the different reported findings may reflect the technique being used rather than the intrinsic properties of the cells being analyzed. Consistent approaches to the isolation of clearly defined ovarian CSC populations are paramount to the successful use of these cells as valid drug targets for generating improved therapeutic strategies. A brief discussion of the methods currently in use is provided here in an effort to outline the benefits and limitations associated with each and perhaps suggest the most important features that need to be considered going forward.

9. USE OF ESTABLISHED HUMAN OVARIAN CANCER CELL LINES

Immortalized cell lines initially derived from various primary tumor biopsies have been used for many years in cancer studies. Though they provide an unlimited supply of material for analyses, extended periods of cell propagation in serum-based media conditions can result in irreversible phenotypic and genotypic alterations (146, 147). Consequently they typically comprise divergent, highly-mutated populations that may not be truly representative of their parent primary tumors, with chromosomal and (epi)genetic differences frequently observed among cultures of the same cell lines maintained in different laboratories.

Though the use of transformed cell lines has been instrumental in unraveling key molecular pathways, the undefined (or highly varied) serum-based media conditions traditionally used to propagate these cells do not represent

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the growth conditions *in vivo* and may be inadequate to maintain the expansion of certain heterogeneous populations. Indeed, it remains to be determined whether these cell lines retain the stem-like hierarchy of their parent tumors of origin or have gained a heterogenic hierarchy as a result of culture conditions.

As cautioned by van Staveren and colleagues (148), the extrapolation of data derived from established cell line based assays to their equivalent human tumors *in vivo* might not be valid, given the (epi)genetic divergence of cell lines over time *in vitro*. For example, changes in oxygen tension alone have been demonstrated to alter CD133 expression *in vitro* in human glioma cell cultures (149). For this reason, we and others (35) would argue that for evaluation of stem-like CSC populations in human tumors, the use of primary tumor cells is essential for confirmation of conclusions drawn from analysis of established cell lines.

10. *IN VITRO* PROPAGATION OF PRIMARY OVARIAN TUMOR CELLS AS NON-ADHERENT SPHEROIDS

The propagation and enrichment of tumor-initiating cells as non-adherent spheroids in serum-free *in vitro* culture conditions has been utilized by several groups to enrich for cell populations that possess stem-like features from various tumor sources, including ovarian (46, 58, 63, 81, 82, 147). This *in vitro* culture methodology was originally developed by Reynolds and colleagues (150) who reported that the culture of CNS cells on non-adherent surfaces in the presence of epidermal growth factor (EGF) led to the formation of spheroid colonies which contained cells with both self-renewal and multi-potential differentiation capacity.

Several laboratories have utilized this method successfully to identify tumorigenic, putative CSC populations across various tumor subtypes, including ovarian (63, 81). This methodology is a significant improvement over the propagation of primary cells in serum-containing media on a monolayer. Although the three-dimensional culture of primary cancer cells as non-adherent spheroids in serum-free media conditions does not completely recapitulate the *in vivo* microenvironment, these cells retain many of the properties of their original tumors that are lost upon two-dimensional culturing (151). The enrichment of cells using this methodology also provides ample material for injection and continued functional analyses and thus represents a convenient platform for CSC culture.

However, the propagation of cells under these conditions may not be ideal given the media components present. Growth factors such as EGF, basic fibroblast growth factor (bFGF) and insulin that are known to stimulate specific signaling pathways are included in the culture medium at concentrations significantly higher than *in vivo* physiological levels. Additionally, as is the case for established cell lines, three-dimensional *in vitro* culturing cannot faithfully or completely reproduce the tumor-

stromal microenvironment. The exclusion of key growth factors and signaling components provided by the ECM and surrounding microenvironment may similarly alter the expression of relevant cell surface markers. For more accurate identification of CSC populations, *in vitro* culture methods incorporating stromal cells with the tumor cells have been proposed (152).

Previous studies indicating aberrant induction of signaling pathways and/or selection of cells showed that serial passage of glioblastoma multiforme tumors in mice produced neurospheres that grew at a faster rate in culture, suggesting that the culture conditions or *in vitro* acquired mutations selected for rapidly proliferating cells (153). Additionally, Ince and colleagues (154) showed that when normal breast tissue cells isolated from a single patient are cultured by two different means, transformed equivalently to generate tumorigenic cells and injected into immunocompromised mice, the resulting tumors differed greatly with respect to histopathology and metastatic potential. Discrepancies in marker expression using different propagation methodologies (*in vivo* versus *in vitro*) have already been described for human breast CSC (56, 63) and primary ovarian CSC (81, 86) with regard to EpCAM and CD117 expression, respectively. Such differences are likely a reflection of the methods used for CSC propagation and maintenance, and the heterogeneity of tumor types.

11. *IN VIVO* PROPAGATION OF PRIMARY HUMAN TUMORS IN XENOGRAFT MOUSE MODELS

Previous studies have utilized the direct xenograft transplantation model for propagating human tumors *in vivo* (56, 58, 86). In these studies, both orthotopic and ectopic injection sites have been utilized to successfully recapitulate the histotype of the parent tumor in immunocompromised mice. The advantages of using direct injection of primary human tumor material versus pre-cultured human tumor cells in xenograft transplantation assays include the absence of artificial stimulation of gene expression by cell culture media components, an important consideration since prospective isolation of CSC populations has been based on specific cell surface marker expression. Conversely, there are some limitations to the use of the mouse ovarian tumor explant model that require careful consideration. These can include 1) direct xenotransplantation of tumor material in a subcutaneous site which may prevent the selection of cells that are capable of anchorage-independent versus -dependent growth; 2) the limiting amount of primary ovarian tumor tissue (as opposed to omental cake or tumor growing immediately distal to the primary site) typically recovered at the time of surgery; 3) the decreased tumor formation rate of primary cells in immunocompromised mice; and 4) the time to generation of a detectable tumor in a immunocompromised mouse model, which can be greater than 6 months (86). This is in contrast to tumors generated following injection of *in vitro* propagated spheroids that are detected at 3-4 months post-injection (81).

12. ADDITIONAL CAVEATS OF THE XENOGRAFT MODEL

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Several additional caveats regarding the use of the xenograft model have been discussed in the literature particularly with regard to tumor type, site of injection, immunotype of the injected mice and potential differences between human and mouse *in vivo* environments. These issues have led to serious questions regarding the usefulness of the xenotransplantation model.

12.1. Solid tumors

Unlike in hematopoietic malignancies, analyses of CSC populations in solid tumors are difficult, given the requirement for both the interplay of supportive stromal cells and chemokines/cytokines and the ability to generate adequate tumor vasculature. The techniques currently used for the dissociation of solid tumors to single cell suspensions required for FACS and xenotransplantation assays may be excessively harsh and may abolish key associations present in the intact tumor that cannot be adequately recapitulated in the *in vivo* environment. Therefore, these technologies generate obvious technical hurdles that may compromise the clinical relevance of the CSC markers identified (155).

12.2. Site of injection

It is generally accepted that a functional microenvironment is required to maintain and support CSCs and provides the necessary signals for tumor initiation and growth. With the exception of breast (56) and brain (46) tumors, most studies assaying tumor formation have relied upon ectopic injection of cancer cells, usually in Matrigel™ complexes, primarily due to the lack of sufficient *in vivo* monitoring technologies capable of accurately accessing tumor formation and growth over time. Delivery of human ovarian cancer cell line-derived cells orthotopically into the ovarian capsule has been performed and resulted in tumor formation 4 weeks post-injection (156). However, for studies involving primary human ovarian tumor material, the obvious advantages of subcutaneous tumor cell injection include avoiding invasive surgery, the ease of monitoring tumor formation and growth, and straightforward extraction of tumors.

Although the effect of non-orthotopic injection of cancer cells is as yet unproven with regard to any potential positive or negative selection of certain cell populations, it is likely that the use of orthotopic sites of injection would be more relevant in accurate assessment of putative CSC function. Additionally, it has been argued that lack of tumor formation at non-orthotopic sites could result from both an insufficient capacity to sequester an adequate blood supply (55) and the lack of relevant microenvironment signals. The success of renal capsule transplantation for growth of non-orthotopic tissues is likely due to its provision of a rich, highly vascularized environment but it does not completely represent the microenvironment provided by the tissue of origin. One interesting report has questioned whether identified CSC surface markers distinguish cells that have increased tumorigenic capacity or those with increased angiogenic and/or vasculogenic capacity that garner the factors necessary for cell maintenance and growth that facilitate tumorigenesis (157).

Further study aimed at deciphering the exact functions of the CSC markers identified to date will determine whether such a possibility is plausible.

12.3. Human versus mouse microenvironments

Normal stem cells and niche cells communicate with one another through adhesion molecules and other factors thereby providing the molecular signals necessary to maintain the unique functional characteristics of stem cells (48). As with normal stem cells, CSCs may also rely on secreted factors from their surrounding microenvironment as well as interaction with their non-tumorigenic progeny to maintain their CSC identity and regulate tumor homeostasis and progression (158). Therefore, one of the biggest challenges in investigating ovarian CSC continues to be the provision of a biologically relevant microenvironment that can closely mimic that present in cancer patients.

Expansion of primary human tumor cells both *in vitro* and *in vivo* introduces additional unknown variables, as both approaches expose the cells to artificial environments that may affect their (epi)genetic integrity and surface marker phenotype. Though xenotransplantation does recapitulate the three-dimensional tumor architecture and provide supportive stromal cells, it fails to obviate potential differences between the human and mouse environment, particularly with regard to cross-species differences in cytokine/chemokine expression, the presence of adhesion molecules and responsiveness to specific growth factors. These likely differences may influence the normal tumor initiation capability of the injected tumor cells.

Several questions, therefore, have been raised about the legitimacy of the mouse xenotransplantation model as a tool for assaying tumorigenicity of marker-defined cell populations (159-161). Does this model select for cells that are more suited to survive in immunocompromised mice? In addition, given the obvious heterogeneity of cancer, is the lack of a comparable microenvironment responsible for selecting cell populations most adapted to survive independently of signals provided by the cancer patient? Conversely, does this model fail to identify populations of cancer cells capable of tumor formation in human patients that are unable to grow in the mouse environment? Given that the behavior and frequency of CSC populations may also be influenced by their surrounding microenvironment, it is also possible that cross-species differences may lead to an underestimation of the frequency of the CSC population or may stimulate non-CSC cells to express a CSC phenotype in a foreign (mouse) environment (48, 161).

12.4. Mouse strain used

To date, several genotypes of immunocompromised mice have been utilized for xenograft transplantation assays to evaluate the tumorigenicity of specified cell populations *in vivo* (Table 1). For analyses of ovarian CSC populations, these have included non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (85, 86), SCID mice (77) and various strains of nude mice including NCr (82), BALB-c/nu/nu (81), Swiss (75)

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and BALB-cAnNCr-nu/nu (84). Though considered immunodeficient, these mice may still generate subtle cell-mediated immunity. For example, NOD/SCID mice may produce natural killer (NK) cells (162) whereas athymic nude mice may still generate residual T lymphocytes and NK cells (163).

The relevance of the immunocompromised mouse host used in determining the tumorigenicity of defined cell populations in xenotransplantation experiments has recently been highlighted in studies on primary melanoma tissue. These analyses indicated that the observed frequency of melanoma cells with tumor-forming capacity increased several orders of magnitude when the tumor cells were analyzed in more highly immunocompromised NOD/SCID interleukin-2 receptor gamma chain null (Il2rg^{-/-}) mice that lack natural killer T cell activity (164). Additionally, several studies have indicated that the number of injected cells may determine whether an immune response is induced in these mice which could have significant implications for experiments aimed at determining CSC frequency (for commentary, see (60)). For these reasons, the use of more immunodeficient recombinaise activated gene 1/2 (RAG1/2) knockout nude mice, which lack T, B and NK cells (165) may be more suitable for such studies.

Though the caveats of the xenotransplantation model would appear to lessen the relevance of the CSC model in human cancer, data from similar transplantation assays performed using syngeneic mouse models demonstrate the existence of specific subpopulations of tumorigenic cells and would appear to support the CSC model in some solid tumors (166, 167). Although these caveats should always be appreciated when interpreting data, the xenograft transplantation model of human tumors most closely reflects the natural three-dimensional tumor environment present in human patients. Tumors generated in this model retain the histopathological integrity of their parent tumors, as shown for ovarian cancer (86), and are exposed to vascular and stromal influences lacking in *in vitro* models. This model also provides a sufficient platform for assessing the efficacy of novel CSC-targeted therapeutics in combating tumor growth.

13. CLINICAL STRATEGIES AIMED AT CANCER STEM CELLS

How can we best move forward in our efforts to develop effective detection and treatment strategies to combat ovarian cancer? Clinical data to date suggest that simply targeting dividing cells in ovarian cancer is insufficient, necessitating the need for more pathway- or cell-specific targeted therapies (6). While current therapies focus on eliminating tumor bulk, recurrent disease in ovarian cancer and other malignancies is presumed to be in part due to the inability to treat remaining tumor cells. The identification of CSCs driving ovarian tumorigenesis, progression and/or recurrence of disease in patients could have important clinical implications if the disease that remains following first-line treatment consists predominantly of CSCs. It is likely that a combination

approach utilizing first round chemotherapy and surgery to reduce tumor burden followed by CSC targeted drug therapies will be required to effectively hinder cancer growth and halt disease progression (168).

The development of improved targeted therapies will require the direct application of the CSC model to the clinical setting. Purification of near-homogeneous populations of ovarian CSC would permit detailed molecular and genetic study of these specialized populations. Such analyses may offer new insights into the key regulatory pathways governing ovarian CSC function. The challenge then lies in the development of drugs that can accurately target and destroy the ovarian CSC population while causing minimal damage to the surrounding tissue. To date three principal methods for eradicating these cells have been proposed: (i) direct targeting of CSCs, (ii) induction of CSC differentiation/proliferation, and (iii) destruction of the supportive niche/stromal microenvironment. The current potential of each of these methods in sufficiently eradicating CSC populations is discussed below.

13.1. Direct targeting of cancer stem cells

Further refinement of the cell surface marker profiles that define ovarian CSC populations is of paramount importance, as drugs designed to target cells expressing identified markers may eliminate these causative populations. Similarly, drugs that target stem cell pathways implicated in cancer development may also be effective therapeutic agents. However, there are several challenges currently facing the development of drugs to target CSC populations. First, since there is overlap in cell surface marker expression and signaling pathways associated with normal stem cells and CSCs, these drugs must sufficiently discriminate between these populations to prevent off-target effects. Our current understanding of the pathways involved in the regulation of CSC homeostasis is limited and it is unclear if inhibition of one or several of these crucial pathways would be sufficient to kill CSC (152). In addition, designing drugs to target specific CSCs in various tumors may be inadequate, given the potential for additional mutational events to select more potent CSC with different phenotypes (59, 60). The likelihood of high patient-to-patient variability in terms of CSC marker expression also needs to be considered.

Despite these potential challenges, several recent reports have indicated favorable responses to treatment of tumors with CSC-targeted therapies. Targeting stem cell factor (SCF)-c-kit signaling in human lung CD133⁺ CSCs using SCF neutralizing antibodies or the c-kit inhibitor imatinib inhibited CSC proliferation and survival *in vitro* (169). Gupta and colleagues identified salinomycin as a selective inhibitor of CD44^{high}/CD24^{low} breast CSCs that inhibited mammary tumor growth *in vivo* and induced increased epithelial differentiation of tumor cells (170). Additionally, Hirsch and colleagues determined that metformin selectively targets CD44^{high}/CD24^{low} breast CSCs can block tumor growth and prolong remission in combination with chemotherapy (171).

Similar strategies in ovarian cancer have been recently reviewed (172). Treatment of slow-proliferating

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ovarian cancer cells with 7-hydroxystaurosporine was cytostatic. A similar effect was observed when these cells were grown as spheres under stem-cell selective conditions (173). Slomany and colleagues blocked the hyaluronan-CD44 interaction in CD133⁺ primary human ovarian tumor cells using small hyaluronan oligosaccharides. This inhibition reduced the association of drug transporters and receptor tyrosine kinases with CD44 and inhibited tumorigenesis of the treated cells (174).

Certain drugs have been developed to target specific pathways associated with normal stem cell maintenance that may be aberrantly induced or repressed in cancer. In particular, the Hh, Wnt and Notch signaling pathways, which have been implicated in promoting tumorigenesis in various organs, have been targeted.

13.1.1. Hedgehog signaling pathway

Evidence implicating a potential role for this pathway in cancer was provided by two reports that indicated loss of negative regulation of Hh signaling due to *Patched1* mutation could result in Gorlin's syndrome, a disease typified by a high frequency of sporadic tumor formation (175, 176). Several studies have since indicated that dysregulated Hh signaling may tip the balance in favor of aberrant cell proliferation and tumor promotion (for review, see (177)). Indeed, Hh signaling has recently been reported to be essential for the maintenance of CSCs in myeloid leukemia (178, 179). In ovarian cancer, cyclopamine, a plant-derived steroidal alkaloid and specific Hh pathway inhibitor, blocks the growth and proliferation of ovarian cancer cells *in vitro* and tumor formation *in vivo* (180). More recent studies have indicated that Hh signaling may exert its tumor-promoting effects indirectly through paracrine Hh activation in surrounding stromal cells resulting in a more favorable environment for tumor growth ((181-183); for review see (184)). Current efforts are focused on more stable derivatives of cyclopamine such as IPI-926, which has been shown to reduce tumor growth by adversely affecting tumor-associated stromal cells through Hh pathway inhibition (185). Also, a multicenter clinical trial has been initiated by Genentech using ovarian cancer patients in second or third round complete remission following chemotherapy evaluating the efficacy of Hh inhibitor GDC-0449 as maintenance therapy to improve progression-free survival (<http://www.clinicaltrials.gov/ct2/results?term=hedgehog>). Importantly there are a number of other commercial entities (Infinity Pharmaceuticals, Pfizer Pharmaceuticals, Bristol-Myers Squibb and Novartis) with their version of Hh pathway inhibitors in various stages of trials or development that appear promising.

13.1.2. Notch signaling pathway

The Notch signaling pathway plays a key role in cell fate determination by influencing cell proliferation, differentiation and apoptosis (for review, see (186)). In human cancer, Notch signaling has been paradoxically implicated in both tumor promotion (187-189) and suppression (190-193) across various tumor subtypes. In ovarian cancer, Notch signaling has been reported to promote cell proliferation and tumor progression (for

review, see (194)) and drugs aimed at targeted inhibition of this pathway are currently being evaluated for therapeutic potential. The main signaling molecule in this pathway is the intracellular domain of the Notch receptor (NICD), which is generated by gamma-secretase cleavage of the receptor in response to ligand binding and subsequent receptor activation. Gamma-secretase inhibitors (GSIs) have been shown to inhibit tumor growth in pancreatic and sarcoma cell lines *in vitro* (195) and are already under investigation in early Phase I clinical trials against a variety of advanced solid tumors including breast cancer (<http://www.clinicaltrials.gov/ct2/results?term=gamma+secretase+inhibitors>). Furthermore, gamma-secretase inhibitors have been found to specifically target CSC populations, such as CD133⁺ cells and SP cells, when Notch signaling was effectively blocked in embryonal brain tumor cells (134). Park and colleagues reported *Notch3* gene amplification in high-grade serous ovarian carcinomas and demonstrated that inactivation of Notch3 with a gamma-secretase inhibitor resulted in suppressed cell proliferation and apoptosis in cell lines with increased Notch expression (196). Further preclinical investigations will help to further elucidate how the Notch signaling pathway contributes to the pathogenesis of ovarian cancer.

13.1.3. Wnt signaling pathway

Several groups have implicated the Wnt signaling pathway in promoting ovarian tumorigenesis. Under normal circumstances, the Wnt pathway performs vital functions in embryonic development of the ovary, follicular development and ovarian function (for review, see (197)). Rask and colleagues reported significant over-expression of several Wnt pathway proteins in ovarian cancer compared to normal ovarian tissue (198), which suggests that this pathway could provide useful therapeutic targets for treatment. Interestingly, ovarian endometrioid carcinomas have been reported to be particularly susceptible to mutations in the beta-catenin gene that lead to constitutive Wnt pathway activation (199-201). However, to date there is a paucity of information regarding the effects of Wnt inhibitors on ovarian tumor growth. Imatinib mesylate (*Gleevec*), a Bcr-Abl kinase inhibitor, has been shown to effectively inhibit beta-catenin signaling and repress cell proliferation of colon cancer cell lines (202) and could provide similar effects as a treatment therapeutic in ovarian cancer. Endogenous Wnt receptor antagonists such as secreted frizzled related protein (SFRP) and Wnt inhibitory factor 1 (WIF1) may also serve as useful targets for therapy, though specific drugs targeting activation of these proteins have not yet been developed.

13.1.4. Additional targets for therapy

Other efforts aimed at targeting CSC populations have focused on the use of small molecules that directly target ABC transporters. Disruption of these transporters could increase the chemosensitivity of CSC populations by preventing their ability to efflux toxic chemotherapeutic drugs, thereby sensitizing these cells to current chemotherapeutic regimens. Results of *in vitro* and *in vivo* studies would appear to broadly support a putative role for these inhibitors in targeted destruction of cancer cells, and possibly CSC (for review, see (203)). However, a Phase III

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clinical trial utilizing the P-glycoprotein modulator PSC-833 in acute AML patients indicated no significant improvement in patient survival (204). Future trials based on this approach will require the development of drugs with reduced patient side effects and increased target cell specificity.

13.2. Induction of cancer stem cell differentiation/proliferation

Stimulating the differentiation and/or proliferation of CSC populations could potentially aid in their eradication. Driving CSCs to differentiate would deplete tumors of the drug-resistant population. Similarly, inducing CSC proliferation would make the cells sensitive to destruction by standard chemotherapeutic strategies. Several of the pathways that could potentially be targeted to drive these processes include those already described above (i.e. Notch signaling pathway) and some significant findings have been made in support of this putative course of therapy.

A report by van Es and colleagues indicated that inhibition of the Notch signaling pathway in normal colon resulted in differentiation of intestinal crypt cells and adenoma cells into goblet cells (205). Piccorillo and colleagues have demonstrated that BMPs can drive the differentiation of CD133⁺ CSC in glioblastomas, thereby reducing their capacity to reconstitute tumors (138). Also, treatment with CD44-specific monoclonal antibodies *in vivo* disrupted AML leukemic stem cell function, in part by altering leukemic stem cell fate and driving them to differentiate (102).

13.3. Destruction of the cancer stem cell niche/stromal supportive environment

The impact of the microenvironment in both promoting and inhibiting tumor growth has been demonstrated by several groups (for review, see (206, 207)), though its precise role in the maintenance of specific CSC populations *in vivo* remains to be determined. It is as yet unclear whether CSCs occupy a microenvironmental niche, similar to that of normal stem cells such as HSCs, upon which they rely for factors necessary to maintain tumor homeostasis. The extracellular environment provides the structural platform necessary for cell growth and intercellular communication, in addition to various growth factors and chemokines that may enhance tumor cell proliferation and invasion. In contrast, the microenvironment may also stimulate production of anti-angiogenic proteins and certain matrix metalloproteases that can inhibit tumorigenesis (208). As mentioned above, the Hh signaling pathway may promote tumor growth through paracrine activation of its surrounding stromal microenvironment and thus may provide a putative target pathway. Whether this pathway is specific to CSCs within these malignancies requires further investigation.

Clearly, a multi-targeted approach aimed at destroying bulk tumor cells, CSCs and their supportive microenvironment may provide the most efficient way to treat ovarian cancer. Given its relatively normal phenotype, the stromal cell milieu should also be less likely to acquire

therapeutic resistance to treatment regimens and may represent a suitable target for adjuvant treatment (207). The caveat to this approach is the necessary targeting of such therapies to the tumor environment to minimize off-target effects on the surrounding normal cell milieu.

14. PERSPECTIVE

Although the hypothesis that CSCs are contributing to the pathogenesis of ovarian cancer has gained increased acceptance, evidence in support of CSCs in ovarian cancer to date is modest. Regardless the collective evidence would suggest that there are cells of ovarian or tubal origin that have the capacity to give rise to a CSC population with multi-potential capabilities. It seems unlikely that CSCs in all ovarian tumors will be defined by a single cell surface marker given the heterogeneity of ovarian cancer. We anticipate that a more refined definition of these populations as truly functional ovarian CSCs will benefit from further studies of previously identified CSCs in other solid tumors. Moreover, identification of these cells in ovarian tumors will allow the definition of the intracellular pathways that are critical to self-renewal and/or mediate communication with the tumor microenvironment. Distinguishing CSC-associated antigen profiles may elucidate novel, more sensitive biomarkers for earlier tumor diagnosis and provide molecular targets for the development of alternative treatment modalities in ovarian cancer.

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Abbreviations: ABC: adenosine triphosphate (ATP) binding cassette, AML: acute myeloid leukemia, bFGF: basic fibroblast growth factor, BMP: bone morphogenic protein, BRCA1/2: Breast cancer 1/2, Bcrp1: breast cancer resistance protein 1, CIC: cancer-initiating cell, CML: chronic myelogenous leukemia, CNS: central nervous system, CSC: cancer stem cell, EGF: epidermal growth factor, EOC: epithelial ovarian cancer, ECM: extracellular matrix, FACS: fluorescence-activated cell sorting, GSI: gamma-secretase inhibitors, GFP: green fluorescent protein, Hh: Hedgehog, HSC: hematopoietic stem cell, IFN-alpha: interferon-alpha, IKK-beta: I kappa B kinase beta, Il2rg: interleukin-2 receptor gamma chain, MyD88: myeloid differentiation factor 88, NF-kappa B: nuclear factor-kappa B, NICD: Notch intracellular domain, NK:

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natural killer, NOD/SCID: non-obese diabetic/severe combined immunodeficient, OSE: ovarian surface epithelium, RAG1/2: recombinase activated gene 1/2, ROS: reactive oxygen species, SCF: stem cell factor, SFRP1: secreted frizzled related protein 1, SP: side population, TIC: tumor-initiating cell, TLR: toll-like receptor, VEGF: vascular endothelial growth factor, VEGFR: vascular endothelial growth factor receptor, WIF1: Wnt inhibitory factor 1.

Key Words: Ovary, Human, Cancer, Stem Cells, Xenograft, Transplantation, Review

Send correspondence to: Bo R. Rueda, Vincent Center for Reproductive Biology, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, 55 Fruit Street, THR-901, Boston, MA 02114, Tel: 617-724-2825, Fax: 617-726-0561, E-mail: brueda@partners.org

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