ADVANCES IN EPITHELIAL OVARIAN CANCER: MODEL SYSTEMS, MICROENVIRONMENTAL INFLUENCES, THERAPY, AND ORIGINS

EDITED BY: Viive Maarika Howell, Ben Davidson, Tian-Li Wang and Christina Annunziata

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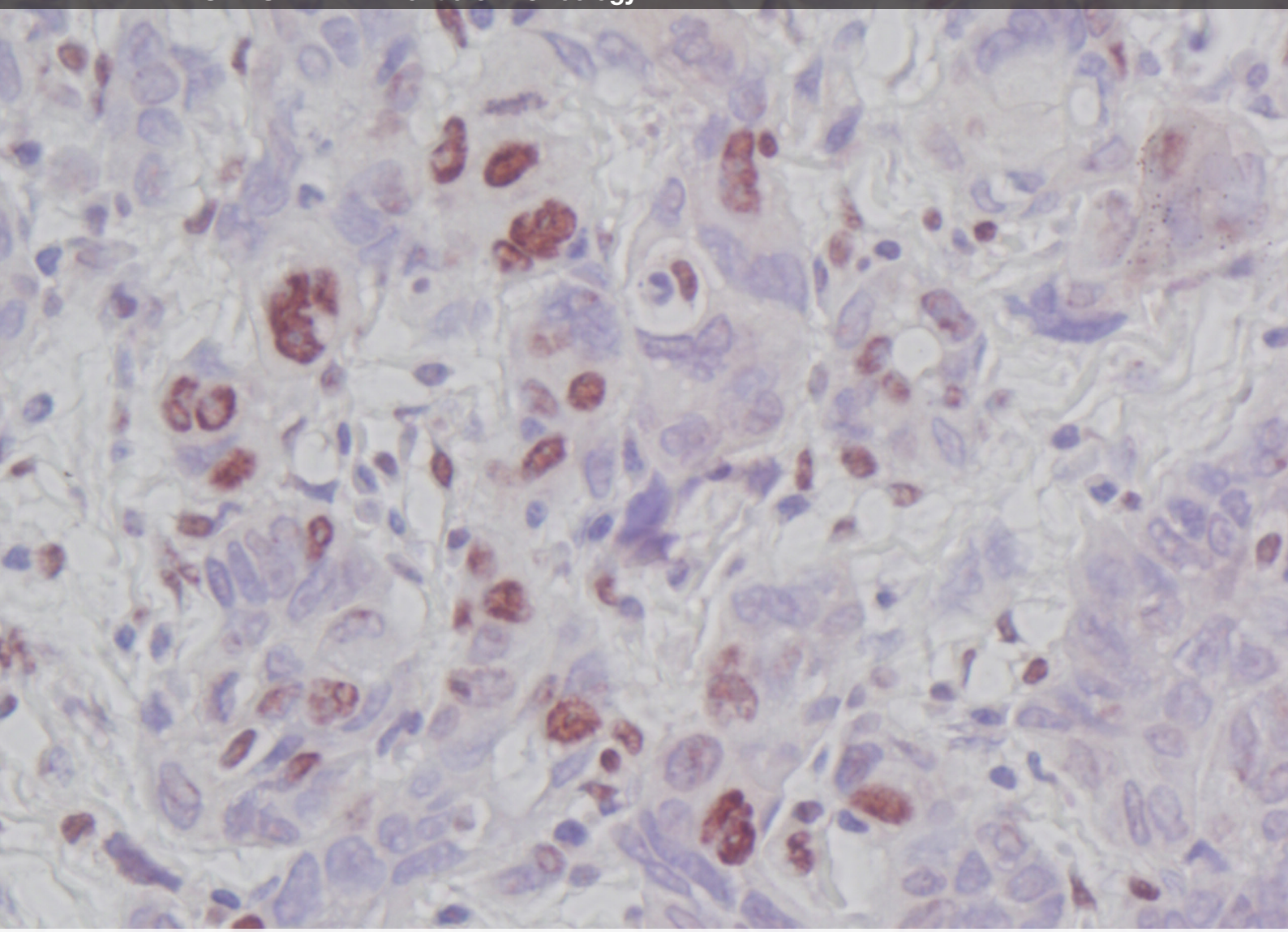

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ADVANCES IN EPITHELIAL OVARIAN CANCER: MODEL SYSTEMS, MICROENVIRONMENTAL INFLUENCES, THERAPY, AND ORIGINS

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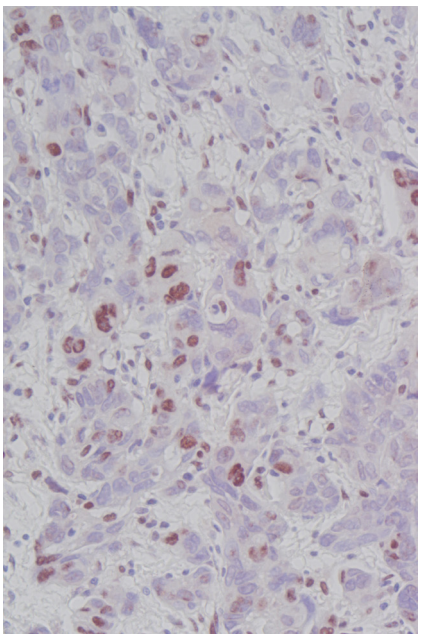

BRCA1 immunolabelling (brown) of high grade serous ovarian carcinoma, original magnification 100X.

Image by Viive Maarika Howell.

This eBook provides a compendium of the current state-of-the-art in research tools for, and understanding of, the critical research areas in epithelial ovarian cancer (EOC) with a strong emphasis on (HG-SOC). Research areas covered include therapy response and development, microenvironmental influences and the etiology and progression of EOC. Ten articles detail established and novel in vivo and in vitro model systems. These include primary and immortalized cell culture in 2D and 3D as well as genetically engineered, transgenic, spontaneous, syngeneic, classical xenograft and patient derived xenograft mouse models. The generation of genetically engineered mouse models of HG-SOC has been a major dilemma as models with the oncogenic aberrations common in the human malignancy do not accurately recapitulate HG-SOC. Conversely, commonly used HG-SOC cell lines have been found to not harbor the expected genetic changes. These issues as well as the rapid acceptance of patient derived xenograft models are reviewed. Five articles discuss different aspects of the tumor microenvironment including its role in therapy resistance, disease progression and metastasis. Mutation of BRCA1/2 continues to be

the best defined risk factor for HG-SOC. Three articles discuss BRCA-loss in the context of disease development, targeted therapies and changes in preventative measures proposed for mutation carriers in light of the recent advances in knowledge regarding the origins of this malignancy. An image of HG-SOC with patchy BRCA1 expression is featured on the cover (image by VM Howell). A major clinical issue for patients with HG-SOC is the development of therapy resistance. Five articles focus on therapy resistance and different ways to overcome resistance. Overall, this eBook is an outstanding resource to aid researchers design their programs of research and determine the most appropriate and up-to-date EOC model systems to address their research questions.

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EDITORIAL
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Editorial: Advances in epithelial ovarian cancer: model systems, microenvironmental influences, therapy, and origins

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Keywords: ovarian cancer, mouse models, microenvironment, chemoresistance, ascites, 3D culture models

Improving outcomes for women with epithelial ovarian cancer is a major health issue worldwide as 5-year survival has not improved significantly over the last two decades.

The urgent need to increase our understanding of high-grade serous ovarian cancer (HG-SOC) led to it being chosen for the pilot project of The Cancer Genome Atlas (TCGA) and the genomes of over 400 HG-SOC samples are now freely accessible for interrogation. These data are being used for the discovery of biomarkers as well as the generation of hypotheses to understand the natural history of this malignancy and develop effective targeted therapies. In this Research Topic, Lisowaska and colleagues used gene expression profiling to identify that reduced expression of *CLASP1*, a regulator of microtubule dynamics essential for mitotic cell cycling was positively associated with survival, either overall or disease free (1). Their data clearly highlighted that different histological subtypes of ovarian cancer had very different molecular signatures, although undifferentiated and high-grade serous ones were indistinguishable. These results are consistent with other findings that together have led to the understanding that epithelial ovarian cancer (EOC) is not a single entity but rather a number of distinct malignancies with different etiologies and molecular aberrations. Of these, HG-SOC is the most aggressive and common, accounting for 60–70% of all cases of ovarian cancer.

Notwithstanding the unprecedented *in silico* resource now available via TCGA and other web-based portals, research in HG-SOC is hampered on a number of fronts. Comparison of results from cancerous or cancer-associated stromal cells with each non-cancerous (normal) equivalent is a fundamental research question, but what to use for normal cells is unclear. Recent evidence suggests that HG-SOC has its origins in the secretory cells located in the fimbrial end of the fallopian tube. This is contrary to the prevailing notion that HG-SOC arises from the epithelium lining the ovary and inclusion cysts. The contribution of each site to serous ovarian carcinogenesis is currently under debate. Jones and Drapkin recount the evolution of evidence for each site of origin and review the use and limitations of primary cell culture model systems developed from each site (2). In support of an ovarian surface epithelial (OSE) origin for HG-SOC, McCloskey and colleagues describe the tumor-initiating characteristics of a novel spontaneously transformed mouse OSE cell line (3). Ahmed and Stenvers draw attention to ascites, an underutilized yet readily accessible source of primary cancer cells from EOC patients and provide a detailed review of its use as a clinically relevant model system (4). Different methods of isolation and culture of primary ovarian cancer tumor cells from ascites and other sources are reviewed by Cunnea and Stronach who also advocate for universal standardized protocols for improved reproducibility and interpretation of results between studies (5).

In conjunction with the development of new cell models is the move to three-dimensional (3D) culture conditions. 3D culture systems feature in several articles in this Research Topic indicating the enthusiasm for this culture type and recognition of the deficiencies of the monolayer systems (2, 5, 6).

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It is hoped that models with more physiologically relevant microenvironments will lead to increased clinical translation of findings. 2D and 3D culture systems are compared by Fuller and Howell and the characteristics of the different matrices available reviewed (6).

Resistance to platinum therapy continues to be a major issue in HG-SOC (5, 7, 8). Cunnea and Stronach review the currently available immortalized cell lines in the context of sensitivity and resistance to platinum and highlight deficiencies in *in vitro* development of treatment resistance (5). In particular, they illustrate the power of having serial cell lines from the same patient which have been able to show that the development of treatment resistance was due to the selection of resistant sub-clones rather than evolution of new lineages. They note the need for more such matched cell lines, which is now one of the goals of the European OCTIPS (Ovarian cancer therapy – innovative models prolong survival) consortium.

In light of the inevitable development of platinum resistant or refractory disease (7), new strategies and agents to reduce tumor burden and improve patient outcomes are in high demand. Essential for preclinical development are accurate and robust EOC model systems. The current status of EOC models specifically for preclinical development is reviewed by Konstantinopoulos and Matulonis (9). Different theories for overcoming platinum resistance are detailed in this Research Topic. Evidence from genetic and functional studies of HG-SOC point to the homologous DNA repair (HR) system, in particular BRCA1 and BRCA2, as critical determinants of response to platinum therapy. Wiedemeyer and colleagues discuss the concept of disrupting HR capacity via BRCA1/2 to reverse platinum resistance in BRCA-proficient cancers (8). They provide a comprehensive overview of available agents and the genetic aberrations targeted by these agents illustrating how rational therapeutic combinations may be designed to prevent or delay the onset of platinum resistance in HG-SOC. Alternative theories of platinum resistance are proposed by Chien and colleagues that also take into account the paradoxical recurrence of platinum sensitivity observed in patients with HG-SOC (7). They present the cancer stem cell theory for both the development of resistance and sensitivity upon recurrence and highlight the involvement of specific components of the extracellular matrix in the establishment of stem cell niches. Based on this evidence, they suggest that cancer cells are not intrinsically resistant to platinum but, rather, acquire extracellular matrix-dependent platinum resistance.

Targeting cancer stem cells is the basis of a new therapy identified by Abubaker and colleagues (10). They observed that paclitaxel treatment enhanced the expression of cancer stem cell-like markers in surviving cancer cells *in vivo* and coincided with significant activation of the JAK2/STAT3 pathway. In their research manuscript, they report the efficacy of concurrent paclitaxel and JAK2/STAT3 pathway inhibitor, CYT387, in a xenograft model of HG-SOC.

Having a familial *BRCA1/2* mutation remains the best defined risk factor for HG-SOC. The search for HG-SOC precursor lesions led to the discovery of P53 signatures, serous tubal intraepithelial lesion (STILs) and serous tubal intraepithelial carcinomas (STICs) all in the fallopian tubal epithelium, implicating this tissue as a site of origin for HG-SOC. George and Shaw provide a critical review of the literature related to these findings, noting that P53 signatures are found with similar frequencies in *BRCA*-mutation carriers and non-carriers, and that STILs and STICS are uncommon and identified with poor reproducibility (11). They review what is known regarding the involvement of hormones in HG-SOC development with specific reference to the altered reproductive physiology in *BRCA*-mutation carriers and propose that the combination of ovulation-induced chronic inflammation coupled with *BRCA*-mutations may predispose the development of precancerous lesions leading to HG-SOC.

While the changing notion of the site of origin of HG-SOC is critical for understanding the molecular mechanisms of ovarian cancer, it also has "real world" implications for prophylactic strategies for women with familial *BRCA* mutations or a strong family history of breast and ovarian cancer. Shenenberg and Mitchell review the molecular evidence providing rationale for risk-reducing bilateral salpingo-oophorectomy *versus* bilateral salpingectomy *versus* bilateral salpingectomy and delayed bilateral oophorectomy (12). This is elegantly balanced against quality of life issues that each individual faces when determining which risk-reducing procedure if any, is most appropriate for them. Non-surgical alternatives that may reduce risk and advances in diagnostic imaging for improved early detection, including current research assessing folate receptor α and HER-2 for tumor-specific imaging are reviewed by Ohman and colleagues (13).

Understanding disease development requires models of tumor progression and is best addressed by spontaneous mouse models. However, there is a paucity of reproducible spontaneous animal models of HG-SOC. This may be partly explained by the lack of promoters to drive genetic changes in the cell of origin of this disease. However, circumventing this technical difficulty by surgical delivery of Cre recombinase has not always delivered the expected outcomes. Does this again relate to the cell of origin? New models for the study of HG-SOC are being generated to accommodate the changing view of the cell of origin and enable monitoring of tumor progression. House and colleagues and others provide overviews of the results of using different methods to direct genetic changes to the OSE and FTE (2, 13, 14). Smith and colleagues discuss the importance of including menopausal physiology in mouse models of EOC and propose the germ cell-deficient Wv mice (*c-kit* mutant) as a background strain for breeding with relevant genetically engineered changes (15).

While grafted models of HG-SOC are not suitable for assessing early stage disease, their faster time course and the ability to easily add in reporters for *in vivo* imaging provide advantages over the spontaneous models. House and colleagues review the few reported syngeneic models of HG-SOC as well as the imaging modalities available for *in vivo* studies (14). The characterization of an FVB/N strain syngeneic model of HG-SOC by McCloskey and colleagues, reported for the first time in this Research Topic, thus has the potential to be an exciting and useful addition to the armory of reagents available for ovarian cancer research (3).

Xenografts are the most utilized *in vivo* platform for many cancers, including EOC. However, it is well recognized that they have deficiencies in recapitulating the tumors they represent. These include the loss of fidelity through *in vitro* culture, lack of

cellular heterogeneity and lack of an intact immune system. The advent of patient-derived xenograft (PDX) models was aimed at overcoming two of these deficiencies by using pieces of patient tumors that had never been cultured *in vitro* and that retained the microenvironment of the original tumor. PDX models are aimed primarily at testing drug responses. The site of implantation of PDXs can alter the rate of engraftment as well as the characteristics of the model. The advantages and disadvantages of the different sites of implantation are discussed by Scott and colleagues as part of their review of PDX model systems that have been trialed for EOC (16). They and others also assess the utility of PDX models as preclinical models for trialing new therapies for EOC (2, 13, 14, 16).

The rapid acceptance of PDXs as preclinical models underscores the increasing awareness of the contribution of the microenvironment to the pathogenesis and progression of cancer. An overview of the different components of the microenvironment such as proteases and extracellular matrix and their roles in promoting invasion and metastasis in EOC is presented by Davidson and colleagues (17). Dissemination of this cancer by shedding of cells into the peritoneum is a major route of metastases and involves interactions between cancer cells and adipocytes, endothelial cells, fibroblasts, mesenchymal stem cells, macrophages, and other immune cells. The microenvironment of the peritoneal cavity that makes it highly conducive to carcinomatosis and the therapeutic implications of targeting the heterotypic cellular interactions within the peritoneum are the focus of the review by Naora (18). Within the immune population, tumor-associated macrophages represent the most abundant infiltrating immune cell in human ovarian tumors and ascites. They display a unique activation profile in ovarian tumors and are able to create an immunosuppressive microenvironment, allowing tumors to evade immune detection and promoting tumor progression. This cell type is the focus of mini-review by Colvin (19).

In summary, this Research Topic showcases our current understanding of a number of key areas in EOC. It has a strong focus on model systems given the critical importance of having accurate systems and the particular difficulties and dilemmas faced especially in the development of *in vivo* models.

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REVIEW ARTICLE
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Modeling high-grade serous carcinoma: how converging insights into pathogenesis and genetics are driving better experimental platforms

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Recent developments in the study of epithelial ovarian cancer have called into question the traditional views regarding the site of tumor initiation. Histopathologic studies and genomic analyses suggest that extra-ovarian sites, like the fallopian tube, may harbor the coveted cell of origin and could therefore contribute significantly to the development of high-grade serous ovarian carcinoma (HG-SOC). Our ability to validate these emerging genomic and pathologic observations and characterize the early transformation events of HG-SOC hinges on the development of novel model systems. Currently, there are only a handful of new model systems that are addressing these concerns. This review will chronicle the convergent evolution of these ovarian cancer model systems in the context of the changing pathologic and genomic understanding of HG-SOC.

Keywords: ovarian cancer, genetics, pathogenesis, model systems, OSE, fallopian tube

INTRODUCTION

In 2013, the American Cancer Society estimates that 22,240 women will receive a new diagnosis of ovarian cancer and that 14,030 women will die from this disease, making ovarian cancer the most lethal gynecological malignancy in the United States (1). Of these newly diagnosed cases, 80% of the serous ovarian carcinomas are diagnosed at late stage, for which the 5-year survival rate is only 9–35% (2). Despite advancements in technology, this poor survival rate has been consistent over the last 30 years, an indictment of the complexity of this disease. In order to combat this clinical challenge, it is imperative to generate robust early detection methods and novel treatment options.

Many of the characteristics confounding the study of ovarian cancer arise from the disease's heterogeneity. Ovarian tumors can arise from three different cell types; epithelial, germ, and sex cord stromal cells, with epithelial accounting for approximately 90% of all ovarian cancers (1). Epithelial tumors are further grouped into different tumor types: Type I and Type II. Type I tumors include low-grade serous carcinoma, low-grade endometrioid carcinoma, mucinous carcinoma, and a subset of clear cell carcinomas, which develop in a stepwise fashion from well-recognized precursors, in most cases, borderline tumors (3–5) (**Figure 1**). These tumors are slow to develop and are generally confined to the ovary (6). Type I tumors are also genetically stable, with each histologic subtype corresponding to a distinct genetic profile (4–6). In contrast, Type II tumors encompass high-grade serous carcinoma, undifferentiated carcinoma, malignant mixed mesodermal tumor (carcinosarcoma), and some clear cell carcinomas (3) (**Figure 1**). High-grade serous carcinomas are the most common Type II tumor. These tumors progress rapidly, harbor *TP53* mutations, and exhibit widespread DNA copy number alterations (3–7).

This new appreciation of tumor diversity and the rapid development of genomic technologies have helped redefine "ovarian cancer." As the field grapples with these emerging concepts, experimental model systems will likely play a vital role in defining new opportunities for early detection and therapeutic intervention. This review will highlight the recent advancements in ovarian cancer genetics and pathology, and explore the past and present model systems employed to study high-grade serous ovarian carcinoma (HG-SOC).

GENETICS

Until recently, neoplastic transformation was thought to be driven by the sequential acquisition of mutations in critical genes. For many epithelial cancers, including Type I ovarian cancer, this is true. The most prominent mutations present in Type I tumors include alterations to *KRAS*, *BRAF*, *PTEN*, *CTNNB1*, and *TGFBR2* (3, 6, 8). However, besides mutations in the *TP53* tumor suppressor gene and the *BRCA1* or *BRCA2* genes, very few recurrent somatic mutations have been associated with the more aggressive Type II tumors (6). This inability to systematically characterize Type II tumors was addressed by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) in the creation of The Cancer Genome Atlas (TCGA). In the TCGA's pilot study of HG-SOC, microarray analyses and new sequencing technology were used to publish the largest and most comprehensive genetic analysis of HG-SOC. The study encompassed mRNA expression, microRNA expression, DNA copy number, and DNA promoter region methylation for 489 HG-SOC and whole exome DNA sequence information for 316 of these samples (7).

Results from the initial TCGA study characterized HG-SOC as having *TP53* mutations in nearly 100% of tumors and identified low prevalence but statistically significant recurrent somatic

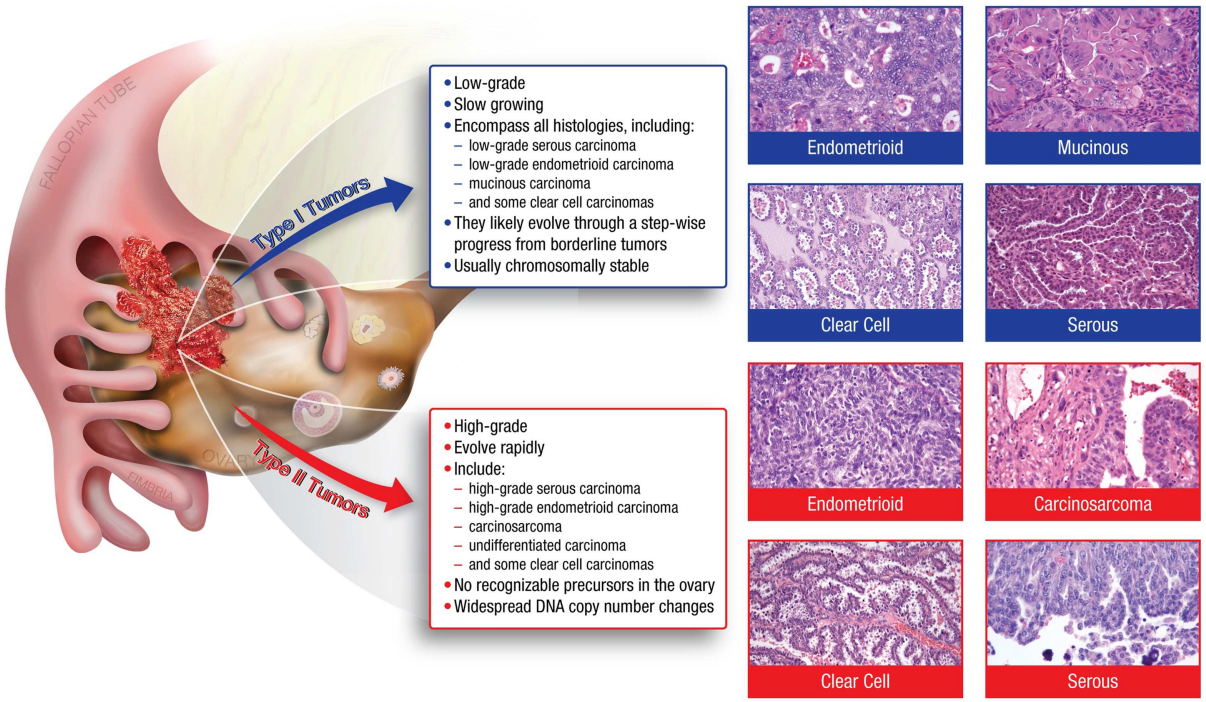

FIGURE 1 | The major histologic subtypes of ovarian cancer fall into two subclassifications. Type I tumors are low-grade, slow growing carcinomas that typically arise from well recognized precursors lesions (borderline tumors) that themselves develop from the ovarian surface epithelium, inclusion cysts, or endometriosis. In contrast, Type II tumors are high-grade and rapidly growing carcinomas. Typically, they have spread well beyond the ovary at the time of diagnosis.

mutations in nine additional genes including *NF1*, *BRCA1*, *BRCA2*, *RB1*, and *CDK12* (7). TCGA also described 113 DNA copy number alterations and implicated 168 genes involved in promoter methylation events (7). Considering the widespread DNA copy number aberrations observed across HG-SOC, it has been suggested that disruption of DNA repair pathways followed by chromosome instability is a viable model for the early progression of HG-SOC (9, 10). The TCGA provides an expanding database that is useful in identify high impact genes. However, because the TCGA studies the advanced state of HG-SOC, determining whether these genes are important to transformation, or instead are related to tumor maintenance, immune evasion, anti-apoptosis, and/or chemoresistance, requires further investigation.

PATHOGENESIS

Historically, ovarian cancer was believed to originate from the ovarian surface epithelium (OSE), where ovulation, follicular rupture, oocyte release, cytokine exposure, and reactive oxygen species introduce DNA damage into the ovarian epithelial layer (11, 12). Proposed back in 1971, the Fathalla "incessant ovulation" hypothesis (13) suggests that over a woman's lifespan, the accrual of DNA damage and the development of cortical inclusion cysts (CICs) results in Mullerian metaplasia of the coelomic epithelium followed by neoplastic transformation (14, 15). This hypothesis attempts to explain the presentation of coexisting serous and non-serous tumor subtypes within ovarian tumors and incorporates the epidemiological data linking ovulatory activity with risk of ovarian cancer (16). However, while precursor lesions have been identified in the OSE that are linked to Type I tumors (17), reproducible pre-malignant lesions have been difficult to identify in the OSE for the high-grade Type II tumors.

A more recent analysis compares the major subtypes of ovarian carcinomas to tumors arising in the fallopian tube, endometrium, and endocervix. Evidence suggests that benign structures derived from these anatomic locations may serve as sites of origin for all tumors that have traditionally been regarded as of primary ovarian origin. Such epithelial structures, which include endosalpingiosis, endometriosis, and endocervicosis, represent non-neoplastic counterparts of serous, endometrioid/clear cell, and mucinous ovarian carcinomas, respectively, and are referred to as extra-uterine Müllerian epithelium (EUME) (15).

The most significant studies supporting the concept of EUME are those implicating the fallopian tube fimbria as the site of origin for high-grade serous carcinomas. Early studies of fallopian tube carcinomas noted *TP53* and *interleukin 6* (*IL-6*) mutations (18, 19). However, a link to ovarian cancer was not proposed until pathologists systematically analyzed fallopian tubes from women carrying mutations in the *BRCA1* tumor suppressor gene. These studies identified preneoplastic lesions localized to the tubal fimbria (20–22), where they displayed secretory cell histology, DNA damage, mutations in *TP53*, and stable p53 protein expression (20, 23). This evidence suggests that HG-SOC tumor progression within the fallopian tube fimbria begins with *TP53* mutations (*p53* signatures), evolves to serous tubal intraepithelial carcinoma

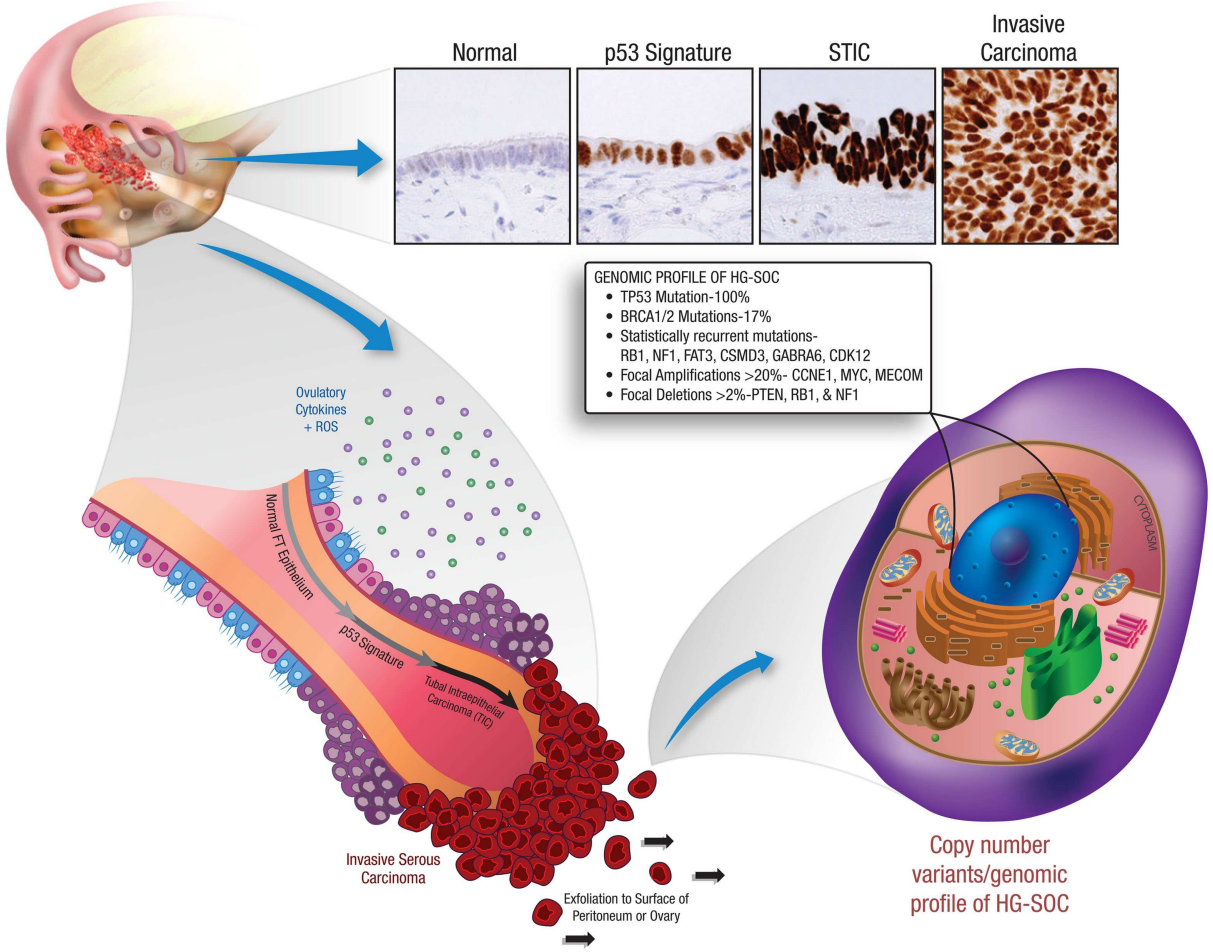

FIGURE 2 | Early tumor progression within the fallopian tube and the resultant genetic profile of HG-SOC. This illustration depicts the recently identified precursor lesions of HG-SOC that are present in the fallopian tube. Mutations in the *TP53* tumor suppressor gene are a very early event in the pathogenesis of HG-SOC, occurring exclusively in benign-appearing secretory cells. These preneoplastic lesions are referred to as 'p53 signatures'. Acquisition of a neoplastic phenotype and proliferative capacity results in the development of serous tubal intraepithelial carcinoma (STIC). Breaching of the basement membrane and localized dissemination to the ovary and/or peritoneal cavity heralds the development of invasive HG-SOC and the associated clinical scenario. HG-SOCs that involve the ovary or peritoneum are characterized by mutations in *TP53* (and *BRCA1* in familial cases) and display a complex genomic terrain with widespread copy number alterations throughout the genome.

(STIC), and eventually transforms and metastasizes to the ovary presenting as HG-SOC (20, 24) (**Figure 2**).

EXPERIMENTAL MODELS OF HG-SOC

Experimental models systems in ovarian cancer biology have evolved significantly over the past 10–15 years. Today there exist a number of useful models that continue to advance translational research in ovarian cancer (**Figure 3**). It is beyond the scope of this mini-review to address all available experimental models. However, in order to demonstrate the utility and evolution of these research tools, a select few of these models will be discussed.

XENOGRAFTS

Xenograft models are possibly the most utilized experimental platform in the field of cancer research. Early developments of this model were reported in the late 1960s when Rygaard et al. found that mice suffering from recessive thymic aplasia could grow mammary and colon xenografted carcinomas (25). This effectively spawned the immunocompromised rodent model, which, due to its ease of application and histological insights, provided an extensive tool to study ovarian cancer tumorigenesis, chemotherapeutics, and biomarkers (26–29).

Despite its utility, questions still remain whether compromising the rodent immune system affects the tumor microenvironment. Various studies have shown that cell lines implanted in immune-compromised mice can lose their histological fidelity (30–33). Likewise, monitoring disease formation and progress is also complicated with immune-compromised mice, as disease and infection rates increase when mice are handled outside their protective environment. To combat this, a small number of syngeneic models have been developed (34, 35). However, this digression from

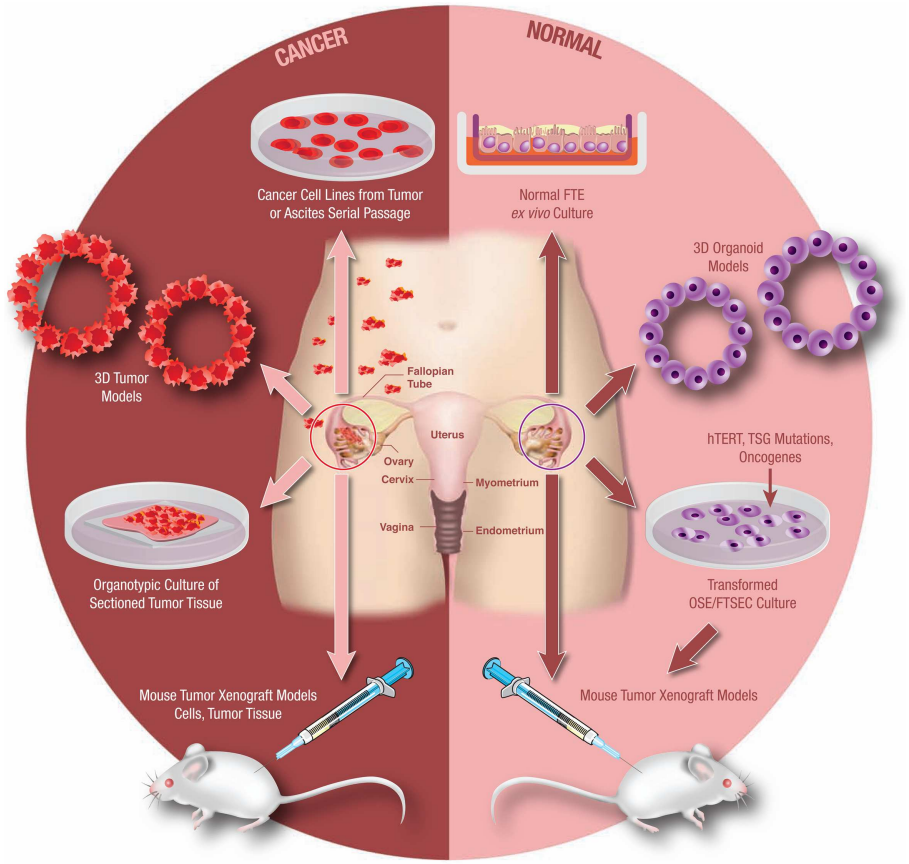

FIGURE 3 | Model systems from primary human tissues. An array of experimental model systems spanning *in vitro* and *in vivo* approaches have been developed to study ovarian cancer. The models include platforms to interrogate the biology of cancer cells as well as for the study of benign epithelium. The expansion beyond traditional two-dimensional (2D) cell culture into 3D and organoid cultures has yielded important insights into the biology of this disease, as has the development of unique animal models. Development of these models is critical as our understanding of this cancer continues to evolve.

human disease presents its own complications when translating experimental results into the clinical setting.

The location of tumor formation and its histologic fidelity to the human disease is also a concern when using xenograft models. The bursal membrane in rodents encapsulates the ovary and creates a unique microenvironment unlike the human equivalent. By acting as a barrier to the peritoneal cavity, the bursal membrane could hinder the development of these tumors (36). In addition, the anatomy of the murine reproductive system departs from that of humans and contains a bicornuate uterus with the fallopian tubes embedded in the aforementioned bursa. Furthermore, the intermittent ovulatory cycle of the mouse corresponds to its rare development of spontaneous ovarian carcinomas (37).

Even with these limitations, xenograft models are still important in translational research and have broad utility. All drug treatments must show promise in animal studies prior to investigation in human clinical trials. In addition, because a high priority has been placed on characterizing the early events of HG-SOC, xenograft models can be effectively paired with *in vitro* transformation studies to characterize preneoplastic and metastatic events (**Figure 3**). Karst et al. demonstrated this by confirming the transformative and metastatic potential of fallopian tube secretory epithelial cells (FT-SECs) in nude mice (38). Considering this versatility and practicality, the future use of xenograft models in ovarian cancer research is a certainty.

CELL CULTURE MODELS

OSE models

Prior to 1981, the isolation of untransformed primary ovarian tissue within the laboratory was unprecedented, making it difficult to discern molecular events related to transformation. This changed in 1981 when Adams and Auersperg isolated and transformed rat OSE (ROSE) cells with the Kirsten murine sarcoma virus (Ki-MSV) (39). The impact of this initial study led to the optimization of cell culture techniques (40, 41) and prompted investigators to start creating a vast cell bank for future studies.

Investigators took advantage of this new technology in the early 1990s when a series of studies simulated incessant ovulation through repeated *in vitro* passaging of rodent OSE cells. Investigators found that primary ROSE and mouse OSE (MOSE) cells that had undergone serial propagation exhibited increased proliferative and tumorigenic properties (35, 42, 43). Further analyses

indicated that these transformed cell lines displayed similar proliferative and genomic patterns observed in human tumors. This was the first comparative analysis between a transformed cell line and its primary parental line and provided supporting evidence for the Fathalla Hypothesis.

While these studies were limited to rodent OSE cells, studies involving isolated human OSE (HOSE) cells were also being attempted (44–46). However, unlike rodent OSE cells, HOSE cells have a limited growth potential *in vitro* and require genetic perturbations to increase cellular lifespan (**Figure 3**). In order to achieve immortalization, two important questions require constant attention; what are the pathways critical to immortalization and how can one alter those pathways without disrupting the normal function of the cell? Initially, these genetic perturbations were achieved via retroviral transduction of either the human papilloma virus E6/E7 oncogenes or the simian virus 40 T antigen (*SV40-TAg*) (46, 47). Cell lines generated through this method displayed increased proliferation without tumorigenicity and remained proliferative after multiple passages (46, 47). Additional retroviral constructs targeting *hTERT*, *TP53*, and *RB* have all been shown to be successful in the immortalization of primary HOSE cells (48–52).

The recent development of small interfering RNAs (siRNAs) has had an impact on ovarian cancer research as well. Primarily used to silence genes through the RNA interference pathway (RNAi), Yang et al. used siRNAs to immortalize OSE cells by targeting *p53* (53) and *Rb* (54), while others have used siRNAs to explore the roles of *PTTG* (55), *CD44* (56), and *STAT3* (57). Certain investigators have even looked into siRNAs as a therapeutic agent. Huang et al. showed that by using a lipidoid-mediated delivery of siRNAs targeting *CLDN3*, OVCAR-3 xenografts showed reduced proliferation, metastasis, and tumor growth (58). The benefits of siRNAs include ease of application and more rapid results. However, specificity and cell toxicity have been a concern.

Fallopian tube models

The first fallopian tube epithelial cell (FTEC) culture system, developed for the purpose of studying the susceptibility of this epithelium to neoplastic transformation, was described in 2010 (59). Unlike traditional two-dimensional (2D) submerged cultures, this "*ex vivo*" system allows FTECs to grow at the air-surface liquid interface (**Figure 3**). This in-turn preserves the natural orientation, architecture, polarity, extracellular features, and biological functions of *in vivo* FTECs, including the retention of ciliated and secretory cells (59). Considering these advantages, this model is ideal to explore the stresses of hormone exposure, ovulation, and inflammatory response. In fact, Levanon et al. reported that in response to DNA damage the FT-SECs display delayed DNA repair kinetics compared to their ciliated cell neighbors (59). This makes secretory cells more sensitive to DNA damage and could explain why FT-SEC are susceptible to neoplastic transformation, especially in the absence of key DNA repair proteins like BRCA1 or BRCA2 (9). Despite the strengths of this model, it has two major limitations. First, it is limited by the dependence on fresh primary FT tissue. Second, the *ex vivo* cultures cannot be further propagated in culture. While they remain viable for weeks, they are not a renewable resource.

To alleviate the need for fresh tissue samples, and to create a long term self-propagating cell population, Karst et al. utilized fresh fallopian tube samples to create the first FT-SEC line (38). By transducing *hTERT* and either *SV40-TAg* or an shRNA targeting *p53* and mutant $CDK4^{R24C}$, FT-SECs were able to overcome senescence and apoptosis (38). Further transduction of either *HRAS* or an shRNA targeting the B56γ subunit of protein phosphatase 2A (*PP2A-B56*γ) and *c-Myc* resulted in an increase in proliferation, anchorage independent growth, and tumor formation in implanted nude mice (38).

Jazaeri and colleagues reported similar results by administering an oncogenic retroviral cocktail containing a myriad of known oncogenes to primary FT-SEC (60). After a period positive selection due to proliferative advantages, the genetic profile of transformed FT-SECs was determined. Increased *c-Myc*, *HRAS*, *hTERT*, and *SV40-TAg* transgene expression and protein accumulation was observed. Further experimentation showed that *hTERT* and *SV40-TAg* expression was sufficient to overcome senescence without tumor formation in nude mice (60). This confirmed the findings of Karst et al. showing that FT-SECs are a possible source for HG-SOC.

Further confirmation of these initial results was reported by Shan et al. These investigators immortalized human FT-SECs by overexpressing *hTERT* and *SV40-TAg* (61). However, when they transduced the cells with oncogenic *HRAS* and implanted them into nude mice, they observed tumor formation that resembled poorly differentiated mucinous adenocarcinomas rather than HG-SOC (61). This is consistent with recent reports showing that Type I, low-grade tumors can emerge from the fallopian tube as well (62, 63).

Recently, FTEC models have even stepped outside traditional human cultures and expanded to baboons and pigs. A recent study used baboon FTECs immortalized with *SV40-TAg* to study the effect of ovulation on FTEC proliferation (64). Likewise, porcine oviductal epithelial cells were used to optimize *in vitro* cell culture conditions to maintain *de novo* FTEC morphological features, i.e., secretory and ciliated cells (65). These new methods could prove useful as investigations into the FTEC continue to increase.

Conditionally reprogramed cells

An alternative to transgene immortalization is a newly developed technique where epithelial cells are "reprogramed" into a stem cell state through conditioned media. Schlegel et al. has been able to show that primary human prostate, liver, lung, and breast epithelial cells, when co-cultured with irradiated fibroblast feeder cells in the presence of the rho-kinase inhibitor Y-27632, can undergo unlimited expansion without senescence or apoptosis (66). This increase in cell proliferation is accompanied by the up regulation of stem cell markers and a decrease in Notch signaling (66). Even more intriguing is that this phenotype is reversible. The removal of Y-27632 and feeders results in the re-differentiation of cells accompanied with their natural polarity and orientation (66). Similarly, Ince et al. showed that human mammary epithelial cells (HMECs) are able to grow indefinitely in a serum-free, chemically defined medium termed WIT (67). The optimization of these techniques for either OSE cells or FT cells would be ideal and may eliminate transgene manipulations and reduce potential off-target effects.

Genome engineering

Despite its successes, certain drawbacks to retroviral transduction and RNAi systems, like oncogenic effects, toxicity, and off-target effects, have prompted investigators to develop targeted genome editing systems. The application of custom DNA-binding proteins, like transcription activator-like effector nuclease (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/cas genome editing systems, have produced a flurry of papers within the last few years. TALENs use a restriction enzyme engineered to recognize specific DNA sequences through the fusion of a TAL effector DNA-binding domain (68). Once a gene is targeted, double strand breaks (DSBs) are introduced and non-homologous end joining occurs (68). However, TALENs are expensive to develop and suffer from off-target effects. The CRISPR/cas system provides a cheaper alternative and works in a similar manner. By utilizing endonucleases that use dual-RNAs for site-specific DNA cleavage, investigators are able to exploit the CRISPR/cas system for RNA-programmable genome editing (69). This had been shown to be very effective and site-specific in controlling gene expression and introducing genetic mutations (70). Overall, despite its lack of validation and limited use with ovarian cancer models, promising results should spark interest and new avenues of investigation.

3D cultures

In addition to conventional 2D culture systems, three-dimensional (3D) culture systems have become increasingly common. There are five major types of 3D culture systems: scaffold free for spheroid growth, scaffolds, gels, bioreactors, and microchips. In these settings, investigators concentrate on creating a more realistic environment where cells of interest can interact with surrounding tissues (71, 72). This seems relevant as studies show that differences in chemosensitivity, cell invasion, and protein expression exist when epithelial ovarian cancer cells are cultured in either 2D or 3D conditions (73–76). Difficulties associated with these models include cell removal, gelling variations, cost, and commercial availability, although the further optimization of these techniques should yield a host of useful tools.

ANIMAL MODELS OF OVARIAN CANCER

Genetically engineered mouse models

In contrast to cell culture platforms, which rely on an artificial environment, genetically engineered mouse models are an efficient alternative for genetic modification and tumor observations *in vivo*. This is important, as questions regarding the identity of cell lines and the selective pressures of cell culture systems continue to surface (77, 78). In addition, investigators have a broad range of techniques to introduce genetic alterations in a temporal or spatial-dependent manner. These methodologies employ transgenic elements, RNAi technologies, and viruses to create both loss of function and gain of function traits within mice.

Limitations to these models include random integration of transgenic elements, limited tissue specific promoters, and difficulties achieving both spatial and temporal control simultaneously. In addition to experimental difficulties, it is also challenging to accurately mimic the human disease in rodents. For example, mice require fewer genetic alterations for tumor induction compared to humans (79–81). Furthermore, rodent tumors that are produced from defined genetic mutations do not always resemble their human counterparts (79–81). The *HRAS* oncogene is a prime example of this anomaly. Hamad et al. showed that the mechanisms of Ras-induced transformation in mice differ when compared to the mechanism of Ras-induced transformation in humans (79). By systematically comparing the murine and mammalian transformation pathways investigators highlighted a critical disadvantage to non-human model systems; the genetic and molecular disconnect between animal models and human disease. However, the ability to validate gene function and test novel therapeutics in a relevant microenvironment, when paired with relevant human studies, still makes these models especially useful (82).

Mouse OSE

Like other model systems, the initial ovarian cancer mouse models focused on the OSE (5, 83–85). The first ovarian cancer transgenic mouse model was developed in 2002 (86). By inducing the expression of the avian tumor virus receptor A (TVA) through the control of the *keratin-5* promoter, these investigators were able to create a cell population within the mouse that was vulnerable to avian retrovirus infection (86). However, the transient expression of *keratin-5* required OSE viral infection to occur *in vitro* with subsequent transplantation. Despite this drawback, infection with different combinations of *c-Myc*, *AKT*, and *KRas*, produced tumors in OSE cells harvested from *TVAp53*$^{-/-}$ mice and provided the first successful transgenic analysis of ovarian cancer in mice (86).

A more specific promoter, the Mullerian Inhibitory Substance Type II Receptor (MISIIR), was later identified and used by Connolly et al. to drive gynecological tissue specific transgene expression of *SV40-TAg* in mice resulting in the formation of ovarian carcinoma in 50% of the transgenic founders (87). However, aggressive tumor formation prohibited the study of early stage tumors and prevented reproduction. Additional studies utilizing the MISIIR promoter explored the oncogenic properties of *PTTG* and *PIK3CA*, however both had difficulties producing tumors (88, 89).

Rather than identify a specific promoter, some investigators have employed the Cre-loxP method to deliver specific genetic alterations (90). Administration of the Cre recombinase can be achieved either through injection of a viral vector (AdCre) or by crossing with a mouse generated to express the protein. This model is a clever way to circumvent problems inherent to typical transgenic models and has been used study *TP53* and *Rb* (91), *KRas* and *PTEN* (92), *PTEN* and *APC* (93), and *BRCA1* (94) within the context of ovarian cancer.

Most recently, Flesken-Nikitin and colleagues applied the AdCre system to perturb *p53* and *Rb* in a stem cell niche in the transitional zone of the bursal cavity of mice. With *p53* and *Rb* inactivated, these stem cells in the hilum region showed the earliest signs of transformation (95). However, perhaps the more interesting aspect of this study was the reporter mouse developed to characterize the fate of the hilum stem cells. A stem cell marker (*LRG5*), specific to the hilum region, was used to drive specific expression of Cre*ERT2*. In turn, subsequent tamoxifen (TAM) administration created a traceable knocked-in fluorescent probe.

Results indicated that hilum cells do have the potential to repopulate the ovarian surface and suggest that stem cell niches could contribute to HG-SOC (95). Whether the hilar cells are OSE cells, or a different cell type altogether, remains to be seen. It is also worth noting that, since there is no bursa surrounding the human ovaries, it is not clear whether there is an equivalent structure or cell type in humans.

Overall, the mouse models developed thus far have focused on the OSE and some have exhibited difficulties with tumorigenicity, female reproduction, anatomical anomalies, and transient expression. Likewise, while these models have offered insight into genes that are important to transformation, they have not provided insight into HG-SOC preneoplastic lesions as such lesions have yet to be identified in the ovary. We anticipate that animal models that target the fallopian tube secretory cell will provide additional insights.

Mouse fallopian tube

The first mouse model targeting the extra-ovarian Mullerian epithelium was developed by Miyoshi et al. By exploiting the promoter of the murine oviduct-specific glycoprotein, Miyoshi was able to drive expression of the *SV40-TAg* in the oviduct, uterus, vagina, and ovary. Except for the ovary, subsequent tumor formation throughout the female reproductive tract was observed (96). Tumor formation was reduced in ovariectomized mice, but when estradiol was injected subcutaneously a dramatic increase in hyperplasia of the extra-ovarian Mullerian epithelia was observed (96). This suggests that ovarian cancer could originate outside the ovary, and that these preneoplastic lesions are highly reliant on hormone regulation pathways involving the ovary.

More recently, Kim et al. disabled *DICER* and *PTEN* using the anti-Mullerian hormone receptor type 2-directed Cre (*Amhr2-Cre*) (97). HG-SOC with aggressive metastasis was observed in these mice resulting in 100% death. In addition, the fallopian tube displayed the earliest lesions and cancer was prevented when the fallopian tube was removed at an early age (97). However, the first signs of increased proliferation within the fallopian tube appear to reside in the stromal compartment, counterintuitive to the epithelial properties presented in the advanced HG-SOC. Equally vexing was the low *p53* expression in mouse tumors, a protein known to be mutated and highly expressed in almost 100% of human tumors (7).

Other animal models

An alternative to the mouse model, which has dominated the field since its initial use, is the domestic laying hen. The hen is the only animal identified to spontaneously develop HG-SOC that is histologically and morphologically similar to human HG-SOC (98). Likewise, because ovarian cancer of the hen presents so many similarities to human ovarian cancer, there are many opportunities to explore early preneoplastic lesions, chemopreventive trials, and perform genomic analyses (99). Disadvantages include a lack of reagents and genetic manipulation technologies that target the hen, as well as anatomical discrepancies (99).

CONCLUSION

Our understanding of ovarian cancer has dramatically changed in the last 10 years. In our search for a cell of origin, our evolving knowledge about the pathogenesis of the disease has led us to sites neighboring the ovary. At the same time, we now appreciate that this is a heterogeneous disease with a complex genomic landscape. In particular, HG-SOCs are marked by surprisingly few recurrent somatic mutations. Instead, this tumor exhibits a complex genome marked by copy number alterations so widespread that few other cancer types mirror its complexity. The challenge now is to elucidate the key alterations related to tumorigenesis, tumor viability, and chemotherapy resistance. In order to achieve this goal, experimental model systems must take center stage and continue to evolve to meet the demanding needs of the scientific community.

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BRCA and early events in the development of serous ovarian cancer

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Women who have an inherited mutation in the BRCA1 or BRCA2 genes have a substantial increased lifetime risk of developing epithelial ovarian cancer (EOC), and epidemiological factors related to parity, ovulation, and hormone regulation have a dramatic effect on the risk in both BRCA mutation carriers and non-carriers. The most common and most aggressive histotype of EOC, high-grade serous carcinoma (HGSC), is also the histotype associated with germline BRCA mutations. In recent years, evidence has emerged indicating that the likely tissue of origin of HGSC is the fallopian tube. We have reviewed, what is known about the fallopian tube in BRCA mutation carriers at both the transcriptional and translational aspect of their biology. We propose that changes of the transcriptome in BRCA heterozygotes reflect an altered response to the ovulatory stresses from the microenvironment, which may include the post-ovulation inflammatory response and altered reproductive hormone physiology.

Keywords: BRCA, fallopian tube epithelium, high-grade serous carcinoma

INTRODUCTION

In 2013, about 22,240 women in the United States would have been diagnosed with invasive epithelial ovarian cancer (EOC) and an estimated 14,000 women with EOC would have died (1). There are five major histotypes of EOC and they are distinct epidemiologically, phenotypically, and molecularly, namely: mucinous, endometrioid, clear cell, low-grade serous, and high-grade serous carcinoma (HGSC). Of these, HGSC is the most prevalent histotype in the Western Hemisphere, the most lethal, typically is diagnosed at an advanced stage, and there are no effective cancer screening strategies. More than 75% of women with this diagnosis will succumb to the disease after combined first line treatment, which includes surgery and adjuvant platinum-based chemotherapy, with a 5-year survival of <30% (1, 2). HGSC is a genetically unstable tumor, characterized by a varied histomorphology unified by marked pleomorphism, a high mitotic rate, and biomarker expression reflective of the most common molecular alterations. The latter includes the near ubiquitous presence of a mutation in the tumor suppressor p53 (TP53), resulting in either over accumulation of p53 protein by immunohistochemistry (missense – 60% of analyzed cases) or complete loss of protein expression (frameshift/splicing junctions/non-sense – 39% of analyzed cases) (3). Mutations of p53 are present in early stage HGSC, and mutant TP53 is likely an essential driver mutation required for the early pathogenesis of HGSC (4). Other recurrent mutations in HGSC are infrequent, but most prominently include BRCA1 and BRCA2, with BRCA germline mutations seen in 13–16% (5), and somatic mutations seen in about 6% of cases.

High-grade serous carcinoma is the predominant histotype associated with hereditary breast-ovarian cancer (6, 7). Women with inherited mutations of BRCA1 or BRCA2, have a lifetime risk of 40–60% (BRCA1) and 11–27% (BRCA2) (8–12). Women known to be at increased genetic risk based on family history and/or genetic testing are offered risk-reducing salpingo-oophorectomy (RRSO), which reduces the risk of malignancy by up to 96% (13, 14) and is usually performed after completion of childbearing and while the woman is still pre-menopausal (13, 15). An unexpected finding on histopathology review of the resected fallopian tubes in this population was the presence of clinically undetected, occult carcinomas in the fallopian tubes, a tissue previously thought to develop carcinomas only rarely. These were seen more frequently than in the ovarian tissues (16). This discovery was followed by careful review of the fallopian tube tissues, and subsequent studies have reported histological lesions purported to be HGSC precursors in the fallopian tube epithelium – these had not been found in the genetic high-risk ovarian tissues (16–22). Hence, detailed histo-pathological examination of the resected ovaries and fallopian tubes in BRCA mutation carriers has led to a radical change in existing paradigms of serous carcinogenesis. Because loss of BRCA function is frequent in HGSC, study of the effect of BRCA, including heterozygosity/haploinsufficiency and loss of function in the fallopian tube epithelium prior to the development of HGSC, offers opportunities to better understand HGSC pathogenesis, and should lead to the development of novel and more effective preventative, and possibly, screening strategies.

BRCA1 AND BRCA2 AND HIGH-GRADE SEROUS CANCER

Molecularly, the breast cancer susceptibility genes (BRCA) BRCA1 and BRCA2 can sense DNA damage and are involved in DNA repair via interactions with RAD51 (23–25); these three proteins are essential for genomic stability in normal cells predominantly through the homologous recombination pathway (HR) (26). BRCA1 is a known modulator of the cell-cycle at the G2-M checkpoint (27) operating through co-activation with p53 (28)

and has also been shown to epigenetically regulate the oncogenic microRNA 155 and to maintain heterochromatin structure via ubiquitylation of H2A (29, 30). Inherited mutations in BRCA1/BRCA2 confer an autosomal-dominant effect and range from being deleterious to protein function to being of uncertain significance (31).

Breast cancer susceptibility gene mutation carriers develop cancers in hormonally regulated tissues, most frequently in breast and ovarian/tubal tissues, but a unifying mechanism of early malignant transformation in these tissues is not known. The BRCA associated carcinomas share some common features including a high-grade phenotype, frequent mutations of TP53, and other copy number landscape features like Cyclin-E amplification and deletion of Rb (32). Altered BRCA function in HGSC does not only occur in the setting of hereditary disease. Dysfunction of BRCA1 or BRCA2 is prevalent in patients with HGSC via 6% somatic mutations (5, 33–35); 13–31% promoter hypermethylation (5, 36–38); 7.9–17% amplification of EMSY (5, 39, 40); or 13.2% promoter hypermethylation of FANCF (41). The sum of these genomic alterations predominantly in the HR pathway of HGSC has led to determining the "BRCAness" profile in patients (42, 43). BRCAness is defined as a phenotype determined by deficiencies in the double strand break (DSB) repair pathways, as seen in tumors associated with germline BRCA mutations and a subset of sporadic high-grade serous ovarian cancers. An understanding of the early molecular changes in genetic high-risk patients may therefore also be of importance to many of the sporadic cancers. Patients with the BRCAness profile most likely will benefit from treatments affecting other DNA repair pathways – specifically PARP inhibitors (43). Outcome data suggests that patients with loss of function of BRCA have improved survival, but recently a study by McLaughlin and colleagues determined that although BRCA mutation carriers have a short-term (up to 5 years post diagnosis) benefit and response to platinum-based therapy, there is a lack of long-term (up to 10 years post diagnosis) survival benefit (44). Most promisingly, the loss of function of BRCA1/BRCA2 whether genetic or epigenetic by mechanisms including promoter hypermethylation, offers the possibility of improved therapies with poly (ADP-ribose) polymerase (PARP) inhibitors (43).

BRCA1 AND BRCA2 AND THE FALLOPIAN TUBE EPITHELIUM

The mechanisms underlying malignant transformation in these estrogen responsive tissues are poorly understood, but likely involve loss of heterozygosity of the remaining wild type BRCA allele (45) in addition to inactivation of p53. During ovulation, it is thought that high levels of reactive oxygen species (ROS) are released via the cytokine surge accompanied with lysis of the ovum (follicular fluid). These species have a complex role in the development and progression of cancer (46). The high ROS levels are likely a source of "carcinogens," which cause DNA damage in the

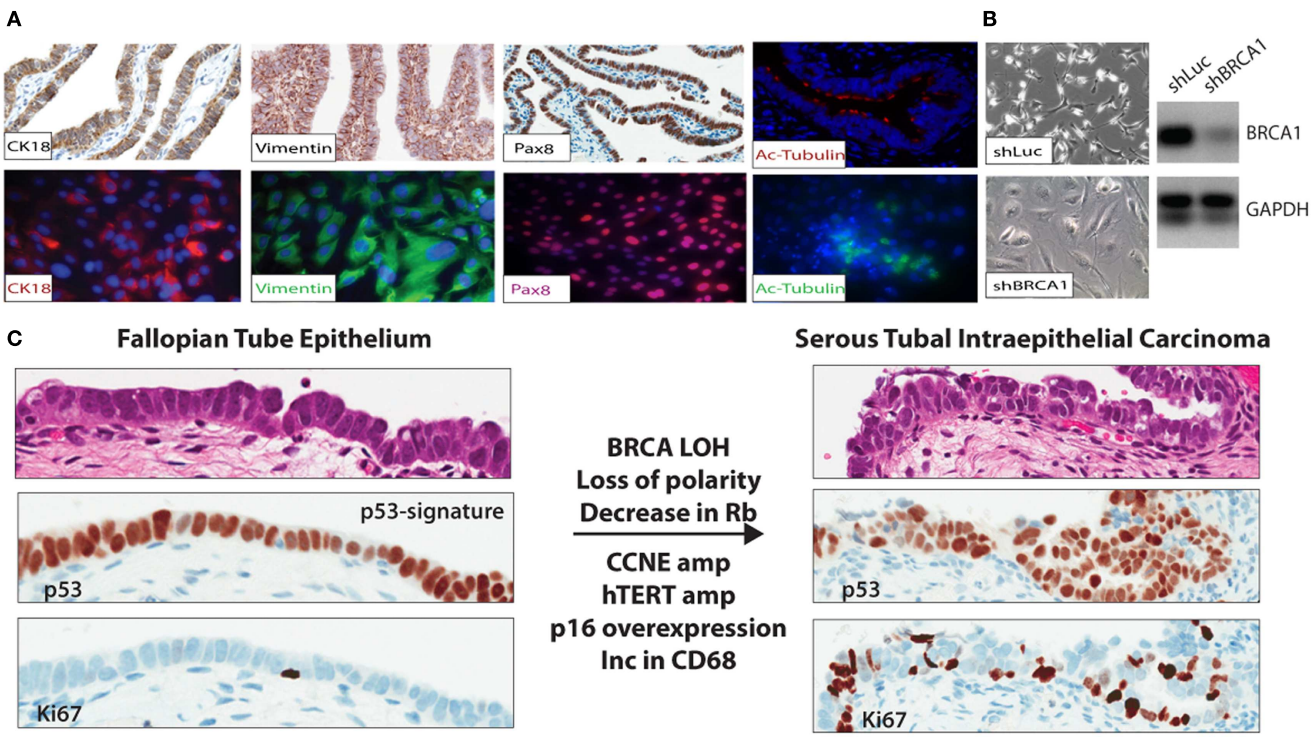

FIGURE 1 | (A) FTE cell lines were established to study gene specific effects in relation to BRCA abrogation in BRCA mutation carriers and other aberrations identified in the precursor lesions and malignant lesions observed *in situ* in the distal end of the FTE. **(B)** FTE cell lines established from normal FTE tissue were infected with a short hairpin to BRCA1 (shBRCA1). The FTE cells with BRCA loss have the classic phenotype of senescent cells – flat, enlarged, and vacuolated. PCR confirmed knockdown. **(C)** In the p53 signature in the normal FTE, low proliferation, normal cell polarity, and over-expression of p53 are observed. Thus far, BRCA loss-of-heterozygosity (in mutation carriers), decrease in Rb, and increase in p16 (immunohistochemistry), CCNE1 amplification, and over-expression (FISH and immunohistochemistry); hTERT amplification (FISH), common in HGSC are also observed in the STIC lesions.

FTE and possibly contribute to the mutations in TP53. In normal cells repair of DNA damage results in cell-cycle arrest through senescence or death as demonstrated in epithelial cell lines established from FTE (**Figures 1A,B**). This process must be overcome for transformation to occur (47). In high-grade serous ovarian cancer cells, 99% of tumors have a mutation in TP53, indicating that the mutation likely occurs early in disease progression (3, 5). This combination – TP53 mutation and BRCA loss, can provide an escape or by-pass through the cell-cycle checkpoints to allow additional cancer promoting mutations, amplifications, or deletions. Therefore, BRCA1/BRCA2 deficient cells [lacking ATM/ATR-CHK2 pathway (48)] cannot sense DNA damage in order to transduce signal to the already TP53 mutant cells. In this setting, cells can overcome the barriers for cell-cycle progression, however this may not be sufficient for transformation into a tumor.

In normal cells of mutation carriers, only one allele is mutated, and BRCA1 function is presumed to be intact. This may however not be true, as evidence in support of BRCA1 haploinsufficiency accumulates. For example, in normal human mammary epithelial cells from BRCA1 heterozygotes, DNA homologous repair is suppressed (49). BRCA1 haploinsufficiency may be an early but not a sufficient step of BRCA1-mediated breast carcinogenesis. In HGSC, it is uncertain when during malignant transformation of FTE, loss of BRCA1 function occurs. In contrast to breast cancer, it seems likely altered p53 function resulting from p53 mutation occurs prior to loss of the wild type BRCA1 allele in FTE transformation. Loss of BRCA1 protein and loss of heterozygosity is seen once malignant transformation has occurred but, according to Norquist et al. not in early precancerous lesions (45). The p53 mutation is thought to promote genomic instability, a hallmark of high-grade serous cancer, and cooperates with BRCA1 loss or a dysfunctional HR pathway to mediate the extent of genomic amplifications and gains so commonly seen in HGSC.

p53 SIGNATURE AND SEROUS TUBAL INTRAEPITHELIAL CARCINOMA

For many years, in the absence of a reproducible histological precursor lesion of HGSC, the cell of origin was presumed to be the ovarian surface epithelium (OSE), a modified type of mesothelium. Detailed histo-pathological examination of tubal epithelia (FTE) in the genetically high-risk population undergoing risk-reducing surgery has led to the discovery of putative cancer precursor lesions in the fallopian tube, some of which, i.e., the p53 signature – described as a string of 10–12 histologically normal secretory (non-ciliated) cells expressing the TP53 protein with a low proliferation rate (Ki67) (50), are found with a similar frequency in BRCA mutation carriers and non-carriers. Two independent studies reported similar findings albeit at different frequencies of p53-signatures between the two study cohorts: 11 and 19% (51) and 24 and 33% (52) in women with germline BRCA mutations and population control, respectively. The cells within the p53 signature are Pax8 positive and up-regulate phosphorylated – γH2AX, reflective of concomitant DNA damage. Women with an inherited mutation in the TP53 gene – the Li Fraumeni syndrome, have an increased risked in developing between five and six different cancers (breast, brain, soft tissue sarcomas, and blood cancers) throughout their lifetime (52). These patients, however, do not have an increased incidence of developing high-grade serous ovarian cancer, but have an increased number of p53-signatures compared to the rest of the population. In addition, in a small epidemiological study, p53-signatures were not associated with the traditional risk factors of breast-feeding, parity and tubal ligation, bringing into questions whether the p53 signature is a true cancer precursor lesion (53). However, it can be said that loss of normal p53 function is necessary, but not sufficient to promote carcinogenesis of epithelial cells in the distal fallopian tube.

Occult invasive carcinoma and serous tubal intraepithelial carcinomas (STICs) were identified in the fallopian tubes of mutation carriers undergoing risk-reducing surgery, with an incidence of about 4–6% for occult cancers (16, 54, 55). Importantly, STICs are found not only in BRCA mutation carriers, but are also detected in about 60% of sporadic HGSC (19, 56). STICs are thought to have progressed from the p53 signature and are characterized as being highly proliferative (>10% Ki67) (57), show loss of apical to basal nuclear polarity and, in common with HGSC, demonstrate: over-expression of cyclin-E (58), amplification of hTERT (59), p16 over-expression (CDKN2A), loss of Retinoblastoma protein (Rb) (60), and up-regulation of the PI3K pathway (61) (**Figure 1C**). In mutation carriers undergoing RRSO, STICs were identified in at least 8% of cases, a higher frequency than seen in patients at low genetic risk (51, 52, 62, 63).

Like HGSC, the frequency of STIC lesions increases with age, is increased in BRCA1/2 mutation carriers, and is lower with oral contraceptive use, all features providing further evidence that STIC is an immediate precursor of invasive and clinically detectable carcinoma (53). These intraepithelial carcinomas should not be considered as only *in situ* carcinomas, because in at least some cases while tumor cells do not invade underlying stroma, they can detach, and because of the accessibility of the ipsilateral ovary and other peritoneal surfaces to the tubal fimbria, cells may implant and establish tumor growth in other sites. Currently, little evidence exists that patients with only a diagnosis of STIC require adjuvant therapy (64). Further molecular and genetic characterization of STIC is ongoing, but molecular evidence to date indicates that alterations commonly seen in HGSC are also present in STIC. Lesions that precede the STIC, are not well characterized, but currently the term serous tubal intraepithelial lesion (STIL) is given to lesions according to criteria recommended in a proposed diagnostic algorithm. The STIL is described as a lesion, which has abnormal p53 expression by immunohistochemistry and increased proliferation relative to background (tubal epithelium) but <10% Ki67 positive. (57, 65). Ongoing studies are required to further define this lesion as current definitions lack diagnostic reproducibility. Other than the changes associated with the p53 signature, molecular changes which precede the establishment of an intraepithelial cancer are not well documented. Indeed, these lesions are uncommon, and identified with poor reproducibility.

NORMAL TUBE EPITHELIUM IN BRCA MUTATION CARRIERS

Hormonally responsive epithelia, from breast, ovary, and fallopian tube, are the preferential targets for malignant transformation in BRCA1/2 mutation carriers and the mechanisms are increasingly being determined primarily through studying breast epithelia (66). Evidence is emerging nonetheless that morphologically normal

fallopian tube epithelium from women with inherited mutations, differs significantly from the tubal epithelium of women at low cancer risk. Differences in morphologically normal epithelium from BRCA mutation carriers have shed light into the effects of heterozygosity and predisposition to high-grade serous ovarian cancer. In five previous reports, we have used morphologically normal fallopian tubal epithelium from BRCA1 and BRCA 2 (FTE-BRCA) mutation carriers and non-mutation carriers (FTE-normal), to compare gene expression profiles to identify differences conferred by the presence of one mutant allele (67–71). In addition to family history, a major risk factor is number of lifetime ovulations, and oral contraception use and increase in parity lead to a reduction in EOC risk (72). The formerly prevailing incessant ovulation hypothesis first described by Fathalla suggested that continuous disruption and surrounding inflammation of the OSE during ovulation led to the development of carcinoma in the ovary (73). It is likely however that the effects of ovulation are still important in malignant transformation, but the effects are on fimbrial, not ovarian, epithelium.

Therefore in the design of experiments, the patient tissues analyzed were controlled for not only age and menopause but also stage in the ovarian cycle – follicular (proliferative phase) and luteal (post-ovulatory phase) at the time of surgery (70, 71). We showed that the BRCA mutation in morphologically normal fallopian tube epithelium confers a significantly altered gene expression signature. Some of these altered pathways include the TGF-β pathway, MAP kinase pathway, the adipokine signaling pathway, inflammatory pathway, and the p53-signaling pathway (70, 71). In particular genes involved in DNA damage and inflammation were validated as both having transcriptional and translational differential expression in the normal fallopian tubes (ampulla and fimbria) of BRCA mutation carriers. Namely, DAB, NAMPT, C/EBP-δ, GADD45β, and NF-κB are genes involved in the Jak/Stat, DNA damage, and TGF-β pathways and are prominently differentially expressed in mutation carriers and in HGSC. In these studies, we noted, that BRCA mRNA levels were not substantially different between carriers and non-carriers, indicating that the wild type allele was still intact. In an independent study, Press et al. reported significant differences in proliferation and cell-cycle regulation in BRCA mutation carriers (with and without occult carcinoma) (74).

We subsequently analyzed the distal end FTE (the fimbria), the anatomical region of highest risk and the ampulla for: (1) the presence of immune infiltrates ($CD3^+$ and $CD8^+$ lymphocytes and $CD68^+$ macrophages) and (2) the proliferation status of FTE cells in both BRCA mutation carriers and population control. This study although not exhaustive, revealed that independent of BRCA mutation status: (1) macrophages were more prevalent in the luteal phase than the follicular phase of the ovarian cycle and (2) proliferation in FTE cells is predominantly an effect of the follicular phase rather than BRCA mutation status in histologically normal tissue (**Figure 2**). However, a small subset of FTEs

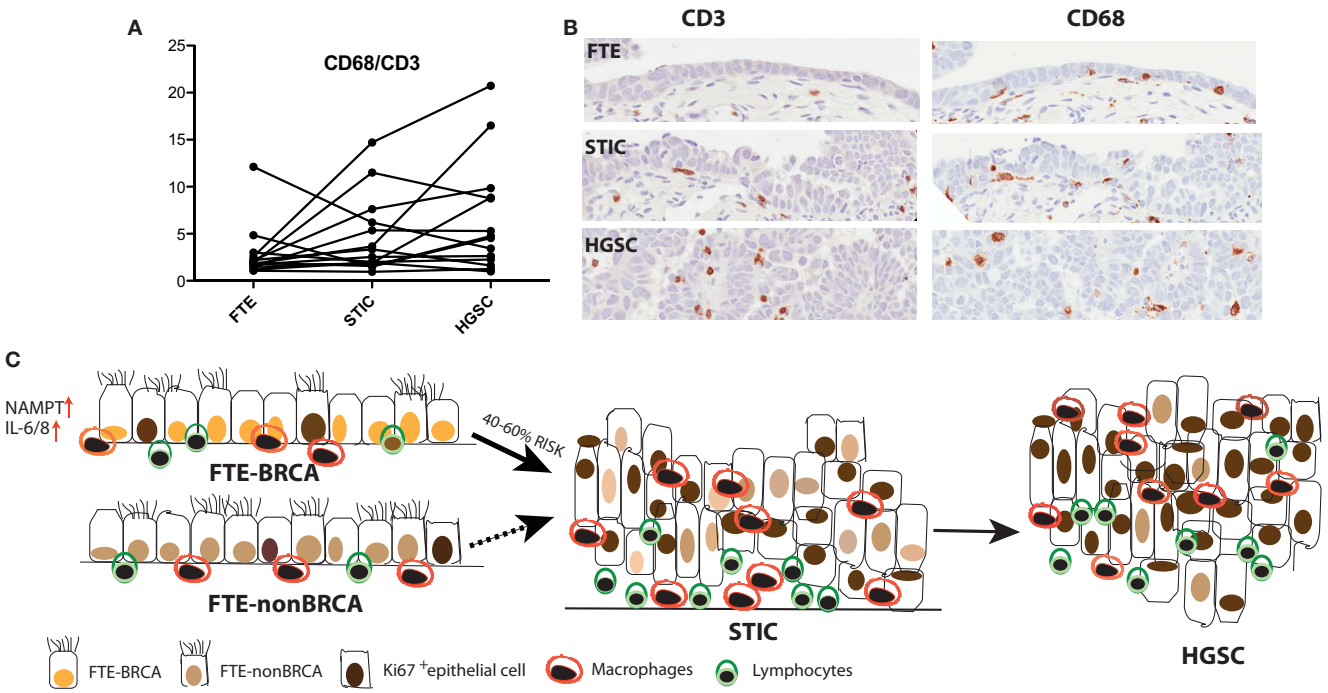

FIGURE 2 | (A) A trend is observed, where in some cases the ratio of $CD68^+$ macrophages to $CD3^+$ lymphocytes increases from the normal FTE to STIC to the concomitant cancer. **(B)** Immunohistochemistry of $CD3^+$ and $CD68^+$ in normal FTE, STIC (same STIC as depicted in **Figure 1**), and HGSC. **(C)** High-grade serous carcinoma is the most common type of ovarian cancer and women with BRCA1/2 mutations have a 40–60% increased lifetime risk for developing the disease. Interrogation of the normal FTE microenvironment demonstrates that there is no inherent difference in proliferation or in some immune cell populations within the histological normal tissue in FTE of BRCA1/2 mutation carriers compared to the normal population. An increase in proliferation and lymphocytes and macrophages occurs later in tumor progression when the FTE have already lost cell-cycle progression barriers and there is histological evidence of a precursor lesion.

from BRCA2 mutation carriers had a diffuse increase in proliferation in the absence of histological lesions, but overall there was no statistical difference in proliferation compared to the control tissues (68). Therefore, we propose that chronic inflammatory states through cyclical ovulation and the presence of a mutated BRCA allele can predispose the normal FTE to develop lesions, which may lead to serous carcinoma. We hypothesize that this occurs through deregulation of DNA damage response genes and synergistically through up-regulation of cytokines, pro-inflammatory, and proliferation genes. It is possible that changes demonstrated in gene expression profiles reflect the earliest alterations in cancer development, and/or that they are markers of increased cancer risk.

OVULATORY CYCLE AND BRCA IN THE FALLOPIAN TUBE EPITHELIUM

Most women who develop sporadic cases of EOC are peri- or postmenopausal with a mean age of 58 years (75); however, BRCA1 mutation carriers develop the disease earlier with a mean age of 51 years and BRCA2 mutation carriers a bit later, with a mean age of 57 years (75–78). In addition to family history, the major epidemiological risk factors for EOC indicate a strong influence of reproductive factors and reproductive hormones. Risk factors including nulliparity, early age of menarche, late age of menopause, hormone replacement, obesity, and protective factors including oral contraceptive use, indicate an association with increased lifetime ovulations and/or greater lifetime exposure to estrogen. A higher risk of ovarian cancer has been reported with cyclical use of hormone replacement therapy rather than continuous use or any use of estrogen or progestin after menopause (79) for both BRCA mutation carriers and non-carriers (72).

The influence of sex hormones on tubal/ovarian malignant transformation is not well understood, but seems likely that the BRCA1/2 associated changes in reproductive hormones and their receptors play a role in tumor formation, in addition to the alterations in DNA damage repair. BRCA1/2 mutation carriers do not have menopause at an early age (80). Higher circulating estradiol is associated in the general population with a pre-menopausal breast cancer risk, and BRCA2 carriers with breast cancer do have higher estradiol levels in the early follicular phase, but a similar association with circulating progesterone is not seen. It has recently been shown that mutation carriers have higher levels of both estradiol and progesterone during the luteal, not follicular phase, leading the investigators to suggest a defect in steroid hormone regulation potentiates the mutagenic effect of the BRCA mutation (80, 81). In mice, it has been shown that granulosa cells in mice lacking functional Brca1 are exposed to increased estradiol stimulation due to a combination of a prolonged pre-ovulatory (proestrus) phase of the estrus cycle and increased levels of circulating estradiol. In addition, estrogen biosynthesis in granulosa cells is altered in mice not only with a deleterious homozygous mutation but also in mice with a heterozygous Brca1 mutation (82), a state which mimics the BRCA1 mutation carriers. This provides further evidence that heterozygous BRCA1 mutations are associated with phenotypic changes.

The role of estrogen and progesterone in early malignant transformation in the FTE is not yet well understood. Estrogen mediates its action primarily through the estrogen receptor (ERα and ERβ). Estrogen stimulates the expression of a number of genes that promote cell proliferation, motility/invasion, and inhibition of apoptosis namely: IL6, TGF-α, EGF, PI3K/Akt, IFG-1, and Bcl-2 (which is predominantly expressed in secretory FTE) (78). The estrogen dominant phase during the ovarian cycle is the follicular (or proliferative) phase and is associated with an increase in FTE proliferation (68) and promotion of ciliogenesis (83). In contrast, progesterone receptor activity is associated with a decrease in cell proliferation (68), an increase in apoptosis, possibly mediated through the down-regulation of CDK1/cyclin B1 complex, which impedes the G2/M transition. Conversely, in the breast, it is known that progesterone elicits proliferation through Cyclin D1 in PR positive cells (a cell intrinsic autocrine loop) and in PR negative cells via NF-κB ligand RANKL secretion (paracrine) (84, 85). Progesterone mediates its activity through the progesterone receptors (PR-A and PR-B are isoforms with differential translational start sites). On progesterone binding PR translocates to the nucleus to direct an antagonist effect on ERα signaling. Both ciliated and secretory FTE cells express the estrogen and progesterone receptors (69) and undergo cyclic changes in growth and differentiation throughout the ovarian cycle; these changes are most evident in the fimbriae (86) the "high-risk" zone of the tube (86). The fallopian tube epithelia in the luteal phase of the ovarian cycle have significantly lower levels of the progesterone (PR-A) (69) and estrogen [ERα) receptors (87)] (**Figure 3**).

During ovulation, there is a surge of estrogen released into the FTE microenvironment with release of follicular fluid, which contains high estrogen levels. This effect might be exacerbated (88) in fallopian tube epithelia of BRCA mutation carriers under the direct influence of the relevant DNA repair pathways, which are potentially dysfunctional. In addition to its well-established roles in regulation of DNA damage response, the Brca1 protein inhibits ERα transcriptional activity through direct action of BRCA1 and ERα proteins and down-regulation of p300, a nuclear receptor co-activator (89). Brca1 protein also regulates estrogen receptor action through suppression of aromatase, the enzyme required for estrogen biosynthesis from androgen. Gorrini et al. recently showed that an antioxidant estrogen target gene – Nrf2, can mediate a pro-survival effect in the absence of normal BRCA1 protein, in which cells would otherwise undergo cellular senescence or death (66). BRCA1 loss in mammary epithelium therefore alters the estrogenic growth response, and increased estrogen signaling collaborates with Brca1 deficiency to accelerate preneoplasia and cancer development. Although this has not been tested in FTE, this is an interesting concept that may have implications in serous carcinogenesis.

A decrease in the transcription and translation (by immunohistochemistry) of PR-A and PR-B were observed in the luteal phase of both BRCA mutation carriers and population control (69). PR gene signatures were identified in a subset of FTE cases in the luteal phase that had a similar profile to HGSC, however, PR target genes were not differentially expressed between BRCA mutation carriers and controls (69). In HGSC, PR expression is predominantly decreased/lost, a finding in 70–80% of patients (69, 90). PR expression in greater than 50% of tumor cells has been recently reported to have an overall survival benefit, and this benefit was independent of germline BRCA1/2 mutation status

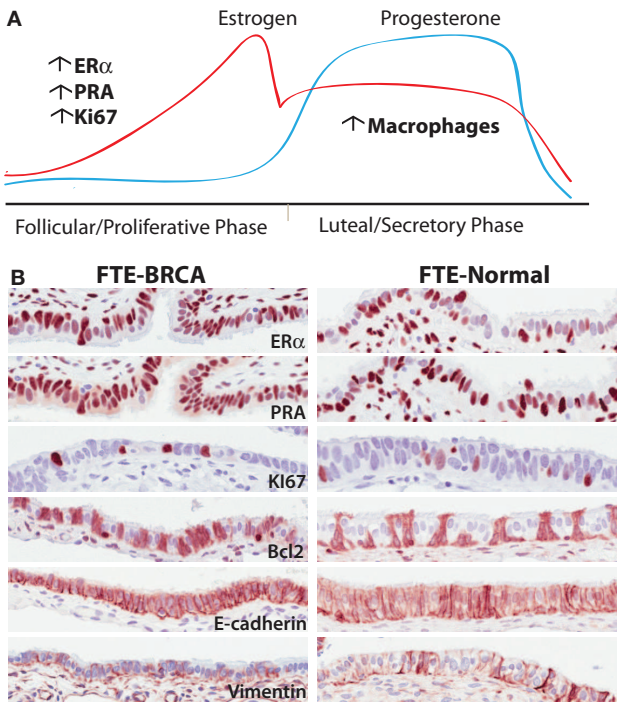

FIGURE 3 | (A) The different phases of the ovarian cycle in pre-menopausal women is the dominant effect on gene transcription in epithelia of normal fallopian tubes in BRCA mutation carriers. Translationally, there are more cells expressing ER, PR in the follicular phase of the cycle independent of mutation status. Similarly there are significantly more cells responding to the mitogenic effects of estrogen observed by an increase in proliferation. In the post-ovulatory phase, there is an observed increase in macrophages in both BRCA mutation carriers and non-carriers. **(B)** Representative images of proteins expressed in normal FTE-BRCA and FTE-normal that seemingly look and express these proteins similarly. Underlying these morphological similarities is a potential haploinsufficiency predisposing FTE-BRCA to cytotoxic stresses.

(90). In contrast, 70–80% of HGSC patients express ERα (>50%) but ER expression has not been shown to be associated with a significant recurrence free progression or survival benefit (87, 90).

Epidemiological data indicate that HGSC risk is closely linked to the events of ovulation, and these risk factors and protective factors for the most part are true for both sporadic and hereditary HGSC. In addition, evidence suggests that the risk for EOC increases during the pre-menopausal years, and that menopause is protective against ovarian cancer (91). The role of sex hormones in ovarian cancer development is complex however, and early evidence suggests that endocrine function may differ in BRCA1 heterozygotes. The mechanisms of altered hormone function and impact of genetic mutations on endocrine production and receptivity in the FTE of high-risk patients is not yet understood, but it remains possible that the underlying growth stimulatory effects of estrogen are altered in a BRCA mutation carrier.

CONCLUDING REMARKS

In conclusion, there are many epidemiological studies linking ovulation, parity, and hormonal use to the development of EOC. About 60% or more of HGSC demonstrate a BRCAness profile predominantly through a dysfunctional homologous recombinant pathway, which synergizes with the ubiquitousness of the p53 mutations found amongst these tumors. In the normal fallopian tube of BRCA mutation carriers, transcription profiles reveal predominant differences in DNA damage and inflammation pathways. Interestingly and may be not surprisingly, FTE-BRCA samples are transcriptionally indistinguishable from FTE-normal samples when transcription profiles undergo unsupervised hierarchical clustering (70). Instead, the sample alignment is dependent on the estrogen or progesterone dominant phases of the ovulatory cycles, lending biological support to the known epidemiological risk factors and providing evidence for a possible haploinsufficiency of the functional allele in the normal FTE.

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Prophylactic bilateral salpingectomy as a prevention strategy in women at high-risk of ovarian cancer: a mini-review

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Risk-reducing bilateral salpingo-oophorectomy is a proven strategy to reduce the risk of serous ovarian cancer associated with germline *BRCA* mutations. It is most effective when performed before natural menopause, but it will render a woman prematurely menopausal. The tubal hypothesis of serous ovarian cancer brings with it the possibility of the alternative surgical approach in younger women comprising of risk-reducing bilateral salpingectomy while conserving their ovaries until nearer the age of natural menopause, when a delayed bilateral oophorectomy can be performed. This article will review the evidence behind the tubal hypothesis of serous ovarian cancer and explore the opportunities for translating this into clinical cancer prevention practice.

Keywords: BRCA, bilateral salpingectomy, ovarian cancer, tubal cancer, bilateral salpingo-oophorectomy, cancer prevention

Women at a high-risk of developing serous ovarian cancer due to their inheritance of a germline mutation in a cancer predisposition gene, such as *BRCA1*, *BRCA2* (1), are strongly advised to have prophylactic surgery to remove their ovaries and fallopian tubes (risk-reducing bilateral salpingo-oophorectomy RRBS0) once childbearing is complete (2, 3). Screening for ovarian cancer in high-risk populations is not recommended although a recent report suggests a degree of tumor down-staging with a strict adherence to an intensive screening protocol (4). No mortality benefit has been shown for ovarian cancer screening, even with strict adherence to screening protocols, in contrast to a clear mortality reduction with RRBSO in this population (5). Premenopausal BSO also brings a 50% reduction in breast cancer incidence in this high-risk group (5) reinforcing the recommendation for early RRBSO.

The timing of an RRBSO is crucial as the stakes are high. On one hand there is the risk of death from cancer, but this needs to be balanced by the potential for significant morbidity and occasional mortality as a consequence of the procedure itself. These are often young patients without cancer and if guided to the wrong prophylactic strategy, they could develop invasive and potentially incurable cancer. However, the risks of the procedure itself also need to be considered, including the immediate surgical and anesthetic risks but also the medical and psychological complications of plunging a woman into a premature menopause.

RRBSO can be a morbid procedure, particularly for younger premenopausal women, although the majority report a positive outcome overall (6–8). In the non-high-risk population, a bilateral oophorectomy at a younger age is associated with increased all-cause mortality (9, 10), predominantly related to the increased risk of cardiovascular disease (11). Reportedly, there is also an increased risk of Parkinsonism, cognitive impairment or dementia (12–14), and osteoporosis (15). While there are good prospective data to support a short-term improvement in mortality for RRBSO in high-risk women (5), the very long-term effects on morbidity and mortality in this group are unknown (6). Obviously, any option to prevent women experiencing an early menopause is going to be attractive to both clinicians and patients. Since the tubal hypothesis of ovarian cancer was first published in 2007 (16), there has been increasing discussion about a staged approach of initial bilateral salpingectomy (RRBS) once childbearing is complete, followed by a delayed oophorectomy (RRBO) closer to natural menopause (17–19).

Prior to 2001, the hypothesis underlying the pathogenesis of ovarian cancer implicated the ovarian surface epithelium or cortical epithelial inclusions that occur during ovulation, with the different ovarian cancer subtypes due to cellular metaplasia. Once initiated the ovarian cancer would then spread to the fallopian tube and other gynecological organs and the wider pelvic and abdominal cavities. In 2001, reports of a high rate of tubal neoplastic lesions in the RRBSO specimens from high-risk women were published (20, 21). In these reports, fallopian tubes of high-risk women were carefully examined and preinvasive cancerous lesions were found leading to other reports with similar findings (22, 23) and the unifying hypothesis by Crum et al. suggesting that the fallopian tubes were the site of origin of many serous ovarian cancers (16). These precursor lesions – tubal intraepithelial carcinomas (TICs) – had no correlating precursor lesions within the ovary. When specimens from women with serous ovarian cancers, untested for *BRCA* mutations, were examined these lesions were also found in at least 40–60% of cases and the fimbrial end of

the fallopian tube obliterated in another 20% (24, 25). Further support of the tubal origin hypothesis came from the highly similar cytological features and striking molecular similarities between TICs and invasive high grade ovarian cancers (25). These include identical *TP53* mutations, a high proliferation rate, chromosomal instability, and gene expression profiles, which all support a clonal origin (26–28).

A refinement to the tubal hypothesis is that the fimbrial ends of the tubes appear to be most vulnerable to malignant transformation, which may explain why tubal ligation provides some ovarian cancer protection in *BRCA* mutation carriers as well as women in the general population (29). In 2006, researchers from Boston described a protocol for sectioning and extensively examining the fimbrial end of the fallopian tubes (SEE-FIM). The fimbria were an area of interest as they are exposed to the peritoneal cavity, are in close proximity to the ovarian surface, merge with the serosal mesothelium, and often contain transitional metaplasia (26). It was found, and subsequently confirmed by others using the same sectioning technique, that the fimbria were the most common place for precancerous and non-invasive malignant precursor lesions within the fallopian tubes (26, 30, 31). Molecular analyses confirm these observations; within the non-neoplastic mucosa of the distal tubes was a benign precursor entity consisting of foci of strong p53 immunostaining (indicative of a *TP53* mutation), subsequently termed the "p53 signature." The p53 signature was equally common in non-neoplastic tubes from *BRCA* mutation carriers and controls, but was observed more frequently and was multifocal in fallopian tubes that also contained TIC. Like the prior studies of TIC, p53 signatures predominated in the fimbriae (23, 30). However, despite the predilection for the fimbriae, approximately one-third of TIC lesions have been observed elsewhere in the tube reinforcing the need for total removal of the tube for risk-reducing purposes (32).

From these data a plausible biological model for the pathogenesis of what might be a large proportion of high grade serous ovarian carcinoma has emerged. The hypothesized pathway begins with areas of non-neoplastic distal fallopian tubes developing *TP53* mutations. The hypothesis then suggests that this leads to a non-invasive malignancy that eventually dedifferentiates into invasive malignancy that subsequently implants into the ovary. A prospective review of RRBSO specimens from women at high-risk of ovarian cancer due to their family history or known *BRCA* mutations is supportive of this hypothesis. Of 360 RRBSO specimens reviewed, four invasive malignancies and four TICs were identified – all of which were associated with the tubal epithelium (33).

Clearly this is a compelling theory with a persuasive, although still incomplete, body of evidence behind it and could provide a rationale for risk-reducing bilateral salpingectomy (RRBS). However, it may not be the only route for the pathogenesis of ovarian cancer because the timeframe of the pathogenic process and the point of transfer of malignant or potentially malignant tubal cells to the ovary are not known. It is clear that even when utilizing the FEE-SIM protocol to examine RRBSO specimens there are still ovarian cancers identified that are not associated with any obvious fallopian tube malignancy/pre-malignant lesion. It may be that the tubal primary is too small to be found and/or that another, intra-ovarian, pathway also leads to ovarian cancer and/or that the tubal cells can be transferred to the ovary at a much earlier time point. For example, it may be that during ovulation cortical inclusion cysts are formed incorporating normal tubal epithelial cells (endosalpingiosis), which can then cause carcinoma with an underlying molecular signature consistent with the fallopian tubes (25). If any of these additional theories are correct then high-risk women may be done a serious disservice by neglecting to perform an oophorectomy with salpingectomy.

The evidence supporting the tubal hypothesis of ovarian cancer has already led to calls for bilateral salpingectomy to be added to hysterectomies performed for benign reasons in women at average population risk of ovarian cancer. This was first proposed in 2009 by Salvador et al (34) and has led to a 20× increase in salpingectomy with hysterectomy in Canada (25) although there are still barriers to its routine implementation (35–37). Adding salpingectomy to hysterectomy does not appear to have any immediate increase in complications (38). Additional proposals to perform salpingectomy rather than tubal ligation for women seeking permanent contraception have also been proposed (25).

While the tubal hypothesis is an intriguing one and can be easily integrated into routine care of women at population risk of ovarian cancer requiring hysterectomy or contraception, is the risk:benefit balance tipped in favor of a staged RRBS followed by risk-reducing bilateral oophorectomy (RRBO) at a later date in younger women at high-risk of ovarian cancer? A Canadian group has developed a Markov Monte Carlo simulation model to compare three strategies for risk reduction in women with *BRCA* mutations: (1) RRBSO; (2) RRBS; and (3) RRBS with delayed RRBO (18). The model estimated the number of future breast and ovarian cancers and cardiovascular deaths attributed to premature menopause with each strategy. RRBSO was the most effective risk-reducing strategy but RRBS with delayed RRBO was still cost effective for those women unwilling to have a RRBSO.

Despite the evidence presented above, unfortunately the point has not yet been reached where the tubal hypothesis of ovarian cancer can be reliably used to guide decision-making around prophylactic surgery in high-risk women (39). To safely change current recommendations, we need prospective evidence that the strategy of a staged approach is not inferior to upfront RRBSO. A randomized controlled trial comparing these strategies is unfortunately not feasible. The difficulties inherent in this approach are obvious, recruiting from a highly selected group of patients will take an international effort over many years in order to give sufficient statistical power to detect a state of non-inferiority, but there is also the ethical dilemma for clinicians offering randomization to an untested procedure against one, which has proven mortality benefits in a young population – would enough clinicians be in clinical equipoise in order to recruit sufficient numbers of participants? A prospective cohort study following high-risk women selecting RRBS over RRBSO (risk-reducing bilateral salpingo-oophorectomy) is a more practicable approach to answer the question but would still require a large population to give a statistically significant result. It is unlikely that a single international cohort study will be proposed and funded to answer this question but there are a number of prospective cohort studies in *BRCA* mutation carriers across the world that could provide the necessary outcome data in the future provided the required data can be

collected systematically. Furthermore, many familial cancer clinics follow up mutation carriers and would also be in a position to contribute prospective outcome data in the future. Provided that all of these groups can be brought together to pool data, an answer may be forthcoming.

So, what to advise a young *BRCA* mutation carrier who has completed her family while still in her 30s, or is in her 40s and declines RRBSO? Careful counseling is necessary to ensure that she is fully informed about the range of surgical prevention options, explaining the risks, and benefits, of all surgical approaches. It is necessary to emphasize the known mortality and breast cancer risk reduction benefits of RRBSO, and ensure that she is aware of the range of strategies to manage any sequelae arising from a premature surgical menopause. The advantage of the alternative of a staged procedure starting with bilateral salpingectomy then a bilateral oophorectomy at or approaching the age of natural menopause is that it avoids morbidity of premature menopause but this comes at the cost of uncertain impact on overall mortality, ovarian cancer-specific mortality and abrogation, or complete loss of breast cancer risk reduction. The Markov model (18) concluding that RRBS with delayed RRBO salpingectomy followed by delayed oophorectomy yields the highest quality-adjusted life expectancy (18) is intriguing, however, it is essential for a fully informed decision that it is made clear to the high-risk woman that no prospective data yet exists on the efficacy of bilateral salpingectomy in reducing mortality in high-risk women. However, in the end, it is a woman's decision based on her own preferences and life experiences and it is the role of her medical team to support her in her choices in order to maximize their benefit and minimize their risk. Some prophylactic surgery in the form of bilateral salpingectomy is probably better than no surgery in this high-risk population.

AUTHOR CONTRIBUTIONS

Tess Schenberg and Gillian Mitchell, article concept, article drafting, and final approval of manuscript.

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Gene expression analysis in ovarian cancer – faults and hints from DNA microarray study

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The introduction of microarray techniques to cancer research brought great expectations for finding biomarkers that would improve patients' treatment; however, the results of such studies are poorly reproducible and critical analyses of these methods are rare. In this study, we examined global gene expression in 97 ovarian cancer samples. Also, validation of results by quantitative RT-PCR was performed on 30 additional ovarian cancer samples. We carried out a number of systematic analyses in relation to several defined clinicopathological features. The main goal of our study was to delineate the molecular background of ovarian cancer chemoresistance and find biomarkers suitable for prediction of patients' prognosis. We found that histological tumor type was the major source of variability in genes expression, except for serous and undifferentiated tumors that showed nearly identical profiles. Analysis of clinical endpoints [tumor response to chemotherapy, overall survival, disease-free survival (DFS)] brought results that were not confirmed by validation either on the same group or on the independent group of patients. *CLASP1* was the only gene that was found to be important for DFS in the independent group, whereas in the preceding experiments it showed associations with other clinical endpoints and with *BRCA1* gene mutation; thus, it may be worthy of further testing. Our results confirm that histological tumor type may be a strong confounding factor and we conclude that gene expression studies of ovarian carcinomas should be performed on histologically homogeneous groups. Among the reasons of poor reproducibility of statistical results may be the fact that despite relatively large patients' group, in some analyses one has to compare small and unequal classes of samples. In addition, arbitrarily performed division of samples into classes compared may not always reflect their true biological diversity. And finally, we think that clinical endpoints of the tumor probably depend on subtle changes in many and, possibly, alternative molecular pathways, and such changes may be difficult to demonstrate.

Keywords: epithelial ovarian cancer, gene expression profiling, oligonucleotide microarrays, tumor histology, survival time, molecular markers, genomic medicine, *CLASP1*

INTRODUCTION

Since the report describing the use of microarray technique in cancer research by Golub et al. (1), great expectations were born concerning better cancer classification, discovery of new molecular markers and finally, individualization of patient's treatment. Disappointingly, after 15 years of research, most potential genomic medicine tools remain at experimental stage and their clinical validity and utility has not been established (2). Although some new biomarkers have emerged from the microarray studies, very few were introduced into clinical practice [e.g., Ref. (3–18); reviewed recently in Ref. (19)]. For ovarian cancer, only one single new biomarker, HE4 was cleared by FDA and one multi-marker test OVA1 (Vermillion Inc.) was developed. HE4, similarly to CA125, is accepted for monitoring and recurrence of the disease, while OVA1 is approved for women with undefined ovarian mass, to assess whether they should be referred to the oncology specialist. None of these biomarkers are suitable for ovarian cancer screening.

We performed a microarray study, which was carefully designed and based on relatively large collection of well characterized clinical samples. Our primary goal was to dissect the molecular background of tumor chemoresistance and to find molecular markers suitable for prediction of therapy failure as well as patient's outcome (prognosis). In addition, we performed a number of systematic analyses of gene expression patterns related to several defined clinicopathological and molecular features. However, in most comparisons we obtained low numbers of statistically

significant genes, majority of which were not validated by real-time RT-PCR. Nonetheless, our results allowed for some considerations concerning biology of ovarian cancer and brought some important hints concerning the analysis of expression data.

MATERIALS AND METHODS

CLINICAL SAMPLES

Surgical samples of ovarian cancer were obtained during primary surgery, then snap-frozen in liquid nitrogen and stored at −80°C. Only samples from patients without neo-adjuvant chemotherapy were used. The tissue samples were collected at the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warsaw, Poland. Altogether, we analyzed 97 ovarian cancer specimens: 71 serous, 11 endometrioid, 9 clear cell, and 6 undifferentiated [classified according to the criteria of the World Health Organization (20)]. The tumors were graded in a four-grade scale, according to the criteria given in Ref. (21).

The majority of clinical analyses were performed on a group of 72 samples (68 serous and 4 undifferentiated) with complete clinical data (**Table 1**). Of those, 32 patients were treated with platinum/cyclophosphamide, while 40 patients were treated with taxane/platinum regimen. Since it was not possible to obtain a group uniform as to residual tumor size, we chose samples from patients in whom the residual tumor apparently did not influence treatment results, e.g., sensitivity to chemotherapy in a patient with large residual tumor or progression in a patient with small residual tumor. Detection of hereditary mutations in BRCA1 gene was done according to Ref. (22). For external validation of the selected genes, we used an independent set of 30 serous ovarian cancers. Detailed criteria of evaluation of the tumors and clinical endpoints were given previously (23).

RNA ISOLATION

Total RNA was isolated from three to five sections (20 μm thick) of frozen tumor using RNeasy Mini Kit (Qiagen) with simultaneous on-column DNase I digestion. RNA purity and concentration were estimated with ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was assessed using Agilent platform: RNA 6000 Nano LabChip Kit, RNA Integrity Number software, and the Agilent 2100 Bioanalyzer (Agilent Technologies). The samples with RIN values above 7 (full range: 0–10) were accepted for further processing.

OLIGONUCLEOTIDE MICROARRAYS

We used HG U133 Plus 2.0 Gene Chip oligonucleotide arrays (Affymetrix). The hybridizations were carried out as described in Ref. (24). Briefly: total RNA (8 μg) was used for synthesis of double stranded cDNA. Biotinylated cRNA was synthesized with the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics). Both cDNA and cRNA were purified with Gene Chip Sample Cleanup Module (Affymetrix). cRNA (16 μg) was fragmented and hybridized to the microarray for 16 h at 45°C. The microarrays were stained, washed, and subsequently scanned with GeneChip Scanner 3000 (Affymetrix). Data were acquired using GCOS 1.2 software (Affymetrix). The preprocessing was performed by Robust Multi-array Analysis (RMA, Bioconductor).

REVERSE-TRANSCRIPTION AND QUANTITATIVE PCR

Half a microgram of total RNA was taken for cDNA synthesis using Omniscript RT Kit (Qiagen), random primers (4 μM, Sigma-Aldrich), oligo(dT) primer (1 μM, QBiogene Inc.), and RNase inhibitor (10 U, Fermentas). The reaction was performed in 20 μl of total volume, according to manufacturer's protocol, using thermocycler UNO II (Biometra). The cDNA was diluted 10-fold and a 5-μl aliquot was taken for real-time PCR performed using Taqman 2× PCR Master Mix (Roche), Exiqon probe (100 nM), and appropriate primers (200 nM each; Data Sheet 1 in Supplementary Material) designed using dedicated software from the Roche web site. The reaction was carried out using ABI PRISM7700 Sequence Detection System (Applied Biosystems) and the following thermal conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 72°C. The experiments were performed in triplicates. The relative amount of cDNA was calculated using comparative ΔC_t method. ΔC_t values of the samples of interest were compared with a calibrator (RNA of known concentration pooled from several samples). The C_t values of both the calibrator and the samples of interest were normalized to the expression of three control genes, *ATP6V1*, *HADHA*, and *UBE2D2*.

Table 1 | Characteristics of the group of patients and tumor samples.

Characteristics	Numbers of samples (*n*)							
	Status	***n***	**Status**	***n***	**Status**	***n***	**Status**	***n***
Histology	Serous	71	Endometrioid	11	Clear cell	9	Undifferentiated	6
CHT-response	CR	48	PR	14	SD	3	P	7
Platinum-sensitivity	Highly sensitive	12	Moderately sensitive	27	Resistant	33		
FIGO stage	FIGO II	3	FIGO III	59	FIGO IV	10		
Tumor grade	G2	9	G3	49	G4	19		
Residual tumor	R0	15	R1	36	R2	21		
BRCA1 mutation	Mutation	19	No mutation	53				

R0, residual tumor less than 1 cm; R1, residual tumor between 1 and 5 cm; R2, residual tumor larger than 5 cm. Chemotherapy (CHT) response described as clinical status of the patient after first line treatment: CR, complete response; PR, partial response; SD, stable disease; P, progression. Platinum-sensitivity: tumors were classified as highly sensitive when DFS was > 732 days, moderately sensitive when 180 > DFS > 732 and resistant when DFS < 180 days.

METHODS OF DATA ANALYSIS

Gene expression comparisons by Welch t-test were performed using GeneSpring 7.2 software (Agilent), with non-corrected threshold of p-value <0.001. False Discovery Rate (FDR) was estimated by Benjamini–Hochberg algorithm. Two-way analysis of variance (ANOVA), with random variance assumption and global testing were carried out by procedures implemented in BRB Array (developed by Richard Simon and Amy Peng Lam; available on the National Cancer Institute website). Class prediction procedure was carried out using support vector machines (SVM) class prediction engine with leave-one-out cross-validation (BRB Array Tools). Sensitivity and selectivity of classification as well as positive predictive values (PPV) and negative predictive values (NPV) were assessed. Biological significance of the differences in gene expression pattern was analyzed using Gene Ontology and Biocarta[1] databases. Gene lists were analyzed using GOHyperG[2] and Bioconductor Package[3]. Three types of tests were used for estimation of signaling pathways statistical significance: least squares, Kolmogorov–Smirnoff, and Hotelling test. Statistical significance of real-time PCR results was estimated using non-parametric Kolmogorov–Smirnov test by SPSS 13 software (SPSS), with two-sided p-value threshold of $p < 0.05$.

DATA ANALYSIS WORKFLOW

In the majority of the analyses, we used Welch test for selection of genes with changed expression. When we compared more than two classes, we used one-way ANOVA, while for selection of genes in pairwise comparisons, we used *post hoc* Tukey test. For estimation of statistical significance of each gene, two types of selection criteria were applied: uncorrected p-value <0.001 and FDR <10%. Biological significance of gene lists obtained in consecutive comparisons was analyzed by searching for over-represented functional gene classes (according to Gene Ontology database) and signaling pathways (Biocarta repository). With the usage of linear discriminant analysis, we also checked whether selected gene lists may be used for classification of samples. Global test was used to confirm if a given gene list is statistically significant (25).

VALIDATION OF THE MICROARRAY RESULTS

First, we used qRT-PCR to compare expression level of 18 selected genes in the tissue samples that were used for microarray experiments. This set of samples was called a training set. Then, we analyzed expression level of selected genes in samples derived from an independent group of patients. This set of samples was called a test set. Disease-free survival (DFS) and overall survival (OS) were analyzed by the Kaplan–Meier method and compared between groups using the log-rank test. Differences in characteristics between groups of patients according to the quantitative real-time PCR estimated gene expression levels were evaluated by the χ^2 test. A p-value of <0.05 was considered statistically significant. The analyses of survival time were performed using R Statistical Software.

[1]www.biocarta.com
[2]www.geneontology.org
[3]www.bioconductor.org

RESULTS

We analyzed global gene expression pattern in ovarian cancer with respect to several defined clinicopathological and molecular features of the tumor. These were: histological tumor type and grade, FIGO (International Federation of Gynecologists and Obstetricians) clinical stage, the volume of residual tumor left after surgery, and a germline BRCA1 gene mutation. Among the clinical endpoints analyzed, there were response to the first line chemotherapy, DFS, and OS. Full lists of genes obtained in these comparisons, the results of hierarchical clustering, as well as the lists of over-represented gene ontology classes and signaling pathways characteristic for each trait are presented as supplementary Data Sheets.

HISTOLOGICAL TYPE OF THE TUMOR

Epithelial ovarian cancers have heterogeneous histology; serous carcinomas are the most frequent ones while endometrioid, mucinous, clear cell, and undifferentiated tumors are relatively rare. All analyses performed in this study by alternative bioinformatic algorithms indicated that histological type of the tumor was the strongest factor affecting global gene expression pattern. When all four histological types were compared using one-way ANOVA, we found 3526 probe sets with significantly changed expression (FDR <10%; Data Sheet 2 in Supplementary Material). This difference was also significant in the global test (3651 probe sets, $p < 0.001$). None of the other features analyzed were associated with that large number of differentially expressed genes.

The annotated genes from the list obtained from ANOVA were taken for analysis of signaling pathways. Among significantly affected pathways were those engaged in cell cycle regulation, apoptosis, ubiquitination and sumoylation, signaling by estrogen receptor, GATA3, Trefoil factor, PTEN, and STAT (Data Sheet 3 in Supplementary Material).

We also performed pairwise comparisons (*post hoc* class comparison, Tukey test) to assess how many genes are differentially expressed between each two histological types of ovarian cancer (**Table 2**). Most pronounced molecular differences were observed between serous and clear cell tumors (625 differentially expressed probe sets with $p < 0.001$ and 40 probe sets with FDR <10%). Endometrioid and undifferentiated types were equally different from clear cell tumors. In the comparison of endometrioid and clear cell tumors, we observed 233 differentially expressed probe sets, $p < 0.001$ (12 probe sets with FDR <10%). Comparison of undifferentiated and clear cell tumors gave 237 probe sets, $p < 0.001$ (11 probe sets with FDR <10%).

Table 2 | Pairwise comparisons of different histological types of ovarian cancer (*post hoc* comparison, Tukey test).

	Endometrioid	Undifferentiated	Serous
Clear cell	233/12	237/11	625/40
Endometrioid	–	38/0	176/0
Undifferentiated		–	2/0

Given in the table are the numbers of probe sets with significantly changed expression (no. of probe sets with p < 0.001/no. of probe sets with FDR <10%).

On the contrary, undifferentiated tumors were characterized by almost identical gene expression pattern to serous tumors (only two differentially expressed probe sets, $p < 0.001$; none of the probe sets with FDR <10%). Also in the global test, the difference between serous and undifferentiated tumors was insignificant (43 probe sets, $p = 0.28$). Taking into account this striking similarity, we decided to merge serous and undifferentiated ovarian cancer samples into one group and excluded clear cell and endometrioid tumors from the subsequent analyses in order to reduce unwanted sources of variability.

We also performed a linear discriminant analysis to check whether we can properly classify tumor samples according to the histological type, based on the expression level of selected genes (3526 probe sets selected in ANOVA were used for this purpose). Results of classification are given in **Table 3**. In total, we observed only 20% of incorrectly classified samples; the best classification rate was achieved for serous cancer (89%). Interestingly, all undifferentiated samples were wrongly classified as serous, again indicating that gene expression pattern of these two histological types is very similar.

Table 3 | Classification of the tumor samples according to the histological type using linear discriminant analysis.

Histology	Sensitivity	Specificity	PPV	NPV	No misclassified/ total no. (% misclassified)
Clear cell	0.778	1	1	0.978	2/9 (22)
Endometrioid	0.667	0.966	0.727	0.955	4/11 (36)
Serous	0.889	0.741	0.901	0.714	8/71 (11)
Undifferentiated	0	0.892	0	0.933	6/6 (100)
All					20

PPV, positive predictive value; NPV, negative predictive value.

Table 4 | Classification of tumor samples according to FIGO stage (linear discriminant analysis).

Stage	Sensitivity	Specificity	PPV	NPV	% Properly classified
FIGO II	0	1	–	0.958	
FIGO III	0.831	0.231	0.831	0.231	
FIGO IV	0.2	0.823	0.154	0.864	
All					71%

PPV, positive predictive value; NPV, negative predictive.

FIGO STAGE

Clinical cancer stage is one of the major prognostic factors. Ovarian cancer, which is the most deadly gynecological cancer, is usually diagnosed at an advanced stage. Our collection of samples was typical in this respect: the majority of patients were diagnosed at FIGO III stage. In order to analyze whether the advancement of the disease may be reflected by the changes in gene expression pattern, we compared 3 samples from patients diagnosed at stage II, 59 samples from stage III, and 10 samples from stage IV tumors (72 tumor samples in total).

When we used one-way ANOVA for comparison of three FIGO classes, we found 541 differentially expressed probe sets passing criterion of $p < 0.001$ (538 probe sets with FDR <10%). Among the most significant genes were *FOXE 1* (Forkhead box E1), *FLRT2* (Fibronectin leucine rich transmembrane protein 2), and *GRK6* (G protein-coupled receptor kinase 6).

However, in the global test the difference between FIGO stages appeared insignificant (25 probe sets, $p = 0.75$). Consequently, when we used the genes selected in ANOVA for classification of samples, the results were poor. Although 71% of samples were properly classified, the specificity was unacceptably low in respect to stage II and stage IV samples (**Table 4**).

Also, in the subsequent pairwise comparisons (Tukey test) we found very low numbers of genes differentiating FIGO classes from each other. There were only one gene differentiating stage II from stage III and two genes showing changed expression between stage II and stage IV. These were *ATH1* (acid trehalase-like 1, yeast) and *AGR2* (anterior gradient homolog, *Xenopus laevis*) in stage II vs. IV comparison; the latter one was also significant for stage II vs. III difference.

For further analysis, we combined stage III and IV and compared this group of samples with stage II. This comparison yielded 714 probe sets, $p < 0.001$ (Data Sheet 4 in Supplementary Material) and 650 probe sets with FDR <10%. To better explore biological differences between early and advanced tumors, we performed analysis of gene ontology classes and signaling pathways that may be affected in these two groups (Data Sheet 5 in Supplementary Material). For this purpose, we used the annotated genes present on the list of 714 probe sets ($p < 0.001$), differentiating stage II from stage III/IV tumors. Among the most significantly over-represented gene ontology classes were those linked to the immunological processes, exogenous signal detection, neural transmission, and differentiation. Signaling pathways (according to Biocarta database), changed between early and advanced ovarian cancer, were those connected with immunological response and inflammation as well as cellular metabolism, apoptosis, PPAR, PKC, and TNFR signaling. These results, although interesting, must be taken with caution: possible bias could have been introduced due to uneven number of samples in the groups (3 stage II vs. 69 other samples).

GRADE

Histological tumor grade is the measure of cancer cells differentiation, with the high grade being a factor indicating bad prognosis. Among 77 analyzed tumor samples, 9 were defined as grade 2 (G2), 49 as G3, and 19 as G4. We were especially interested in defining the molecular difference between G3 and G4 as grade 4 is nowadays not commonly recognized, and most pathologists use the 3-grade scale.

In one-way ANOVA, we found 327 ($p < 0.001$) and 152 (FDR <10%) differentially expressed probe sets. In the global test, this difference appeared to be significant (257 probe sets, $p < 0.001$). However, in linear discriminant analysis only 55% of samples were properly classified; such result may likely be achieved by chance. Also, in pairwise comparisons (*post hoc* class comparison, Tukey test), we found only very limited numbers of differentially expressed genes: in G2 vs. G3 comparison – only one gene with

$p < 0.001$ (10 probe sets with FDR <10%); for G2 vs. G4 and G3 vs. G4 comparisons no genes with $p < 0.001$ were obtained (5 and 1 probe set with FDR <10%, respectively).

These results indicate that although postulated tumor grade 4 may be distinguished histologically, it does not differ in gene expression pattern from grade 3 tumors. Thus, we merged G3 and G4 groups and compared them against G2, using Welch test, that yielded 411 ($p < 0.001$; Data Sheet 6 in Supplementary Material) and 267 (FDR <10%) probe sets, among them there were many uncharacterized or poorly characterized ones. Within this gene set, most over-represented gene ontology classes were associated with hemopoiesis, amino acid metabolism, and MAP kinase pathway (Data Sheet 7 in Supplementary Material). Among signaling pathways from Biocarta database, significantly engaged in this difference were: cdc25/chk1, pRB, src, sonic Hedgehog, G2/M checkpoint, and "role of BRCA1, BRCA2, and ATR in cancer susceptibility."

CYTOREDUCTION

Usually, at the time of diagnosis, ovarian cancer spreads widely inside peritoneal cavity. The state of the art treatment of patients with this cancer is based on maximal possible surgical cytoreduction and adjuvant chemotherapy. The volume of residual tumor left after surgery is one of most important prognostic factors; the smaller is the size or volume of the residual tumor, the better for the patient. The best prognosis is reported for patients with no residual disease, while it is the worst for residual tumor above 5 cm in diameter. It has been already shown by Berchuck et al. that different sizes of residual tumor (<1 and >1 cm) are linked to different gene expression patterns (26). This might indicate that the size of residual tumor may not only be attributable to the successful removal of the tumor masses, but may be partially linked to the underlying biologic properties of the cancer.

Our analysis was done using the data from 72 cancer samples (serous and undifferentiated) for which the appropriate clinical data were available. In 15 cases, the residual tumor had diameter less than 1 cm (R0 group), 36 patients had tumor masses within 1–5 cm range (R1), while 21 cases had residual tumor over 5 cm in diameter (R2). Using one-way ANOVA, we found 349 probe sets with $p < 0.001$ and 63 probe sets with FDR <10%. Interestingly, in the global test, this difference was statistically significant (187 probe sets, $p < 0.001$). However, in *post hoc* Tukey test, only a few genes were found that differentiate the classes in pairwise comparisons. These were: one gene, $p < 0.001$ and seven genes, FDR <10% for R0/R1 difference, zero genes, $p < 0.001$ and two genes, FDR <10% for R1/R2 comparison and none for R0/R2. Thus, we merged groups R1 and R2 and compared it against R0 (a comparison alike that in the study by Berchuck et al.). Two-hundred and twelve probe sets with $p < 0.001$ (Data Sheet 8 in Supplementary Material) but only two with FDR <10% were found in Welch test. Only MAP3K7 gene was common in Berchuck's and in our analysis. Gene ontology assessment revealed functional gene groups connected with embryo- and morphogenesis. The analysis according to Biocarta database showed signaling pathways related with chromatin remodeling as well as pathways regulated by CDK5, AKT, estrogen receptor, CDC25, CHK1, pRB, Fas, TNF, Ras, and NF-κB (Data Sheet 9 in Supplementary Material). The list of 349 probe sets ($p < 0.001$) obtained in Welch test was validated in linear discrimination analysis. Only 57% of the tumors were properly classified into classes R0, R1, and R2, the result likely obtained by chance.

RESPONSE TO CHEMOTHERAPY

Ovarian cancer usually responds well to the first line chemotherapy and patients achieve either complete remission (CR) or partial remission (PR). Fewer numbers of tumors respond poorly, leading either to the stabilization of the disease [stable disease (SD)] or to progression (P). Among 72 tumor samples of serous or undifferentiated histology with sufficient clinical data, 62 were obtained from patients with either CR or PR, as it was established prospectively. These samples were classified as "chemotherapy-sensitive." Another 10 samples were obtained from patients with SD or progression (P) and were classified as "chemotherapy-resistant." Merging of SD and P samples seemed not only biologically valid, but was also justified by the low numbers of samples in these groups, the factor that can cause bias in the results of microarray data analysis. We found 196 differentially expressed probe sets, $p < 0.001$ (9 probe sets with FDR <10%) when comparing CR/PR vs. SD/P samples in Welch test (Data Sheet 10 in Supplementary Material). Majority of the top genes were uncharacterized, except for *SNX8* (sorting nexin 8) and *FGF12* (fibroblast growth factor 12). Gene ontology analysis (done on the annotated genes present at the list of 196 probe sets with $p < 0.001$) revealed only six functional classes significantly changed in this comparison, containing genes related with neuronal development, regulation of cell division, and WNT1 signaling (Data Sheet 11 in Supplementary Material). Analysis of signaling pathways (Biocarta repository) revealed only two affected pathways: "cyclins and cell cycle regulation" and the second one concerned with the neuronal signaling. To check whether the genes selected in Welch test may serve for classification of chemotherapy-sensitive vs. resistant tumors, we applied linear discrimination analysis. Although 81% of tumor samples were properly classified, the test showed unacceptably low specificity (10%) in respect to chemotherapy-sensitive samples and low sensitivity in detecting resistant tumors (10%). Thus, this test is without practical clinical value in respect to prediction of tumor response to chemotherapy.

PROGNOSTIC FACTORS

Among the genes that are differentially expressed in the tumors from patients with short and long survival times, putative prognostic molecular markers may be selected. Potentially, such markers could serve to predict patients' prognosis and individually tailor the therapy in order to improve treatment outcome.

OVERALL SURVIVAL

The genes related to the OS were selected using Cox-regression model. Seventy-two tumor samples with sufficient clinical data were analyzed, all of serous or undifferentiated histology. We found 93 differentially expressed probe sets, $p < 0.001$, however, it must be noted that they were characterized by high FDR values (between 12 and 21%; Data Sheet 12 in Supplementary Material). The most significant were *ATRX* (α-thalassemia/mental

retardation syndrome X-linked, RAD54 homolog) and *PI3KR1* (phosphoinositide-3-kinase, regulatory subunit 1). Odds ratio (OR) estimated for twofold increase in the expression level of those genes were: 6 for *ATRX* and 14.5 for *PI3KR1*. Several genes showed protective effect connected with its increased expression level (OR <1). They were, e.g., tyrosine phosphatases *PTPN2* and *PTPRS* (OR = 0.24 and 0.31, respectively), MRPS10 (OR = 0.22), *KCNC3* (potassium voltage-gated channel, Shaw-related subfamily, member 3; OR = 0.31), and *FBXW7* (F-box and WD-40 domain protein 7; OR = 0.32).

DISEASE-FREE SURVIVAL

The Cox-regression model was also applied to select the genes associated with DFS. We analyzed 72 tumor samples. Eighteen probe sets were selected with $p < 0.001$, however, FDR values were very poor (~85%; Data Sheet 13 in Supplementary Material). Two genes with the best *p*-values and highest OR rates calculated for twofold expression increase were: *CLASP1* (cytoplasmic linker associated protein 1) and *VAV2* oncogene (OD 3.5 and 3.7, respectively). *ATRX* was also present on this list (OR = 3.15). Among the genes with protective effect was *CDC42EP4* (CDC42 effector protein, Rho GTPase binding 4; OR = 0.4).

TECHNICAL VALIDATION OF THE MICROARRAY RESULTS

To verify the microarray results, we analyzed expression of selected genes by quantitative RT-PCR. The same RNA samples were used for qRT-PCR as were previously analyzed in the microarray experiment. Fifteen genes related with OS were chosen for validation; two of those genes (*ATRX* and *CLASP1*) also showed an association with DFS. Four genes were confirmed to be significantly associated with OS ($p < 0.05$; see also **Figure 1**). These were *CLASP1* ($p = 0.005$), *MBNL1* [Muscle blind-like (*Drosophila*), $p = 0.0381$], *SPPL2B* (signal peptide peptidase-like 2B, $p = 0.0271$), and *VAV2* oncogene ($p = 0.0133$), however, correlation of expression of *ATRX* and *CLASP1* with DFS was not validated.

We also analyzed three genes associated with CHT-response, i.e., two cyclins: *CCNB1* and *CCNE1* and cyclin-dependent kinase inhibitor 2A (*CDKN2A*), however, none of them were positively validated by qRT-PCR.

Using the expression data obtained by real-time RT-PCR, we also performed few other comparisons to check, whether the genes previously selected as related to OS/DFS and CHT-response may be significantly correlated with other features (**Table 5**). We analyzed the so-called platinum-sensitivity (classified as follows: DFS

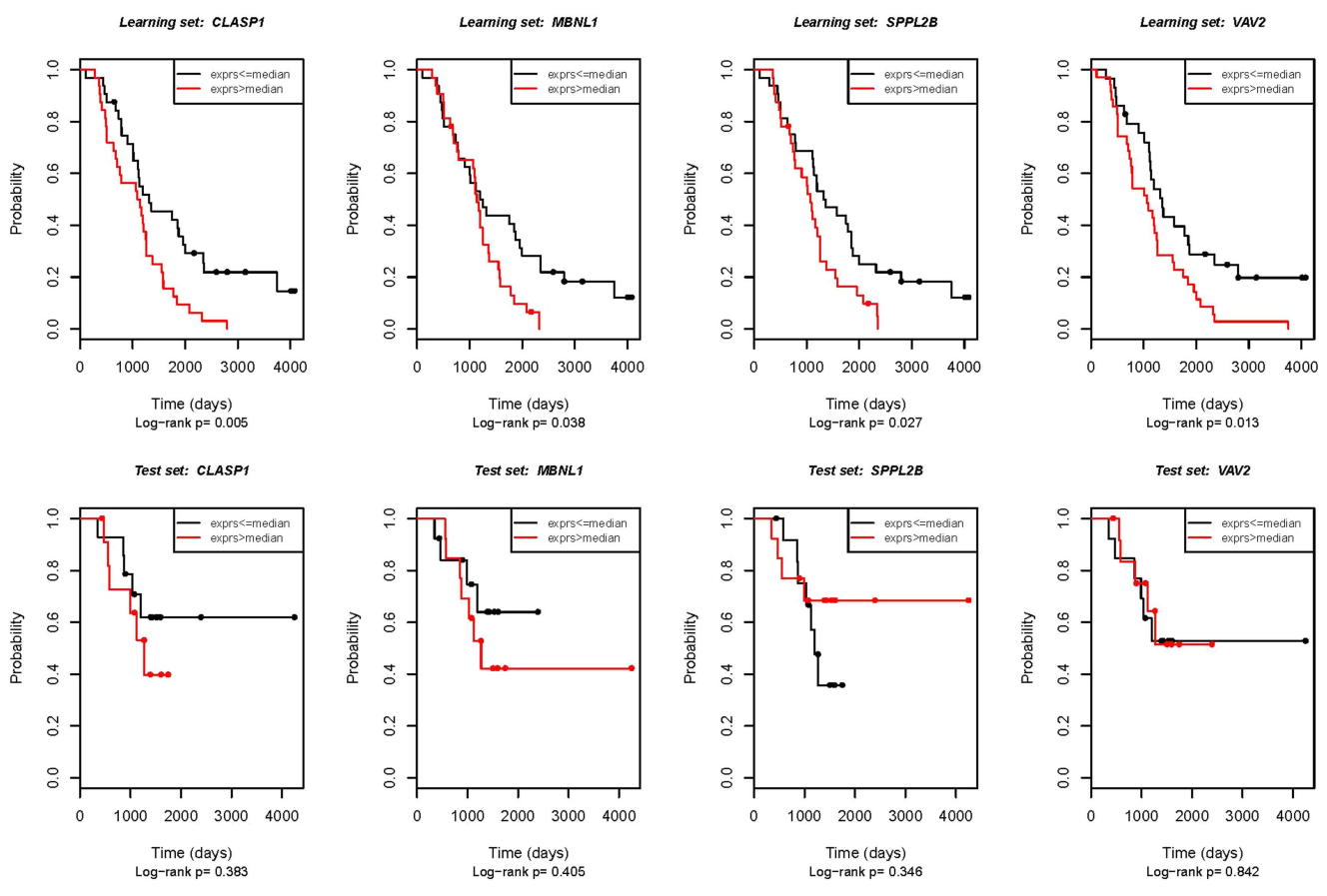

FIGURE 1 | Real-time RT-PCR validation of the genes potentially associated with OS. First row: technical validation in the initial set of samples (the same samples that were used for the microarray experiment). Second row: external validation in the independent patient group. The Kaplan–Meier analysis plot of observed overall survival for patients with ovarian cancer by log-rank test according to real-time RT-PCR estimated gene expression.

Table 5 | Technical validation of microarray results by real-time RT-PCR.

No.	Gene	Related to (in microarray analysis)	Statistical significance in real-time RT-PCR validation (*p*-value)				
			OS	DFS	CHT-response	Platinum-sensitivity	BRCA1 mutation
1	*AGGF*	OS	–		0.0818	**0.0293**	
2	*ATRX*	OS, DFS	–	–			
3	*CCNB1*	BRCA1, CHT-response	**0.0431**		–		
4	*CCNE1*	CHT-response	**0.0342**		–		
5	*CCNF*	OS	–				
6	*CDKN2A*	CHT-response			–		
7	***CLASP1***	OS, DFS	**0.0050**	–	**0.0005**		**0.0349**
8	*CTNND2*	OS	–				
9	*MRPS10*	OS	–	**0.0215**			
10	***MBNL1***	OS	**0.0381**	**0.0273**			
11	*PIK3R1*	OS	–				
12	*PRKCA*	OS, TP53 mutation	–				
13	*PSCD3*	OS	–		**0.0183**	**0.008**	
14	*PTPN2*	OS	–			**0.0248**	
15	***SPPL2B***	OS	**0.0271**	0.0684			
16	*STX7*	OS	–				
17	*USP1*	OS	–				
18	***VAV2***	OS, DFS	**0.0133**				

The third column describes the feature that appeared to be significantly linked with a given gene in microarray analysis. Only statistically significant correlations measured at the validation step are shown. Minus in brackets, i.e., (–) indicates that the given gene was negatively validated in respect to the feature which it was related to in the microarray analysis. OS and DFS were analyzed by the Kaplan–Meier method (log-rank test). CHT-response and platinum-sensitivity were analyzed by Monte Carlo method (Kruskal–Wallis test). Correlations with the germline BRCA1 were calculated using Mann–Whitney U test. Statistically significant correlations are indicated in bold.

<180 days means platinum-resistant tumor; DFS >180 means platinum-moderately sensitive one; DFS >732 days (2 years) means high platinum-sensitivity), as well as CHT-response (measured as CR and PR vs. SD and P). In addition, we analyzed an association of selected genes with hereditary BRCA1 mutation status. There are data indicating that tumors developing in patients with hereditary BRCA1 mutation respond better to DNA-damaging cytostatics than sporadic cancers, and thus BRCA1 testing may be important for therapeutic decisions [e.g., Ref. (27)].

Interestingly, *CLASP1*, in addition to its association with OS, showed also strong correlation with CHT-response ($p = 0.0005$) as well as with BRCA1 mutation status ($p = 0.0349$).

Expression of three genes: *AGGF* (angiogenic factor with G patch and FHA domains 1), *PSCD3* (pleckstrin homology 3), and *PTPN2* (protein tyrosine phosphatase, non-receptor type 2), which was not validated to correlate with OS, was proven to correlate with platinum-sensitivity. One of them (*PSCD3*) also showed correlation with CHT-response. Surprisingly, in this analysis, expression of both cyclins (*CCNB1* and *CCNE1*, not validated in respect to CHT-response) proved to be significantly correlated with OS.

VALIDATION IN THE INDEPENDENT GROUP OF PATIENTS

The clinical importance of potential prognostic and predictive molecular markers must be reproducibly seen in different groups of patients, if the markers are to be used in practice. Thus, four genes that were validated in respect to OS (*MBNL1*, *SPPLB2*, *VAV2*, and *CLASP1*) were further tested in the independent set of 30 ovarian cancer samples. Disappointingly, none of these genes were validated according to OS in the independent set of samples. However, in this experiment, *CLASP1* turned out to be related to DFS again (**Figure 2**).

DISCUSSION

Expression microarrays are used to analyze molecular profiles of cancer in order to better understand the biological background of the disease. Another aim is to find new molecular markers, therapeutic targets, and/or new classification approaches that will enable better treatment of patients. Our study was intended to achieve both goals. We searched for gene expression patterns that may characterize histological types of ovarian cancer and are related to its histological grade, FIGO stage, response to chemotherapy, and survival times.

From the broad spectrum of features that we analyzed in our study, only histological type of the tumor was a factor, which showed a very strong impact on the gene expression pattern. Interestingly, there was one exception: six undifferentiated tumors that were available for this analysis, showed practically no difference in gene expression pattern from serous cancers. If confirmed in other studies, this may be an indication for evaluating these two groups together in microarray analyses.

On the contrary, the differences between serous/undifferentiated, endometrioid, and clear cell cancers were statistically highly significant. Moreover, the gene expression signature selected in respect

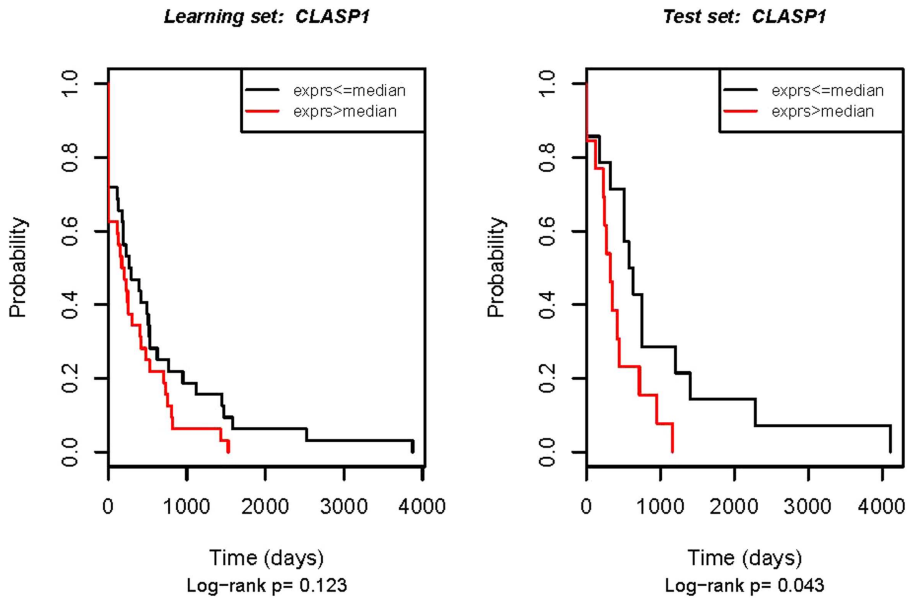

FIGURE 2 | Real-time RT-PCR validation of the *CLASP1* gene in relation to DFS. Left: technical validation in the initial set of samples (the same samples that were used for the microarray experiment). Right: external validation in the independent patient group. The Kaplan–Meier analysis plot of observed DFS for patients with ovarian cancer by log-rank test according to real-time RT-PCR estimated gene expression.

to tumor histology allowed for a very precise sample classification, with the sensitivity and specificity not achieved in any other comparisons. Also unsupervised analysis, performed using the singular value decomposition (SVD) showed that histological type of the tumor is a major source of variability in the gene expression pattern in ovarian cancer (not shown). This large difference in gene expression pattern may be not surprising when we take into account that histological differences are clearly manifested at the morphological level and are easily distinguishable by light microscopy. On the other hand, these results, indicating deep molecular divergence, may support the current knowledge that ovarian cancer has a heterogeneous histological origin (e.g., fallopian, endometrioid, or endocervical) (28–32).

The histology of ovarian cancer was already analyzed in many previous microarray studies (33–40), however, it has not been regarded as a confounding factor in gene expression analysis in respect to other features. Conversely, different factors have been analyzed across various histological types. This may be one of the reasons for discrepancies and low reproducibility of the findings. Thus, a practical conclusion may be drawn that when searching for the genes related to other features of ovarian cancer, the analyses should be carried out on histologically homogenous groups of samples. Alternatively, the influence of the histological type on gene expression may be controlled by multivariate approach.

Except for evaluation of histological type, no other comparison gave such a huge number of statistically significant genes. This was the reason why we decided to use less stringent criteria for gene selection (uncorrected p-value <0.001 and FDR $<10\%$). Analyzing gene expression patterns in tumor samples of different grades, we focused mostly on the difference between grade 3 and 4, as the usage of the latter grade was abandoned in ovarian cancer diagnostics. A study performed by members of our group showed that the recognition of grade 4 might be important from the clinical viewpoint, since patients with grade 4 ovarian cancer had worse response to taxanes than to DNA-damaging agents (23). Thus, we expected that we would find differences between grade 3 and 4 also at the molecular level. However, samples classification was poor and in pairwise comparison we found only one gene with significantly changed expression (FDR $<10\%$). It was surprising, as in ANOVA we found 152 probe sets (FDR $<10\%$) differentiating between three grades (G2, G3, and G4), and this difference was also significant in the global test. In our opinion, this discrepancy may suggest that although tumor grade is generally associated with significant changes in gene expression pattern, the subjectively defined grades 3 and 4 may not reflect these differences. An additional factor influencing the results of this analysis may be the small and unequal size of the groups evaluated.

The problem also occurred when analyzing gene expression profiles in relation to FIGO stages and residual tumor size. These features were significant in the ANOVA and global tests, but the number of genes with different expression found in pairwise comparisons was low and the quality of classification was poor. The difference between FIGO II and FIGO III/IV was statistically significant, however, this result may be an artifact related to uneven samples distribution in the groups being compared.

As far as the residual tumor size is concerned, poor classification of tumor samples may be due to the fact that debulking status did not solely depend on the biological tumor profile, but also on the changing attitude to optimal debulking over several years during which our material was collected. Other factors influencing the

results might be technical issues, such as skills of surgeons and the equipment available. Our samples came from mid 1990s (patients treated with platinum–cyclophosphamide, PC), and from early 2000s (patients treated with taxane–platinum, TP). The group treated with PC had been generally less radically operated than the group treated with TP (23). Thus, this may be the major reason why it was hard to obtain reliable results in gene expression analysis in respect to this parameter. In addition, the arbitrarily outlined classes (R0–2) may not reflect intrinsic biological differences.

The most important, from the clinical point of view, is the search for molecular markers suitable for prediction of tumor response to the therapy. In the presented analysis, we were not able to find a gene signature that would allow for good classification of samples sensitive and resistant to chemotherapy. It seems that chemosensitivity/resistance, in contrast to, e.g., histological type, is a feature that may depend on subtle molecular changes, possibly in many alternative pathways. Such differences may be hard to detect by the methods applied. It has been shown recently, by comparing the data from Cancer Cell Line Encyclopedia and Cancer Genome Project, that discrepancies in drug sensitivity testing are common even when performed on cell lines (41). Another reason for the failure of this analysis may be again the fact that we analyzed two cohorts of patients treated with different CHT regimens. Probably, different molecular pathways were engaged in tumor response to the two regimens and this could affect the results of our analyses. It was not advisable, however, to divide patients into two groups according to the CHT regimen, because this would result in biased results due to small classes of samples.

We also searched for genes that may be related to patients' prognosis, i.e., DFS and OS. Only 4 out of 15 genes, selected in microarray analysis as associated with OS, were positively validated by qRT-PCR, and none were validated for DFS. Our further attempts to validate these four genes in the independent set of samples were unsuccessful. There may be several reasons for this result. First, all genes selected in respect to survival time were of low statistical significance in the microarray analysis. Second, contrarily to the initial group, the independent set of patients used for validation was uniformly treated with TP regimen only. Therefore, it might show results different from those obtained in the initial, mixed group. Indeed, we observed that the initial group of patients had different OS statistics than the test group (**Table 6**).

In general, the results of qRT-PCR validation were surprising. Several genes that were selected as related to one feature appeared to correlate with another factor(s). In our opinion, this observation confirms that many clinical and biological features of the tumor are difficult to define and that arbitrarily assigned groups of samples used in gene expression analyses not always reflect biologically significant differences.

Our attempts to validate selected genes were rather unsuccessful. It should be noted, however, that we performed an external validation on the independent group of tumor samples, while many other studies that claim finding potential biomarkers, were confined just to the internal, technical validation [reviewed, e.g., in Ref. (2, 42)].

One of the most interesting genes selected in our study is *CLASP1* (cytoplasmic linker associated protein 1). It was associated with both OS and DFS in the microarray analysis, although validation results were mixed. In the initial group of samples, it was validated in respect to OS and showed significant association with response to chemotherapy and with the presence of hereditary BRCA1 mutation. Surprisingly, when we tried to validate *CLASP1* in the independent set of samples it was statistically insignificant in respect to OS, but it proved to be associated again with DFS. *CLASP1* is thought to play a role in the regulation of microtubule dynamics in interphase and during cell division (43, 44). Thus, the protein may be important in tumor cell response to taxanes. Possibly, it may also be somehow engaged in differential response to CHT in patients with hereditary, BRCA1 mutation-linked ovarian cancer. Regardless of the inconsistent results of validation, we think that *CLASP1* may be worth further investigation as a potential prognostic and predictive marker.

Table 6 | Characteristics of the two groups of patients according to OS statistics (days).

Group	Minimal OS	First quartile	Median OS	Third quartile	Mean OS	Max. OS
Learning set	104	687	1131	1306	1773	4080
Test set	346	885.5	1199.0	1267.0	1468.0	4250

Learning set, ovarian cancer samples used for the microarray analysis; test set, ovarian cancer samples from the independent group of patients, used for external validation.

CONCLUSION

Our results confirm previous observations that histological type of the tumor is the major source of variability in gene expression in ovarian cancer. This statement does not refer, however, to the difference between serous and undifferentiated tumors. In our analyses, these two histological types showed almost identical gene expression pattern and were evaluated as one group. Taking into account large differences in molecular profile between serous/undifferentiated vs. endometrioid vs. clear cell tumors, we think that it is advisable to perform analyses of other clinical and molecular features of ovarian cancer only on the histologically homogenous groups of samples. In our opinion, the mixed results of quantitative RT-PCR validation shed light on the general problem that is present in supervised analyses of microarray results. In such approach, one arbitrarily defines the groups of tumors to be compared in terms of gene expression pattern. Most likely, arbitrarily performed division of samples may not reflect biological diversity of the tumors. In our opinion, this may be one of the reasons why, the results of such studies are often inconclusive and hard to replicate in different experimental settings.

AUTHOR CONTRIBUTIONS

Katarzyna Marta Lisowska took part in designing the study and preparing grant application; did some microarray experiments; analyzed the results; and drafted the manuscript, Magdalena Olbryt did the majority of microarray experiments, Volha Dudaladava and Jolanta Pamuła-Piłat did some microarray experiments, Ewa Grzybowska took part in designing the study and in preparing grant application, Katarzyna Kujawa did qRT-PCR experiments,

Michał Jarzab did majority of bioinformatic analyses, Sebastian Student did some bioinformatic analyses, Iwona Krystyna Rzepecka did genetic tests, Barbara Jarzab took part in designing the study and helped to analyze the results, Jolanta Kupryjanczyk collected all tumor samples, did pathological assessments, and provided all clinical and molecular data; she also took part in designing the study; preparing the grant application; and writing the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/journal/10.3389/fonc.2014.00006/abstract

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Current status and evolution of preclinical drug development models of epithelial ovarian cancer

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Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy and the fifth most common cause of female cancer death in the United States. Although important advances in surgical and chemotherapeutic strategies over the last three decades have significantly improved the median survival of EOC patients, the plateau of the survival curve has not changed appreciably. Given that EOC is a genetically and biologically heterogeneous disease, identification of specific molecular abnormalities that can be targeted in each individual ovarian cancer on the basis of predictive biomarkers promises to be an effective strategy to improve outcome in this disease. However, for this promise to materialize, appropriate preclinical experimental platforms that recapitulate the complexity of these neoplasms and reliably predict antitumor activity in the clinic are critically important. In this review, we will present the current status and evolution of preclinical models of EOC, including cell lines, immortalized normal cells, xenograft models, patient-derived xenografts, and animal models, and will discuss their potential for oncology drug development.

Keywords: epithelial ovarian cancer, high-grade serous, preclinical models, personalized therapy, cell lines, xenografts, mouse models, patient-derived xenografts

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy and the fifth most common cause of female cancer death in the United States (1). Advanced stage at diagnosis for most women with this cancer and emergence of resistance to conventional chemotherapy are primarily responsible for this dire outcome. Although important advances in surgical and chemotherapeutic strategies over the last three decades have significantly improved the quality of life and median survival of EOC patients, the overall cure rate has not improved appreciably (2–5). EOC is a genetically and biologically heterogeneous disease and is traditionally divided into two types (types I and II) with distinct genotypic and phenotypic characteristics which are summarized in **Table 1** (6–8). Type I tumors frequently harbor somatic mutations in KRAS, BRAF, PIK3CA, PTEN, CTNNB1, and ARID1A genes, and exhibit low genomic instability without genome-wide copy number changes (9) while type II tumors are characterized by high degree of genomic instability with high frequency of DNA copy number changes and p53 mutations (6, 7, 10).

High-grade serous carcinomas (HGSCs) represent the most common type II histologic subtype and account for approximately 70% of all EOCs. These tumors exhibit histological features that are identical to those of primary peritoneal and fallopian tube serous cancers and are treated similarly to these neoplasms. A number of molecular studies and most recently The Cancer Genome Atlas (TCGA) project have shown that HGSCs are characterized by frequent genetic and epigenetic alterations in gene members of the homologous recombination (HR) DNA repair pathway, including the BRCA1 and BRCA2 genes (10). Furthermore, the NOTCH, FOXM1, RB, and PI3K/RAS signaling pathways have also been implicated in the pathogenesis of HGSCs (10). These important advances in our understanding of the molecular pathogenesis and heterogeneity of EOC hold promise for the development of novel therapies against these tumors. However, for this promise to materialize, appropriate preclinical experimental platforms that recapitulate the complexity of these neoplasms and reliably predict antitumor activity in the clinic are critically important. In this review, we will discuss the current status and evolution of preclinical models of EOC focusing on their potential for oncology drug development.

CELL LINES

Historically, ovarian cancer cell lines have been the most frequently used tumor models to prescreen experimental anticancer agents *in vitro* and to select specific histologic subtypes of EOC for further exploration of these agents. These cell lines have undergone a high degree of evolutionary selection pressure *in vitro* as they have been in passage for several years (or even decades in some cases). As a result, their genomic profiles have been irreversibly altered and rarely recapitulate the genetic and pathologic characteristics of the parental cells (11–13). Furthermore, cancer cell lines lack the molecular heterogeneity of the parental tumor and are molecularly skewed toward affinity to grow in monolayers.

In a recently published study, Domcke and colleagues used available molecular profiles (copy number changes, mutations, and mRNA expression profiles) of cell lines from the Cancer Cell Line Encyclopedia (CCLE) and of tumor samples from the TCGA to evaluate the suitability of 47 EOC cell lines as *in vitro* models of HGSCs (14). The investigators showed significant differences in the molecular profiles between commonly used EOC cell lines and HGSC samples and reported that the presumed histologic subtype for several of these cell lines did not correspond to their

Table 1 | Molecular and clinical characteristics of EOC subtypes.

Histology	Type	Molecular characteristics	Clinical characteristics
Low grade serous carcinoma	I	KRAS, BRAF mutations	Frequently arise from serous cystadenoma-borderline sequence Relatively indolent growth Poor response to platinum based chemotherapy
Low grade endometrioid carcinoma	I	CTNNB1, PTEN, PIK3CA, and KRAS mutations Microsatellite instability	Frequently arise from endometriosis Relatively indolent growth Association with HNPCC[a] Poor response to platinum based chemotherapy
Clear cell carcinoma	I	PIK3CA, ARID1A mutations MET amplification	May arise from endometriosis Association with HNPCC[a] Worse prognosis and response to platinum based chemotherapy
Mucinous carcinoma	I	KRAS mutations HER2 amplification	May arise from cystadenoma-borderline sequence
High-grade serous and high-grade endometrioid carcinoma	II	P53 mutations (almost universal), BRCA1, BRCA2 mutations Genomic instability and very high degree of somatic copy number alterations	May arise from fallopian tube intraepithelial carcinoma (TIC) Association with HBOC[b] Rapid growth Very good response to platinum based chemotherapy

[a] *HNPCC, hereditary non-polyposis colorectal cancer syndrome due to germline mutations in mismatch repair genes.*

[b] *HBOC, hereditary breast ovarian cancer syndrome due to germline BRCA1 or BRCA2 mutations.*

molecular profiles. Of note, the two most frequently used cell lines, SKOV3, and A2780 were deemed unsuitable as HGSC models, while other rarely used cell lines such as KURAMOCHI, OVSAHO, and SNU119 closely resembled the molecular profiles of HGSC samples. Interestingly, the suitability of these cell lines as HGSC models did not correlate with time of their derivation suggesting that number of passages may not correlate with model suitability. Among the cell lines deemed most suitable to use as HGSC models, three cell lines harbored BRCA mutations i.e., KURAMACHI (BRCA2), COV362 (BRCA1), and JHOS2 (BRCA1) and therefore may be useful as *in vitro* models for BRCA-associated EOC.

This study may provide molecular explanation for the challenges of translating preclinical observations from ovarian cancer cell lines into the clinic, a problem that is not unique to ovarian cancer but transcends multiple tumor types (14, 15). However, this study also highlights that certain EOC lines may still hold value as HGSCs models and underscores the importance of evaluating and screening them to confirm their origin and molecular resemblance with HGSC. This is now feasible given the increasing availability of large scale genomic data from studies such as the TCGA, the CCLE, and the Sanger Cancer Cell Line project (10, 16). Cell line models whose molecular identity has been confirmed using targeted sequencing and copy number profiling may be extremely valuable as preclinical models, particularly those with well defined molecular alterations such as BRCA1/2 or PI3K mutations in order to assess the potential of experimental drugs in patient populations with specific molecular alterations. In this regard, the promise of PARP inhibitors in the management of BRCA-deficient EOC was first realized in BRCA1/2 deficient cell lines (17, 18). In the era of advanced molecular profiling, using cell lines with molecular similarities with patient samples may increase the possibility that *in vitro* observations will be eventually translatable to the clinic.

IMMORTALIZED NORMAL CELLS AND STEM CELLS

Several investigators have reported isolation, *in vitro* propagation and immortalization of human ovarian surface epithelial (OSE) and fallopian tube epithelial (FTE) cells which are considered the cells of origin of ovarian carcinomas. Retroviral transduction of either the human papilloma virus E6/E7 oncogenes or the simian virus 40 T-Antigen (SV40-TAg) in human OSE cells leads to increased and sustained proliferation even after multiple passages but does not induce transformation (19, 20). For immortalization to occur, additional retroviral constructs targeting TP53, hTERT, or RB are required (21, 22). Besides retroviral transduction, RNA interference technology has been successful in immortalizing human OSE cells as exemplified by the work of Yang and colleagues who successfully immortalized OSE cells via siRNA knockdown of p53 and Rb (23, 24). As with human OSE cells, Karst and colleagues immortalized normal human FTE cells via retroviral transduction of hTERT and either of SV40-TAg or an shRNA targeting p53 and mutant $CDK4^{R24C}$, while transformation occurred via further ectopic expression of either MYC or HRAS oncogenes (25). When injected in immunocompromised mice, these cells developed tumors resembling HGSCs both histologically and clinically. Shan and colleagues used a similar approach of hTERT and SV40-TAg overexpression for immortalization and of additional ectopic HRAS expression for transformation of human FTE cells while similar results have been reported by Jazaeri and colleagues (26, 27).

Although presence of ovarian cancer stem cells has been reported, definite characterization of these cells is still lacking (28). Furthermore, the stem cell niche of the OSE which regenerates after each ovulation has not been determined. There have been several reports of ovarian cancer stem cells isolation which have been based on markers and protocols used to define stem cells in

other tumors including leukemia, colon, and breast cancers (29–31). In a seminal study, Flesken-Nikitin and colleagues proposed that the hilum region of the mouse ovary is a stem cell niche of the OSE (32). Specifically, the investigators showed that hilum cells express stem cell markers ALDH1, LGR5, LEF1, CD133, and CK6B, display long-term stem cell properties *ex vivo* and *in vivo* and exhibit increased transformation potential after inactivation of TP53 and RB1.

XENOGRAFTS

Xenograft models have been extensively used in ovarian cancer research and are still very important experimental platforms for preclinical drug development (33–36). These models require use of immunodeficient mice strains, i.e., athymic nude mice lacking T lymphocytes, severe combined immunodeficient (SCID) mice which lack functional B and T lymphocytes, or the NOD/SCID/IL2Rγ^{null} mice which also exhibit inactive innate immunity due to abrogation of maturation of natural killer (NK) T cells (37). The requirement of immunodeficiency has often been cited as one of the main reasons why xenografts have shown limited predictive value in the clinic (38, 39). Specifically, tumor xenografts in immunocompromised mice cannot recapitulate either the contributions of immune factors on tumor development and progression or the extensive interactions of the human host tumor microenvironment (stroma, extracellular matrix, and vasculature) with the tumor cells.

Traditionally, xenograft models rely on implantation of established EOC cell lines subcutaneously, intraperitoneally, or orthotopically. Subcutaneous implantation offers the advantage of easy quantification of tumor volume which is ideal for assessing antitumor efficacy of experimental agents, but rarely results in ascites formation or intraperitoneal (IP) seeding of the tumor, and thereby fails to reflect the clinical course of human EOC. Conversely, IP and orthotopic implantation (OI) frequently result in peritoneal carcinomatosis and development of malignant ascites. The most commonly used xenograft model in ovarian cancer was developed by IP injection of a subpopulation of the drug resistant cell line NIH:OVCAR-3 (40) (isolated by serial *in vitro* and *in vivo* selection of cells) into athymic mice which resulted in development of ascites and peritoneal carcinomatosis (33). The NIH:OVCAR-3 cell line has been molecularly ranked as possibly of HGSC origin on a rank of likely, possibly and unlikely, and this xenograft model is still widely used today (14). The OVCAR-3 and other xenograft models have been used in the preclinical evaluation of antiangiogenic agents (41, 42). Specifically, these models demonstrated the ability of a monoclonal antibody (mAb) to human vascular endothelial growth factor (VEGF) to prevent ascites formation and that combination therapy with inhibitors of VEGF plus paclitaxel exhibits synergistic reduction of tumor growth and ascites in ovarian cancer. These observations were subsequently confirmed in clinical trials of bevacizumab as single agent and in combination with paclitaxel in EOC (43–45).

Orthotopic implantation involves injecting EOC cells into their natural position adjacent to the ovaries which in mice corresponds to the ovarian bursa, a thin membrane that encapsulates the ovaries (46). OI is usually accomplished by direct injection within the ovarian bursa via the infundibulum (47, 48). OI recapitulates initiation of EOC growth in the ovaries, does not require selection of EOC cell lines, and preserves tumor histology and the potential for peritoneal dissemination and ascites formation. Furthermore, several studies have indicated increased tumor take rates with OI thereby reflecting a more favorable microenvironment for tumor growth and metastatic dissemination (48, 49). Unlike subcutaneous xenografts, orthotopic and IP xenografts pose a challenge for accurately quantifying tumor volume and monitoring disease progression thus making them less appealing as models for preclinical drug development. However, this challenge may be overcome by advances in non-invasive imaging of tumors in mice [magnetic resonance imaging (MRI), ultrasound (US), positron emission tomography (PET), computed tomography (CT), and single photon emission computed tomography (SPECT)] and/or use of fluorescent or bioluminescent reporters with optical imaging [fluorescent imaging (FLI) or bioluminescent imaging (BLI)] and/or use of serum tumor biomarkers such as CA125 (50).

PATIENT-DERIVED XENOGRAFTS

Patient-derived xenografts (PDXs) represent an evolution of the cell line xenograft model whereby fresh tumor tissue, obtained directly from patients, is implanted subcutaneously or orthotopically into immunodeficient mice (51, 52). After a variable period of time, PDXs enter a logarithmic growth phase which allows for harvesting and reimplantation in successive mice generations with reported tumor engraftment rates higher than 75% (53–55). The time to engraftment depends on the individual tumor, the site of implantation and the type of immunodeficient mice used (NOD/SCID/IL2Rγ^{null} mice are associated with superior engraftment efficiency) and is generally between 2 and 4 months. PDXs have been successfully established from primary or metastatic tumors (56, 57), from untreated or heavily pretreated tumors (58, 59) thereby potentially capturing chemotherapy-refractory tumor populations and permitting the study of molecular changes that occur at the time of development of resistance.

A growing body of literature suggests that PDXs hold significant promise as models for preclinical drug development because they closely resemble and recapitulate tumor growth in humans (**Table 2**). In a seminal study by Hidalgo and colleagues, the investigators treated PDXs from 14 patients with various advanced solid tumors with 63 drugs in 232 treatment regimens, and showed that there was an excellent correlation between response in the PDX models and patient response to these regimens (60). Of note, in some cases, the treatment administered to patients based on the PDX response was not the first choice of the oncologist treating these patients. This study highlights the potential of PDXs as experimental platforms for preclinical drug development. PDXs represent significant improvement over the standard cell line xenografts because they maintain the principal characteristics of the original patients' tumors including histology, mutational status, DNA copy number changes, gene-expression patterns and clinical behavior while they remain biologically stable when passaged in mice. Specifically, genome-wide expression analysis in non-small cell lung cancer has demonstrated that PDXs exhibit similar gene-expression profiles and maintain the key gene and pathway activity of the primary tumors (61). Furthermore, mutational and expression analysis in pancreatic PDXs has shown that

Table 2 | Advantages and disadvantages of PDX models.

Advantages	Disadvantages
Unlike cell lines, PDXs do not undergo evolutionary selection pressure from *in vitro* culture	Immunocompromised mice cannot adequately capture the intact human immune component of primary tumors and thus may not recapitulate the complex cross talk between tumor cells and the human immune system
PDXs maintain the characteristics and heterogeneity of the original tumor i.e., histology, mutational status, DNA copy number changes and gene expression	Human stroma is eventually replaced by murine stroma thereby limiting the ability to recapitulate tumor-stroma interactions in late passages PDXs
PDXs maintain their molecular similarity with the primary tumors during sequential passage	Orthotopic implantation is technically challenging
PDXs include a component of the primary tumor's stroma including microvasculature, stem cells, and memory T cells, although it is unclear for how long this is maintained	Expensive to establish and maintain PDX banks thus requiring significant funding resources or institutional support
PDXs offer the opportunity to evaluate tumors from metastatic sites or tumors that have developed resistance to multiple treatments	Establishment of PDX banks requires prompt processing of primary tumor and significant coordination between departments
Studies have shown very good correlation between response in PDX models and clinical response in patients	Possible regulatory challenges i.e., IRB approval and HIPPA and intellectual property issues

there is excellent concordance between primary tumors and PDX models (62). Several studies have also shown that PDXs maintain their molecular similarity (histology, protein expression, tumor biomarkers, genomic, and genetic status) with the primary tumors during sequential passage (63–65). This molecular similarity is even higher when PDX models are generated using patient tumors that are immediately implanted into immunocompromised mice without an intermediate *in vitro* culture step (66, 67). Another key feature of PDXs is the maintenance of the original tumor architecture and histopathological characteristics, including a component of human stroma as well as tumor microvasculature although there is a controversy over how long this is maintained. Specifically, in one study of pancreatic PDXs, vessels with human endothelial cells were maintained or even increased over time while in a similar study with renal cell cancer PDXs, a decrease in human-derived tumor microvasculature was observed (68, 69). Of note, maintenance of human tumor-associated leukocytes including memory T cells for up to 9 weeks after implantation has been reported in lung cancer PDXs implanted into NOD/SCID/IL2Rγ^{null} mice. Furthermore, preservation of pluripotent CD133+ stem cells in PDXs following repeated orthotopic subtransplantations has been reported and in these studies the CD133+ cells continued to exhibit multi-lineage differentiation capacity *in vitro* (70–73). PDXs (particularly early passage PDXs) may therefore be excellent preclinical platforms to study stromal-tumor interactions and cancer stem cell biology as well as to assess novel anticancer agents or drug combinations.

Several limitations of PDXs exist (**Table 2**). A major limitation of PDXs is the requirement to use immunodeficient mice which limits the number of drugs that can be evaluated (i.e., alternative models are necessary for immune-modulating agents) (74, 75). Furthermore, severely immunocompromised mice cannot adequately capture the intact human immune component of the primary tumors and thus may not recapitulate the complex cross talk between tumor cells, stroma, and the human immune system. One approach to circumvent this problem may be transplantation of human CD34+ cord blood cells enriched for human hematopoietic stem cells that may reconstitute a human innate and adaptive immune system in mice (76). However, development of PDX models in mice with a reconstituted human immune system is technically challenging and would require that the xenografted tumors and the human immune cell component are HLA matched. Furthermore, the eventual replacement of human stroma by murine stroma is an important disadvantage of PDX models given the importance of tumor-stroma interactions in mediating drug response and resistance. Therefore drugs that target the tumor-stroma or microvasculature such as antiangiogenic agents may also require alternative models for evaluation. Murine models are also known to be imperfect models of drug metabolism and distribution in humans. For example, an overestimation of response may occur when drugs are tolerated at higher doses in mice while an underestimation may occur when mice are less tolerant to drugs compared to humans. There also several logistic challenges including financial and personnel resources that are necessary to establish and maintain PDX banks and the ability to freeze and reestablish tumors after months of storage. Compared to the inexpensive cell line experiments, the cost burden of PDX tumor models is substantial and will likely require significant institutional and national funding to support widespread use of PDXs as experimental models.

In EOC, Kolfschoten and colleagues have reported development of a panel of 15 human ovarian cancer xenografts (12 from fresh tumor derived from patients and 3 from EOC cell lines) grown subcutaneously in the flank of athymic nude mice (77). They assessed the sensitivity of these xenografts to six commonly used anticancer agents and showed that their panel reflected the response rates known for similar drugs in ovarian cancer patients. This study, together with several analogous studies in other tumor

types, suggests that PDXs may be used for drug screening in EOC. In our institution, in collaboration with the Belfer Institute of Applied Cancer Research we have embarked on building a platform of ovarian cancer PDXs. The goal of this project is to provide a resource for evaluating efficacy of experimental agents and to identify novel predictive and pharmacodynamic biomarkers. Ovarian cancer cells taken from consented patients are implanted intraperitoneally into immunodeficient mice and these tumors grow and disseminate in the peritoneal cavity similar to human EOC (manuscript in progress, personal communication, Joyce Liu). In order to accurately quantify tumor growth and assess response to experimental therapies, ovarian cancer cells derived from the initial passages are tagged with luciferase and reimplanted into mice for non-invasive BLI. In addition, surrogate biomarkers such as CA125 are evaluated in each of the models to monitor response to therapy.

In the era of personalized medicine, patient-centric PDX models for tumor growth and assessment of drug efficacy may be a valuable resource for the preclinical development of experimental anticancer agents. However, as in the case of cell lines, periodic molecular assessment of these models examining the fidelity to the patients' original tumors in terms of genetics and histology, two factors that are major determinants of their eventual predictive ability.

ANIMAL MODELS

Spontaneous EOC models including the aging hen, the cynomolgus macaque, and the rhesus macaque are rarely used in preclinical drug development due to their low incidence rates and long interval until cancer development (78–80). However, because of its anatomic resemblance to humans, the cynomolgus macaque has been occasionally used to evaluate novel agents such as chimeric antibodies or antibody-cytotoxic conjugates (81, 82). Similar to spontaneous EOC models, chemically or hormonally induced models of EOC are rarely used because their histopathological features are not always predictable and their individual molecular alterations are not well defined (83). Conversely, genetically engineered animal models may be promising platforms for preclinical drug development and will be reviewed below (48, 84).

VIRUS-MEDIATED GENE DELIVERY

The first successful mouse model of EOC using a retroviral gene delivery system was reported in 2002 by Orsulic and colleagues (85) who isolated OSE cells from transgenic mice which carried the avian tumor virus receptor A (TVA) under the transcriptional control of the b-actin or keratin 5. Using this TVA retroviral delivery system, they infected OSE cells from TVA; $p53^{-/-}$ mice with any combination of two or three of the c-MYC, KRAS, and AKT oncogenes, and reimplanted them in the TVA; $p53^{-/-}$ mice resulting in rapid formation of tumors 8 weeks later. The resulting tumors exhibited poorly differentiated histology with areas of papillary structures resembling HGSCs. This model was subsequently used to assess sensitivity to molecular pathway inhibitors; for example tumors with AKT and c-MYC oncogenes or AKT and KRAS were sensitive to mTOR inhibitor rapamycin while tumors with all three oncogenes (KRAS, c-MYC, and AKT) were resistant to rapamycin but sensitive to a combination of mTOR inhibitor and MEK inhibitor (i.e., rapamycin and PD98059). These experiments highlight how such models may be used to test the efficacy of molecular targeted agents in EOC. A similar experimental strategy was also employed for development of a BRCA1-associated EOC model whereby expression of c-MYC resulted in transformation of BRCA1 and p53 deficient murine OSEs (86). When implanted intraperitoneally in mice, these cells developed tumors with several characteristic of BRCA1-associated HGSCs, i.e., papillary architecture, peritoneal carcinomatosis, development of malignant ascites, and enhanced sensitivity to cisplatin.

TRANSGENIC MODELS

A transgenic EOC model was developed by Connolly and colleagues (87) by expressing the early region of SV40-TAg under the transcriptional control of Mullerian Inhibitory Substance Receptor II (MISRII). Fifty percent of the transgenic founder mice developed very aggressive tumors (poorly differentiated carcinomas with rapid development of peritoneal carcinomatosis and ascites) but none of them were fertile. In a subsequent report (88), the same group reported a stable transgenic line from a male transgenic founder (TgMISRII-Tag-DR26) whereby all female offsprings developed bilateral EOCs resembling HGSCs. This is the first transgenic model of HGSC and it has been used for evaluation of experimental agents in clinical trials (89).

CONDITIONAL MODELS

Genetically engineered mouse models using conditional expression of tumor suppressor genes via Cre-recombinase-mediated excision of LoxP flanked sequences have been reported extensively in ovarian cancer literature. Given that there are currently no transgenic mice that express Cre-recombinase only in ovarian epithelial cells, localized delivery of recombinant adenovirus expressing Cre-recombinase in the ovarian bursa of mice is required to achieve Cre-LoxP-mediated gene inactivation solely in the ovarian epithelium. Flesken-Nikitin and colleagues (90) first reported intrabursal administration of Ad-Cre for conditional inactivation of p53 and Rb in $p53^{LoxP/LoxP}$; $Rb^{LoxP/LoxP}$ mice which resulted in ovarian tumor formation in 97% of them (39% low grade serous, 45% poorly differentiated, and 15% undifferentiated carcinomas). Peritoneal carcinomatosis and ascites were present in 27 and 24% of the cases respectively. Dinulescu and colleagues (91) developed the first model of endometrioid EOC by conditional expression of an activating KRAS mutation and inactivation of PTEN via intrabursal administration of Ad-Cre in LoxP-Stop-LoxP-$KRAS^{G12D/+}$; $PTEN^{LoxP/LoxP}$ mice. Endometrioid EOCs developed in all mice as early as 7 weeks after injection and were associated with ascites, peritoneal carcinomatosis, and lymph node involvement. Endometrioid EOCs also developed in $PTEN^{LoxP/LoxP}$; $APC^{LoxP/LoxP}$ mice after conditional inactivation of PTEN and APC using intrabursal injection with Ad-Cre (92). These tumors had short latency, 100% penetrance and were associated with peritoneal carcinomatosis and ascites in 21 and 76% of the cases. Importantly, the gene-expression profiles of these tumors closely resembled those of human endometrioid EOCs, particularly those with mutations in the Wnt/b-catenin and PI3K/PTEN pathways suggesting that these models may be promising preclinical experimental platforms for evaluation of novel anticancer agents for these tumors. Another conditional

model was reported by Kinross and colleagues (93) whereby intrabursal administration of Ad-Cre for conditional activation of the PI3KCA-H1047R mutation and inactivation of PTEN resulted in ovarian serous adenocarcinomas and granulosa cell tumors.

Finally, a HGSC model was reported by Kim and colleagues (94) by conditionally deleting DICER, a key gene for microRNA synthesis, and PTEN using anti-Mullerian hormone receptor type 2-directed Cre (Amhr2-Cre). HGSCs developed from the fallopian tube in $DICER^{LoxP/LoxP}$; $PTEN^{LoxP/LoxP}$; $Amhr2^{cre/+}$ mice and spread to encapsulate the ovaries and then metastasize throughout the abdominal cavity killing all mice by 13 months. These fallopian tube HGSCs exhibited molecular similarity with human high-grade serous ovarian cancers suggesting that they may be used as preclinical models for drug development. Interestingly, removal of fallopian tubes but not of the ovaries prevented cancer formation confirming the fallopian tube origin of these cancers and providing further support to the hypothesis that the fallopian tube is the primary origin of high-grade serous ovarian cancer (95).

LIMITATIONS OF ANIMAL MODELS FOR PRECLINICAL EVALUATION OF EXPERIMENTAL AGENTS

Although certain genetically engineered mouse models of EOC mimic the origin, histopathology, clinical behavior (peritoneal carcinomatosis, ascites formation, lymph node involvement, and sensitivity to platinum), and molecular fingerprints (gene-expression profiling and mutational events) of EOC, there are several limitations of these models particularly relevant to their use for preclinical evaluation of novel anticancer agents (84). The most significant challenge is the species-specific differences between humans and mice. Telomerase is active in most mouse cells (unlike human cells where it is inactive) and therefore mice tumors require fewer genetic alterations for malignant transformation compared to human tumors. Mouse telomerase activity prevents adequate modeling of the genomic instability of human tumors, particularly of HGSCs which are characterized by high degree of genomic instability. Furthermore, fundamental differences in drug metabolism (protein binding, metabolic rate, and pathways of metabolism) between mice and humans represent a major challenge when mouse models are used for preclinical testing.

Another issue is that mouse models rely on specific oncogenes and tumor suppressor genes while ignoring other aspects of tumor development such as the host immune system and the tumor microenvironment. Due to the limited number of genetic alterations that induce the development of mouse tumors, mouse models are relatively homogeneous and thus may not adequately recapitulate the significant molecular heterogeneity of human tumors which is an essential element of a good preclinical model. Finally, logistical issues including cost, technical challenges in generating GEM models especially GEMs with multiple genetic alterations, long interval until development of tumors and variable penetrance are important limitations of GEM models for preclinical evaluation of novel anticancer drugs.

CONCLUSION

Despite significant advances in surgical and medical management, EOC remains a highly lethal malignancy for which new therapeutic strategies are urgently needed. Appropriate experimental platforms that recapitulate the complexity of these tumors are critically important for evaluation of novel therapeutics. **Table 3** presents the cell/animal models used for preclinical evaluation of selected experimental agents in EOC and shows the outcome of clinical phase II/III evaluation of these agents. In the first two cases (antiangiogenic agents and PARP inhibitors), cell lines and xenograft models successfully predicted the activity of these agents

Table 3 | Preclinical evaluation of selected experimental agents used against EOC.

Agents	Preclinical models	Reference	Comments
Antiangiogenic agents e.g., bevacizumab	NIH:OVCAR-3 and other cell line xenografts were used for preclinical evaluation of antiangiogenic agents as single agents and in combination with other cytotoxics e.g., paclitaxel	(41, 42, 96)	Clinical evaluation of antiangiogenic agents as single agents and in combination in phase II and phase III trials in ovarian cancer confirmed the preclinical observations (43, 44, 97, 98)
PARP inhibitors (PARPis) e.g., olaparib	Proof of principle in BRCA-deficient cell lines (embryonic stem cells and Chinese hamster cells) and xenografts from these cell lines	(17, 99)	Clinical evaluation of PARP inhibitors in patients with BRCA-associated tumors confirmed the preclinical observations in breast and ovarian cancers (18, 103, 104)
	In vivo evaluation in PDX model of BRCA2-associated ovarian cancer and in genetically engineered mouse models of BRCA1 and BRCA2-associated breast cancer	(100–102)	PARPis are currently in phase III clinical trials
Anti-CA125 antibodies e.g., oregovomab, abagovomab	Xenografts with the CA125 positive NIH:OVCAR-3 cell line were used for preclinical evaluation of these agents	(105, 106)	No PFS or OS benefit was detected in large randomized phase III trials for either oregovomab and abagovomab (107, 108)
Anti-HER-2 agents e.g., trastuzumab, pertuzumab	NIH:OVCAR-3, SKOV3, and OVCA433 cell lines and associated xenografts were used for preclinical evaluation of anti-HER-2 drugs as single agents	(109, 110)	Limited single agent activity of trastuzumab and pertuzumab in ovarian cancer (111, 112) Improved PFS with pertuzumab and gemcitabine in platinum resistant ovarian cancer (113)

in phase II/III clinical trials, while in the case of anti-CA125 antibodies and anti-HER-2 agents, preclinical evaluation did not correlate with their phase II/III evaluation. These examples highlight the challenges of preclinical evaluation of novel agents in EOC and underscore the need for appropriate preclinical platforms for a wide variety of experimental agents, i.e., immunotherapies, targeted agents, etc.

In conclusion, cell lines with confirmed molecular identity using targeted sequencing and copy number profiling may be extremely valuable as *in vitro* models, particularly those with well defined molecular alterations such as BRCA1/2 or PI3K mutations. Xenograft models of established EOC cell lines are still commonly used in preclinical drug development, but are increasingly giving place to PDXs which offer the important advantage of closely resembling original patients' tumors and adequately capturing the molecular and intratumoral heterogeneity of the original tumors. Finally, genetically engineered mouse models hold promise as they may mimic all major elements of human EOCs including stromal-tumor interactions without the requirement of an immunodeficient background. Clearly, there is no one best preclinical EOC model. Rather, preclinical evaluation of experimental anticancer agents should include multiple model systems in order to increase the possibility of correctly predicting their clinical activity.

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Modeling platinum sensitive and resistant high-grade serous ovarian cancer: development and applications of experimental systems

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High-grade serous ovarian cancer remains the most common sub-type of ovarian cancer and, characterized by high degrees of genomic instability and heterogeneity, is typified by a transition from early response to acquired resistance to platinum-based chemotherapy. Conventional models for the study of ovarian cancer have been largely limited to a set of relatively poorly characterized immortalized cell lines and recent studies have called into question the validity of some of these as reliable models. Here, we review new approaches and models systems that take into account advances in our understanding of ovarian cancer biology and advances in the technology available for their generation and study. We discuss primary cell models, 2D, 3D, and organotypic models, and "paired" sample approaches that capture the evolution of chemotherapy failure within single cases. We also overview new methods for non-invasive collection of representative tumor material from blood samples. Adoption of such methods and models will improve the quality and clinical relevance of ovarian cancer research.

Keywords: high-grade serous ovarian cancer, platinum sensitive, resistant, tumor heterogeneity

INTRODUCTION

Ovarian cancer is currently the fourth leading cause of cancer deaths in women in the UK and the most common cause of gynecological cancer deaths, with approximately 4300 deaths from the disease in 2011 alone (http://www.cancerresearchuk.org/cancer-info/cancerstats/types/ovary/). The mortality rate for ovarian cancer is high as disease largely remains undetected, due to the vague nature of its symptoms and lack of reliable biomarkers, until patients finally present with high volume, disseminated disease. The current standard care for ovarian cancer involves cytoreductive surgery followed by combination chemotherapy with platinum compounds and taxanes. However, chemoresistant disease typically recurs in patients, most commonly in the high-grade serous (HGS) sub-type, with a low 5-year average survival rate of less than 40%. Ovarian cancer is a very heterogeneous disease, with the four most common sub-types of epithelial ovarian cancer (EOC) being serous, endometrioid, mucinous, and clear cell. They can be further divided into low-grade type I (relatively resistant to platinum-based chemotherapy) and the more common high-grade type II (more responsive to initial platinum-based chemotherapy but paradoxically poorer prognosis) tumors. Type I tumors, including low-grade serous and endometrioid, mucinous and clear cell histotypes, make up 10–20% of EOC, present at early stage (FIGO I-II), genetically have near normal gene copy number, are usually wild type for p53 and harbor characteristic mutations in genes such as *Ras* (mucinous and low-grade serous) and *PTEN* (endometrioid) among others. Type II lesions including HGS cancers, undifferentiated cancers, carcinosarcomas, and high-grade endometrioid, typically present at advanced stage (FIGO III–IV), are characterized by high genomic instability (near 50% deficiency in Homologous Recombination repair), near 100% p53 mutation rate, and have extensive DNA copy number changes (1).

The development of platinum-resistant disease is a critical and poorly understood problem in ovarian cancer, especially in the most prevalent HGS sub-type. Broadly, two potential models for the evolution of chemoresistance in HGSOC are proposed; one suggests that treatment with DNA-damaging platinum therapy causes mutations that give rise to resistance and the other suggests that genetically heterogeneous tumor clones exist prior to chemotherapy and subsequent treatment preferentially selects resistant clones for survival while platinum sensitive clones are eradicated by chemotherapy treatment (2). A genomic analysis of cell lines derived from three serous ovarian cancer patients, both before and after acquisition of clinical platinum resistance, indicated that in addition to shared genomic features, sensitive and resistant tumor cells from the same patient also exhibit mutually exclusive genomic characteristics, indicating that rather than a direct linear evolution of resistance from sensitive disease in response to platinum challenge, platinum-resistant clones are present from the outset within the sensitive presenting tumor (3). However, the bulk of research in this area has suffered due to the lack of appropriate models for developing effective therapeutic solutions to counter chemoresistance; and an inadequate sampling of tumor tissue, potentially missing the rich heterogeneity of HGS disease and hence the ability to study its underlying biology. Furthermore, many mechanistic studies investigating platinum

resistance have to date relied on cell lines in which platinum resistance is derived *in vitro*, the mechanisms of which may have little or no relevance in the clinical setting (4).

Therefore, there is an urgent need to develop new models of platinum-resistant and refractory ovarian cancer to help improve outcomes for patients with chemoresistant disease. In this review, we will outline the procedures, technical challenges, and applications of modeling platinum-based chemoresistance in primary tumor cell cultures derived from ascites and solid tumors; the development of new immortalized cell lines and currently available cell line models of platinum-sensitive and -resistant HGS; and alternative systems of capturing tumor biology and heterogeneity in HGS disease.

DEVELOPMENT OF PRIMARY MODELS OF HGS DISEASE

The clinical relevance of cell line models is a topic that is often debated. The use of established cell lines, while certainly not without their merits, may misrepresent responses to targeted therapies and users should research carefully the nature of the cell line models they chose, and how closely they relate to the clinical condition. To uncover the molecular mechanisms driving EOC development and treatment, suitable disease models must be available to faithfully mirror the disease *in vitro* and *in vivo*. For the study of drug resistance, especially when testing novel therapies, *ex vivo* models or cell line models that closely mimic the *in vivo* situation are required. The use of patient material such as ascites (a rich source of tumor cells) or solid tumor allows us to derive primary tumor cell cultures that closely resemble the patient situation, therefore representing a more experimentally accurate model than poorly characterized immortalized cell lines, often of uncertain origin. Primary tumor cell cultures are kept for a relatively short period of time and can be cumbersome to start and maintain in culture, but can be developed into well-annotated secondary immortalized cultures. Different methodologies have been developed for the isolation of EOC tumor cells from ascites and solid tumors. Here, we outline a number of recently published methods to retrieve and culture EOC tumor cells, the methods for development of immortalized cell lines from primary cultures and options available for 3D cell culture systems that attempt to more closely model the *in vivo* setting.

ISOLATION AND CULTURE OF EOC TUMOR CELLS FROM ASCITES

Ascites fluid can be a rich source of tumor cells that are highly accessible following paracentesis from the patient. Isolation and primary culturing of tumor cells from ascites have become more widespread and several different methods have been established to achieve this aim (5–8). A widely used protocol for the propagation of EOC tumor cells was developed by Langdon et al. (6, 9). Freshly drained ascites fluid, mixed with heparin to prevent cell aggregation, is pelleted by centrifugation, resuspended in PBS, and subjected to gradient centrifugation using histopaque or Ficoll-hypaque to remove any contaminating erythrocytes. The resulting interface layer is washed in PBS prior to culturing in appropriate tissue culture media, monitored carefully for fibroblast or mesothelial cell contamination, with the EOC tumor cells sub-cultured upon confluency (6, 9, 10). Alternatively, Shepherd et al. mix ascites 1:1 with M199/MCDB105 growth medium and monitor EOC tumor cell growth in culture, relying on EOC cells adhering to the plastic and contaminating erythrocytes being removed in the first set of media changes approximately 4 days after initial seeding (7). Similarly, methodology favored by Mes-Masson and colleagues directly mixes the EOC cells with growth media with minimal manipulation of the ascites-derived EOC cells (11–14). The different adherence rates of particular cells can also be used to separate EOC tumor cells from ascites (15). In this study, ascites cells were seeded onto low attachment plates for 24 h, following which two distinct populations of cells were observed: multicellular aggregates floating in media; and spindle-like fibroblast cells adhered to the low attachment plates. Further characterization identified the non-adherent cell population to be epithelial cell adhesion molecule (EpCAM) and CA125 positive EOC tumor cells (15), thus indicating that differential rates of adherence to plastic can be used to minimize contamination from other ascites cell types.

Different media can also influence the growth of primary tumor cells. Originally optimized for the growth of primary ovarian surface epithelial (OSE) cells (16), a combination of M199/MCDB105 media has been used for culturing primary ovarian tumor cells derived from ascites or solid tumor (7, 11). Supplements such as EGF and hydrocortisone have also been used in different cell culture media preparations; however, use of these agents in culturing OSE cells have been shown to initiate EMT (17). Continuous growth of primary ovarian tumor cells can also be achieved using commonly used culture media RPMI or DMEM (9). Addition of pre-cleared autologous ascites fluid to EOC cultures may aid in enhanced growth of cultures (9). Successful establishment of ovarian serous carcinoma cell lines has also occurred using serum-free culture media (18, 19).

A significant problem that arises however in the growth of EOC tumor cells following isolation from ascites is the presence of other contaminating cells such as fibroblasts or mesothelial cells. Careful monitoring of tumor cells in culture from any culturing technique is required to prevent contamination of the cultures from these cell types. Proliferation of fibroblasts will cease after a number of passages; however, if not contained from initial culturing, fibroblasts can outgrow the tumor cells and take over the culture. Selective trypsinization with low concentrations of trypsin can be used to remove contaminating fibroblasts or mesothelial cells from primary cultures, as they tend to detach more rapidly from plastic than tumor cells (6). However, this may need to be repeated several times to maintain a fibroblast-free culture.

Recent advances have lead to the use of magnetic enrichment for detection or purification of cells from fluid specimens (20). The EpCAM (CD326) is a transmembrane glycoprotein expressed by particular epithelial cell types in healthy individuals; however, it is over-expressed in most carcinomas (21) and is a target in antibody-based therapies, e.g., trifunctional bispecific antibody catumaxomab was approved by the European Medicines Agency 2009 for the treatment of malignant ascites (21–23). The over-expression of EpCAM in carcinomas has been exploited in the development of purification systems such as CD326 microbeads (from Milteny Biotec, Germany) or BerEP4 Dynabeads (Life Technologies, USA) allowing for the enrichment or depletion of EpCAM positive cell populations from fluid specimens such as ascites

or pleural effusions. An advantage to selectively enriching EOC tumor cell populations prior to culturing is reduced contamination from fibroblasts and other cell types in the initial culture, thus not requiring monitoring for overgrowth of contaminating cell populations. Immunofluorescent staining for EpCAM performed on ascites cells (pre-cleared using histopaque-gradient centrifugation) before and after EpCAM microbead purification can be used to confirm highly enriched EpCAM positive EOC tumor cell populations post purification (authors unpublished observations). However, when using this additional step for isolation of EpCAM positive epithelial tumor cells, one must take into account that tumor cells that do not express EpCAM or express EpCAM at very low levels will be excluded, thus potentially losing important sub-clones of tumor cells. Cells that have undergone EMT, such as circulating tumor cells (CTCs), may have low or absent levels of EpCAM so using such enrichment techniques may fail to isolate these cell populations (24).

DISSOCIATION OF EOC TUMOR CELLS FROM SOLID TUMORS

Several laboratories over the previous decades have developed various methods for EOC tumor cell isolation from solid tumor and metastatic deposits. Langdon et al. have advocated mechanical dissociation of tumor fragments using crossed scalpels, with cell suspensions filtered through sterile gauze to remove any remaining cell clumps before placing in culture (6, 10). The laboratories of Nachtigal and Mes-Masson have employed both mechanical disruption of tumor tissue using cell scrapers and enzymatic disruption of tumor using collagenase and concluded that mechanical dissociation was the more efficient method for their purposes (13, 14). More recently, Sueblinvong and colleagues compared a number of enzymes (collagenase A, hyaluronidase, and dispase II) commonly used for tumor dissociation and digestion times to mechanical disruption, examining viability and proliferation of the isolated tumor cells, and determined that 30 minute incubation with dispase II was optimal for dissociation of viable EOC tumor cells for primary culture and downstream applications (25). As with tumor cell cultures derived from ascites, careful monitoring of tumor-derived EOC cells in culture is required to minimize fibroblast contamination.

DEVELOPMENT OF IMMORTALIZED SECONDARY CELL LINES

Primary cell lines have the disadvantage that they are short lived and may only be sub-cultured for days, weeks, or at most a number of months. A secondary immortalized cell line can be obtained from a primary culture: these have the advantages in that one is studying a pure and expandable population of tumor cells, uncontaminated with fibroblasts or other stromal cells, and are a continuous source to be accessed repeatedly *ad infinitum*. Establishment of a secondary culture can be achieved either by spontaneous or induced transformation of cells, e.g., SV40 T antigen induced immortalization. Spontaneous immortalization of cells can occur as cells maintained in culture over time can overcome senescence without the addition of exogenous agents to induce immortalization (26). Recently, platinum sensitive and resistant HGSOC lines were derived by Letourneau et al. and deemed to be immortalized when passaged more than 50 times (13). Alternatively, cell line models, such as the cisplatin-resistant HEY ovarian cancer cell line, have been developed from xenografted ovarian tumors passaged in immunologically deprived mice (27).

Normal controls of cancer tissues for comparative studies are also required but spontaneous immortalization of cultured normal cells is extremely rare and in the case of normal breast cells has only been observed in epithelial cells (28). *In vitro* immortalization is therefore necessary to induce secondary cultures of normal cells. Different methods have been employed to overcome the growth arrest barrier including transduction of viral oncogenes [reviewed in Ref. (28, 29)], radiation treatment (30), or carcinogenic chemical treatment (31, 32): viral oncogenic transformation methods have been used most commonly and successfully. Immortalization of normal human OSE cells has been induced using various methods, for example, using telomerase and temperature-sensitive SV40 large T antigen (33) and more recently immortalization of fallopian tube secretory epithelial cells (FTSEC) have been established by expressing human telomerase reverse transcriptase and perturbing the p53 and pRb tumor suppressor pathways (34). However, a caveat to the establishment of any secondary culture is that the cell clones that survive and become immortalized may be derived from a subpopulation particularly well adapted to cell culture conditions, but may not necessarily be the best representation of the actual tumor.

3D MODEL SYSTEMS OF HGS DISEASE

The vast majority of data produced on ovarian cancer and therapeutic responses is based on 2D cell culture models, whether they are *ex vivo* primary cells in short-term culture or immortalized cell lines, which both have distinct advantages but ultimately do not represent the three-dimensional nature of the human *in vivo* situation. Within the peritoneal cavity, transformed epithelial tumor cells can freely disseminate and be carried by the flow of peritoneal fluid (35). Spheroids of tumor cells can adhere to peritoneal mesothelial cells, anchor in the submesothelial matrix, and invade to form secondary lesions (36, 37). Thus, mimicking this system of adhesion, migration, and invasion *in vitro*, and thereby creating a more physiologically relevant microenvironment, could improve the concordance between predictions made in the laboratory and the clinical situation.

The 3D cultures can be created using several different methods; culturing cells within extracellular matrix gels (38), on low-adherent plastics (15) or non-adherent (polyHEMA)-coated tissue culture plastics (39); hanging-drop culture methods (13, 40, 41); spinner flasks (42); or rotary cell culture system (39). Recreating the 3D architecture of tissues and solid tumors using these methods better recapitulates primary tumor architecture than 2D monolayer culturing of cells. The 3D system could also be used as a predictive preclinical model, treating tumor cells in this *ex vivo* environment and determining response to therapy. Several studies have emerged recently using 3D models to elucidate mechanisms of drug resistance in EOC (43–47). A large-scale study using 3D models of 31 epithelial cell lines, compared their biological and molecular features with 2D cultures, and determined their response rates to chemotherapy agents, with 3D cultures differentially expressing adherens junction proteins (47), and in concordance with previous studies, 3D cultures were frequently more

chemoresistant than their 2D counterparts (43–46). Furthermore, gene expression profiles of 3D cultured cells differ significantly from their 2D profiles, with 3D cultures resembling more closely the tissue of origin (41, 48, 49). However, one study revealed no major gene expression profile differences in OVCAR-5 cells grown in either 2D or 3D culture (50). The differences between these studies may be due to cell line variability or variability in 3D systems used.

Kenny et al. among other groups, have taken the 3D model a step further and describe a 3D organotypic model of ovarian cancer metastasis, mimicking human peritoneum and omentum (35, 51). This model has the potential to advance our understanding of invasion and metastasis, allowing researchers to work with a highly physiologically relevant model. The 3D model can be assembled to histologically mimic the *in vivo* situation, with primary human omental mesothelial cells, primary human omental fibroblasts, and primary ovarian tumor cells to create patient-specific biology and drug treatment options *ex vivo* (35). As with all model systems, there are advantages and disadvantages: the main disadvantages being that the primary cultures are usually viable for only a short period of time (around 1 week) and that the 3D models lack vasculature, host immune cells, and other *in vivo* factors. However, an *in vitro* 3D model of EOC represents a significantly more complex experimental system than monolayer cell cultures for analysis of tumorigenesis and development of new therapeutic approaches.

In the next section, we will discuss the most relevant current cell line models of platinum sensitivity and resistance in HGS and how their development has aided our understanding of chemoresistance.

CURRENT CELL LINE MODELS OF PLATINUM-SENSITIVE AND -RESISTANT HGS CANCER

Several cell lines exist representing platinum-sensitive or -resistant HGS cancers, derived from patient tumor or ascites prior to chemotherapy/chemoresistance and following resistant relapse. The best known of these are three sets of platinum sensitive and clinically acquired platinum-resistant HGS cell lines established by Langdon et al. (10). These cell lines were derived from the ascites or pleural effusions of platinum-sensitive patients and again following their relapse with platinum-resistant disease. These were the first sets of clinically acquired sensitive and resistant HGSOC models and have become an important resource in the study of chemoresponse and resistance EOC (52–55). In two of the sets, ascites or pleural effusion was obtained prior to chemotherapy (PEO14 and PEA1), with the other lines derived from cells obtained either after chemotherapy (PEO1, PEO4, PEO6, PEA2, and PEO23) or radiotherapy (PEO16). PEO1 cells, while derived following chemotherapy treatment, were done so following chemosensitive relapse. Disappointingly, there have been few paired cell line models that accurately depict acquired resistance to chemotherapy. The laboratories of Mes-Masson and colleagues have also established similar cell lines representing platinum sensitive and clinically acquired resistance from the same patient and have also generated further unpaired platinum-sensitive and -resistant cell lines (13, 56). **Table 1** shows the list of available paired HGSOC cell lines that exist currently.

Cell line models of platinum sensitive and clinically acquired platinum-resistant HGSOC have been used over the past decade examine the hypotheses regarding clonal evolution of tumor heterogeneity and treatment failure in HGS cancer, and to develop novel therapies to reverse resistance. Cooke et al. used multiplex fluorescent *in situ* hybridization and array CGH profiling to characterize the Langdon et al. (10) cell line pairs of platinum-sensitive and -resistant HGS disease and determined, in these three cases, that platinum-resistant disease did not appear to evolve linearly from sensitive disease (3). Rather, their data implied that both cell lines shared a common ancestor from an earlier stage in tumor development. Due to the extent and type of genomic alterations observed between the pairs, they proposed that platinum-resistant disease arose from pre-existing resistant sub-clones that were selected for during chemotherapy treatment (3). Subsequent research in our laboratory using these paired HGS cell lines identified key drivers of chemoresistance including DNA-PKcs-mediated activation of pro-survival AKT following treatment with DNA-damaging platinum-based chemotherapy (55) and HDAC4-regulated STAT1 deacetylation and activation following platinum treatment of resistant cells but not sensitive ones (54).

In vitro platinum sensitive and resistant cell lines, derived artificially in the laboratory by continuous or regular repeated exposure to increasing concentrations of platinum drugs, are widely used to uncover and characterize drug-resistant mechanisms (57, 58). While they have the advantages of being well-established, easy to work with and have identified useful tumor biology, we suggest that due to the non-physiological manner in which resistance is created, they should not be used as a definitive model of platinum resistance for clinical research. We performed a gene expression profiling of clinically acquired resistance versus *in vitro* derived resistance, in cell lines derived from the same patient. This analysis showed very poor concordance in gene expression profiles between the two models (54). Many other unpaired, clinically acquired platinum-sensitive or -resistant EOC cell lines are available, and

Table 1 | Clinical characteristics of paired platinum sensitive and resistant HGSOC cell lines.

Cell line nomenclature	Histology at diagnosis	Treatment course	Reference
PEO1/4/6	Poorly differentiated serous adenocarcinoma	*Cis*-platinum/5-fluorouracil/chlorambucil	(10)
PEO14/23	Well-differentiated serous adenocarcinoma	*Cis*-platinum/chlorambucil	(10)
PEA1/2	Poorly differentiated serous adenocarcinoma	*Cis*-platinum/prednimustine	(10)
OV2295/OV2295(R2)/TOV2295(R)	Serous adenocarcinoma	Cisplatin/topotecan Paclitaxel/carboplatin Doxorubicin	(13)

have been extensively studied but as is the limitation with immortalized cell line models, which inherently develop phenotypic and genotypic alterations over time due to prolonged passaging, many established cell lines do not adequately model the clinical condition they are intended to represent (59). Commonly used epithelial ovarian cell line models such as the platinum-resistant SKOV3 cell line and A2780 have come under the spotlight recently, with multiple studies suggesting that they are poor models of HGSOC (60, 61) as, at the molecular level, they do not closely resemble typical HGS tumors. In a separate study examining drug sensitivity in 3D cultures, SKOV3 lines were shown to have hallmarks of clear cell histology (47). However, limited as models of HGSOC, these cell lines do have a utility as general models of ovarian cancer, for example SKOV3 is a good model of AKT-driven ovarian cancer, harboring an activating point mutation in PIK3CA (61). Significant strides to re-characterize existing cell lines to allow informed experimental design and interpretation of data have been made in recent years. For instance, a panel of 32 reported ovarian cancer cell lines has been systematically classified into their correct histotypes with the aim of definitively identifying more reliable models of clear cell ovarian cancer (60). These recent studies should be taken into account when choosing an ovarian cancer cell line as a model system.

It is clear that new initiatives are required to generate well-annotated and -controlled models of HGS for the ovarian cancer research community.

ALTERNATIVE MODELS OF IDENTIFYING TUMOR CELL HETEROGENEITY IN HGS DISEASE

CIRCULATING TUMOR CELLS

The identification of significant tumor heterogeneity in HGS has raised questions over how representative single biopsy sampling of tumor material is. Alternative and/or complementary methods and models for tumor identification and monitoring of tumor burden are required. Over the last decade, the need for alternative models has seen a focus on CTCs and the isolation and analysis of CTCs in peripheral blood as a model of tumor characterization and evolution. First detected in 1869 by Ashworth in the blood of a patient with metastatic cancer, CTCs have been implicated in the development of distant metastasis (62). Furthermore, the potential prognostic role of CTCs has been demonstrated with the number of CTCs at any given time in peripheral blood, in certain tumor types, e.g., breast (63), lung (64), and prostate (65), predictive of disease progression, thus allowing for monitoring of disease burden during therapy. Several different approaches have emerged to isolate and identify CTCs, for example microfluidics systems for detection of cytokeratin positive or negative CTCs (66) or EpCAM positive CTCs (67–69), or PCR-based methods for monitoring a panel of predefined genes in ovarian cancer (70, 71). However, there are a few key drawbacks to the detection of CTCs in peripheral blood. First, the low number of CTCs present in circulation makes the initial detection of tumor cells problematic. Second, the various methodologies employed to detect CTCs may not be identifying all CTCs in circulation depending on the experimental approach. Methods using EpCAM as the tumor cell selection marker will not detect sub-populations of CTCs that have undergone EMT or lost EpCAM expression by other mechanisms. Additionally, many CTC isolation methods use two-layer density-gradient centrifugation, thus leading to potential isolation of peripheral blood mononuclear cells in addition to CTCs. Altered immune profiles of mononuclear cells could bias PCR-based profiling of gene expression panels (72, 73). A recent study investigating the predictive value of CTCs in newly diagnosed and recurrent ovarian cancer patients was inconclusive, showing no correlation with clinical characteristics or patient outcomes (69), suggesting that more work is required to delineate the prognostic value of CTCs in ovarian cancer while establishing a robust system for CTC isolation. The attractiveness of the use of CTCs as a form of "liquid biopsy" to establish a diagnosis and to monitor cancer burden in patients undergoing therapy and thus a model of disease progression or resistance must be carefully considered. Advances in methodology for reliable isolation of CTCs represent a vital area for progress.

CIRCULATING TUMOR DNA

The last 2 years have seen the emergence of detection of circulating tumor DNA in plasma as a method of tracking the genomic evolution of the tumor in response to therapy (74, 75). Ease of processing and accessibility to samples makes this non-invasive system an enticing prospect for detection of disease, monitoring of tumor burden, and determining evolution of clonal heterogeneity. Whole genome, exome, and targeted deep sequencing of plasma tumor DNA as single or serial samples have demonstrated the validity of this system (76, 77). In particular, the recent study by Murtaza et al. tracked six patients with advanced breast, ovarian, or lung cancer over 1–2 years. They performed exome sequencing on multiple samples collected at different time points and observed changes in copy number (both gains and losses) and gene-specific mutations between samples. The somatic mutations found in plasma prior to and after each treatment course were analyzed to identify changes in mutation profiles that could be attributed to disease progression and drug resistance (78). A further potential advantage of this method, as with CTCs, is that it reduces samples bias that may exist using single-site biopsy sampling as ctDNA is more likely to represent the tumor genome from multiple tumor sites, thus reducing the emphasis of future analyses on single sub-clones that may not represent the most common tumor genome.

Such techniques for detecting CTCs and sequencing of ctDNA from liquid biopsies (blood and plasma) are expected to become commonplace and to be developed and validated for prognostication and patient stratification in future clinical trials without the need for invasive diagnostic procedures.

CONCLUSION

It is becoming increasingly evident that in the study of cancer cells and in particular examining drug resistance in HGSOC, more *ex vivo* and relevant *in vitro* models must be developed that more closely resemble the *in vivo* tumor environment, an opinion shared by others in recent reviews (59, 79). To that end, one of the goals of the European OCTIPS (Ovarian Cancer Therapy – Innovative Models Prolong Survival) consortium is to establish new, paired HGSOC cell lines derived from patients who are platinum sensitive

at presentation but subsequently relapse, in an effort to delineate the mechanisms behind the development of relapse and platinum resistance in HGSOC and furthermore to develop new *in vivo* systems such as the relatively high throughput avian chorioallantoic membrane (CAM) models (80) and patient-derived xenografts (PDXs) (81), to advance novel therapies to combat chemoresistance. A recent study highlighted the utility of the PDX model in HGSOC, as platinum response in the PDXs echoed clinical outcome (82), whereas Lokman et al. highlighted advantages of the CAM system in cost, throughput, and reproducibility, compared to mice, as an *in vivo* model for studying complex phenotypes in ovarian cancer (83). Ideally, a consensus needs to be reached on standardized protocols for the isolation of tumor cells from both ascites and solid tumor or metastatic deposits, both in platinum-sensitive and -resistant disease, and a standardized set of markers that will definitively differentiate EOC tumor cells from contaminating stromal cells, such as cancer-associated fibroblasts. Such efforts would ensure that the subsequent results and conclusions drawn from experiments performed on these primary tumor cell populations and subsequent immortalized tumor cells can be confidently and correctly interpreted.

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Reversing platinum resistance in high-grade serous ovarian carcinoma: targeting BRCA and the homologous recombination system

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Resistance to platinum chemotherapy is one of the main factors driving ovarian cancer mortality, and overcoming platinum resistance is considered one of the greatest challenges in ovarian cancer research. Genetic and functional evidence points to the homologous recombination (HR) DNA repair system, and BRCA1 and BRCA2 in particular, as main determinants of response to platinum therapy. *BRCA*-mutant ovarian cancers are especially sensitive to platinum, associated with better survival, and amenable to poly ADP ribose polymerase inhibitor treatment. Here, we discuss a therapeutic concept that seeks to disrupt HR capacity via targeting of BRCA1 and BRCA2 functionality in order to reverse platinum resistance in BRCA-proficient high-grade serous ovarian cancers (HGSOC). We review the molecular signaling pathways that converge on BRCA1 and BRCA2, their activation status in ovarian cancer, and therapeutic options to modulate BRCA function. Several recent publications demonstrate efficient chemosensitization of BRCA-proficient cancers by combining targeted therapy with standard platinum-based agents. Due to its inherent genomic heterogeneity, molecularly defined subgroups of HGSOC may require different approaches. We seek to provide an overview of available agents and their potential use to reverse platinum resistance by inhibiting the HR system, either directly or indirectly, by targeting oncogenic activators of HR.

Keywords: high-grade serous ovarian cancer, platinum resistance, BRCA1, BRCA2, cyclin-dependent kinases, cyclin E1, cyclin-dependent kinase inhibitors, homologous recombination

INTRODUCTION

Platinum-based chemotherapy agents, such as cisplatin, have been used in the treatment of ovarian carcinoma since the late 1970s. Cisplatin significantly improved the overall survival (OS) of women with ovarian cancer, leading to its adoption as the backbone of most chemotherapeutic regimens (1, 2). Carboplatin, a cisplatin analog, with an improved toxicity profile and equivalent therapeutic efficacy has replaced cisplatin as a standard of care since the mid-1980s (3). The next major advance in chemotherapy for epithelial ovarian cancer occurred with the introduction of the mitotic inhibitor paclitaxel, which further improved OS when combined with platinum (4, 5). Despite these advances, tumor recurrences still occur in the majority of ovarian cancer patients and cures remain too infrequent.

Epithelial ovarian cancer is a heterogeneous disease with multiple histological subtypes and multiple subclones even within a given patient's tumor. High-grade serous ovarian cancer (HGSOC) accounts for the majority of epithelial ovarian cancers (68%). A recent comprehensive analysis by The Cancer Genome Atlas (TCGA) revealed HGSOC to be highly genomically unstable with *TP53* gene mutations in more than 96% of cases, and less frequent mutations in *BRCA1* and *BRCA2*, while mutation frequencies for all other genes are each <5% (6). Other ovarian cancer subtypes, such as clear cell and endometrioid ovarian cancers have fewer *TP53* mutations and are not commonly associated with *BRCA* gene mutation (7, 8). In addition, TCGA identified a plethora of recurrent DNA copy number changes affecting known oncogenes and tumors suppressor genes. The genomic complexity of HGSOC may explain previous failures of targeted therapy approaches in unselected patient populations. HGSOC has a poor prognosis likely due to a combination of factors, including late stage at presentation and the development of chemoresistance (9). Most patients with advanced (stage III and IV) HGSOC undergo cytoreductive surgery followed by combination platinum- and taxane-based chemotherapy (4, 5). While initial response rates are quite high (~80%), the majority of patients ultimately relapse due to the emergence of chemoresistant disease (10). Once patients develop resistant disease, the options for effective salvage treatment are limited. Clinical trials investigating the inclusion of alternative chemotherapeutic and biologic agents in recurrent platinum-resistant ovarian cancer have failed to demonstrate significant improvements in OS (11), and the 5-year survival rate has remained relatively unchanged at 43% for several decades (12). Thus, there is a critical need to identify and understand the molecular mechanisms and biological pathways that contribute to platinum resistance in HGSOC.

Upon entering a cell, platinum-based compounds generate inter- and intra-strand DNA adducts that activate the DNA damage response (DDR) and subsequently induce DNA repair (13). In the absence of a functional DNA repair system, damage accumulates and cell death ensues. Here, we discuss a therapeutic concept that seeks to reverse platinum resistance in HGSOC via targeting the DNA homologous recombination (HR) repair pathway and the *BRCA1* and *BRCA2* genes in particular. We will review the role of BRCA1 and BRCA2 in determining the platinum response of the cell as well as the concept of synthetic lethality that has led the introduction of poly ADP ribose polymerase (PARP) inhibitors for the treatment of *BRCA*-mutant HGSOC. We will then outline pharmacological strategies to mimic "BRCAness" in BRCA-wildtype HGSOC and explore the use of molecularly targeted agents to exploit this pathway and sensitize the cell to platinum-induced lethality.

DNA REPAIR PATHWAYS AND PLATINUM RESISTANCE

Platinum resistance is a complex phenotype characterized by decreased platinum uptake, increased metabolic turnover, inhibition of pro-apoptotic signals, and restored DNA repair capacity [reviewed in Ref. (14, 15)]. Due to this complexity, the development of chemoresistant disease is assumed to be a dynamic process involving multiple mechanisms. As DNA alkylating agents, the cytotoxic effects of platinum drugs are largely dependent on the cell's ability to detect and repair DNA damage. Several DNA repair pathways exist and have been linked to platinum resistance. Nucleotide excision repair (NER) is the primary pathway used for intrastrand platinum adduct removal and is an important mediator of responsiveness to platinum-based chemotherapy (16). High NER activity is correlated with platinum resistance (17, 18). The mismatch repair (MMR) system functions to repair single-base pair mismatches and erroneous insertions and deletions that occur during DNA replication and recombination. Mutation and decreased expression of MMR components, MLH1 and MSH2, have been documented in ovarian and other cancers, and correlated with prognostic indicators including chemotherapy response (19, 20). In ovarian cancer, deficiencies in MMR and subsequent microsatellite instability (MSI) are estimated to account for tumor development in <10% of cases (20). However, the role of MMR inactivation and MSI in platinum response in HGSOC remains controversial, as several studies have reached conflicting conclusions (21, 22).

The most lethal lesions induced by platinum agents are DNA double strand breaks (DSBs), which are a result of platinum-induced interstrand crosslinks. These DSBs are particularly toxic as both strands of DNA are affected and there is no intact complimentary strand to utilize as a template for repair. DSBs are repaired by two major pathways within the cell: non-homologous end-joining (NHEJ) and HR. The preferred method of DSB repair is HR, as NHEJ is inherently mutagenic and can result in undesirable insertions and/or deletions. HR is a highly conserved pathway that provides error-free repair of DSBs by using the intact sister chromatid as a template, which fixes the break while maintaining sequence integrity. Due to this requirement, HR occurs during G_2 and S phases of the cell cycle (23). Two of the most well-known HR proteins are BRCA1 and BRCA2. BRCA1 is a multipurpose protein that participates in DDR activation, cell cycle checkpoint initiation, and DSB repair as a component of several supercomplexes (24). In HR, BRCA1 is localized to DSBs through its association with the abraxas-RAP80 complex, and promotes 5′-end resection of the break in cooperation with other proteins (25, 26). BRCA1 is also required in the later stages of HR, where its interaction with PALB2 and BRCA2 is necessary for the recruitment of RAD51 to DSBs and subsequent strand invasion of the sister chromatid for DNA repair (27, 28). Of note, the only well-documented function of BRCA2 is its direct binding of RAD51 in HR (29).

THE BRCA PARADOX

Mutations in *BRCA1* or *BRCA2* are found in the majority of hereditary breast and ovarian cancers and greatly increase lifetime risk for both cancers. Moreover, somatic mutations in at least one of the *BRCA* genes are present in a significant proportion of sporadic HGSOC, rendering *BRCA1* and *BRCA2* as two of the most frequently mutated tumor suppressor genes that guard against the transformation of serous epithelium to HGSOC. However, once an advanced tumor has developed, *BRCA*-mutant HGSOC are associated with better survival than wildtype HGSOC. This seeming paradox was first described by comparing outcomes of women with hereditary epithelial ovarian cancer to those of women with sporadic ovarian cancer. *BRCA* mutation carriers had significantly prolonged survival compared to patients with sporadic disease (30, 31). A meta-analysis of 26 studies comparing 1213 cases with germline *BRCA* mutations and 2666 non-carriers determined that the 5-year survival rate was 36% for non-carriers, 44% for *BRCA1* mutation carriers, and 52% for *BRCA2* mutation carriers (32). Further, the analysis by TCGA of 316 HGSOC confirmed that *BRCA*-mutant HGSOC (both hereditary and sporadic) are associated with better survival than *BRCA*-wildtype HGSOC (6). Collectively, these studies suggest that *BRCA*-deficient HGSOC respond better to standard therapies, specifically platinum chemotherapy, compared to *BRCA*-wildtype cancers, and further, that an intact HR system seems to be crucial for the survival of platinum-treated ovarian cancer cells.

RESTORED BRCA FUNCTION IN PLATINUM-RESISTANT CANCERS

An independent line of evidence supporting intact BRCA and HR function as one of the main determinants of chemosensitivity emerged from the analysis of platinum-resistant cells in which BRCA function had been restored by secondary mutations. Sakai et al. analyzed cisplatin-resistant subclones of the CAPAN1 pancreatic cancer cell line, which carries a 6174delT frame-shift mutation and lacks wildtype BRCA2 (33). Fifty percent (7/14) of the resistant clones had restored expression of *BRCA2* by intragenic deletions, insertions, or deletions/insertions. In all clones, the reading frame had been restored, and a functional protein was expressed. Similarly, frame-shift mutations in the *BRCA1* gene can be reversed by secondary mutations in cisplatin-resistant ovarian cancers (34, 35). Mechanistically, secondary mutations could be the result of error-prone DNA repair in cells that lack a functional HR system. In the presence of cisplatin, cancer cells with restored HR function are expected to have a strong selection advantage and may thus become the dominant cell clone in recurrent cancers. In a mouse model of mammary tumorigenesis induced by combined

loss of *Brca1* and *p53* (K14-Cre; Brca1$^{flox/flox}$; p53$^{flox/flox}$), *Brca1*-null tumors do not become cisplatin-resistant over the course of at least six cycles of cisplatin treatment (36). In contrast to point mutations or small insertions/deletions found in human cancers, large genetic deletions resulting from Cre-mediated recombination in this mouse model are irreversible. The inability of these murine cancer cells to restore functional *Brca1* expression may explain their sustained platinum sensitivity. Interestingly, platinum treatment cannot fully eradicate the breast tumors in this model, leaving a small fraction of surviving cells that can repopulate the tumor following withdrawal of cisplatin (36). It is tempting to speculate that the few surviving clones escape from platinum-induced death by employing mechanisms related to reduced proliferation, such as acquisition of cancer stem cell properties, or complete exit from the cell cycle [dormancy, reviewed in Ref. (37)].

EXPLOITING LOSS OF BRCA FUNCTION IN A SYNTHETIC LETHAL APPROACH USING PARP INHIBITORS

Synthetic lethality is defined as death resulting from concomitant mutation of two genes if mutation of either gene alone is associated with viability but mutation of both is lethal (38). This concept can be expanded to more than two genes and to pharmacologically modulated gene activity, e.g., loss-of-function following pharmacological inhibition of protein that is critically required in cancer cells. In the context of anticancer therapy, a synthetic lethal approach may take advantage of somatic mutations that render the tumor sensitive to specific chemotherapeutic agents but spare normal cells without the mutation. Alternatively, tumor-specific dependency on individual genes or signaling pathways ("oncogene addiction") can expose synthetic lethal vulnerabilities.

In ovarian cancer, the most prominent example of synthetic lethality involves PARP inhibition in *BRCA*-mutant cancers. PARP is a DNA repair enzyme and part of the base excision repair (BER) pathway. The HR defect in *BRCA*-mutant cancers renders them particularly sensitive to inhibition of other DNA repair pathways that compensate for loss of HR activity. Concomitant defects in the HR and BER pathways are synthetic lethal; DNA damage accumulates in PARP inhibitor-treated *BRCA*-mutant cells and may be repaired by error-prone mechanisms, such as NHEJ. As a result, complex chromatid rearrangements ensue that lead to G_2/M phase cell cycle arrest and subsequent cell death (39).

Based on the concept of synthetic lethality, several PARP inhibitors (PARPi), such as Olaparib, have entered clinical trials for ovarian cancer and other *BRCA*-associated cancers. Ovarian cancer-specific trials in patients with recurrent *BRCA*-mutant cancers showed high response rates between 30 and 60% for Olaparib (40, 41) and increased progression-free survival (42). While *BRCA*-mutant cancers are especially sensitive to PARPi, a significant proportion of BRCA-proficient HGSOC are thought to exhibit a "BRCAness" phenotype, which is caused by HR defects other than *BRCA* mutation (43). HGSOC with the BRCAness phenotype are also predicted to be sensitive to PARPi, and the identification of these cancers within the pool of *BRCA*-wildtype HGSOC could increase the proportion of PARPi-eligible patients.

TARGETING HR FUNCTION AS A CHEMOSENSITIZATION STRATEGY

In addition to identifying cancers with inherent HR defects, the active modulation of HR capacity in BRCA-proficient cancers via targeting of BRCA function is an attractive therapeutic concept. Quinn et al. showed that downregulation of *BRCA1* by RNAi increased sensitivity to cisplatin in ovarian cancer cell lines (44), thus providing a rationale for the use of pharmacological agents in order to inhibit BRCA function. Several recent publications suggest that pharmacological targeting of BRCA1 and BRCA2 can sensitize *BRCA*-wildtype cancers to platinum-based chemotherapy and PARP inhibition. In the following sections, we will outline potential therapeutic strategies that target BRCA loss-of-function as a result of transcriptional downregulation or inhibition of protein activity. We will describe the molecular pathways regulating *BRCA* gene expression and their activation in HGSOC, and the transcription factors that mediate transcriptional activation of *BRCA1* and *BRCA2*. Finally, we will discuss different classes of targeted compounds for their potential use as chemosensitizing agents in BRCA-proficient HGSOC. We hypothesize that molecular targeting of HR function can reverse platinum resistance in HGSOC.

REGULATION OF *BRCA1* AND *BRCA2*

Control of BRCA1 and BRCA2 activity involves transcriptional regulation and post-translational modifications of the BRCA proteins. As part of the DDR, BRCA1 is phosphorylated by CHEK2 and ATM in normal cells and cancer cells following irradiation or exposure to alkylating agents [reviewed in Ref. (24)]. While DDR inhibitors may be able to sensitize cells to DNA-damaging agents, this article focuses on targeting genes and pathways that are activated specifically in cancer cells and required for *BRCA* gene expression and/or activity. Importantly, many oncogenic drivers and their downstream mediators, such as proliferation-associated transcription factors, are positive regulators of *BRCA1* and *BRCA2*. Activation of BRCA1 and BRCA2 by oncogenes offers the opportunity to selectively sensitize cancer cells to platinum by targeting defined genetic alterations that are not present in normal cells. Inhibition of oncogenic drivers may result in downregulation of *BRCA* mRNA and/or inactivation of BRCA proteins.

BRCA1 and BRCA2 are regulated in a cell cycle-dependent manner. In cultured cells, *BRCA1* and *BRCA2* mRNA expression is low under conditions of serum starvation, confluency, or other factors that induce G_0 cell cycle arrest (45, 46). In contrast, rapidly proliferating cells express high levels of both *BRCA1* and *BRCA2* mRNA. This is in line with the documented function of BRCA1 and BRCA2 in HR, which occurs during the S and G_2 phases of the cell cycle, and ensures DNA replication fidelity.

Transcriptional regulation of BRCA1 and BRCA2 by ETS, MYC, and E2F

Several classes of transcription factors are involved in cell cycle progression and have been shown to regulate *BRCA1* and *BRCA2* expression. Initial analysis of the human *BRCA1* promoter identified a core promoter region that extends from about 250 bp upstream of the transcription start site (TSS) into the first exon (47, 48). This <300 bp region, which was associated with the highest

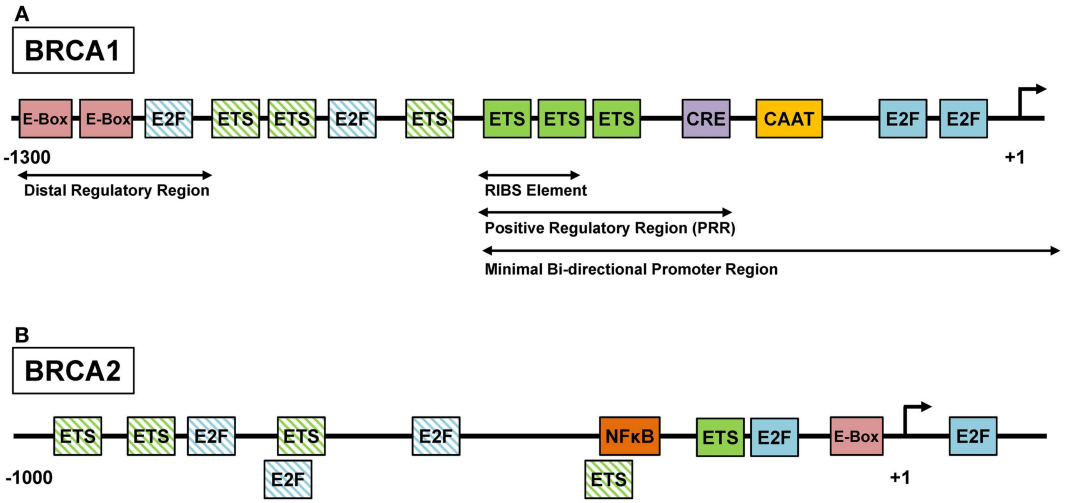


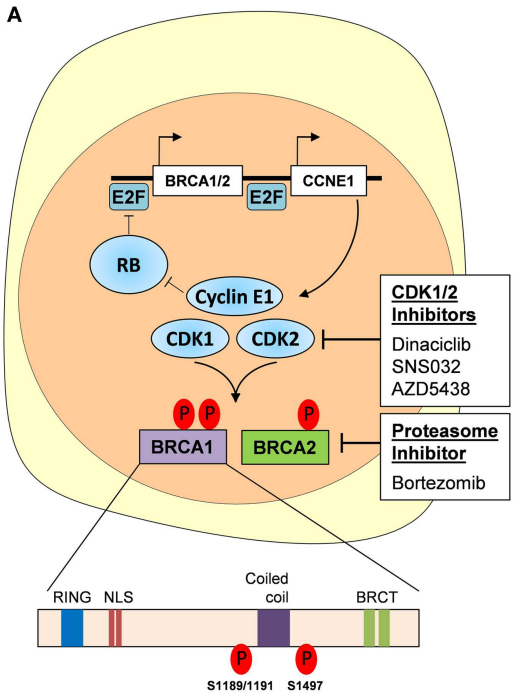
FIGURE 1 | ETS, MYC, and E2F regulate the *BRCA1* and *BRCA2* promoters. **(A)** The regulation of the *BRCA1* promoter is complex and several regulatory sites have been identified. The positive regulatory region (PPR) located at the 5′ end of the promoter has been shown to be necessary and sufficient for *BRCA1* transcription, and contains sites such as the cyclic-AMP response element (CRE). Within the PPR, the three consecutive ETS factor binding sites are known as the RIBS element and have been shown to be bound by GA-binding protein α/β (GABPα/β), an ETS factor family member. **(B)** The *BRCA2* promoter contains several conserved recognition motifs for transcription factors including E-box, E2F, and ETS (49). *BRCA2* gene transcription may be activated by the binding of ELF1, an ETS factor, to the ETS recognition motifs or by the binding of USF1 and USF2, basic helix-loop-helix leucine zipper family members, to the E-box. Additionally, NF-κB can also bind the *BRCA2* promoter and induce genes expression (59). Functionally characterized binding sites are depicted by solid colored boxes. Putative binding sites are depicted by patterned boxes and were identified using the MatInspector software (Genomatix, Munich). Diagrams are not drawn to scale.

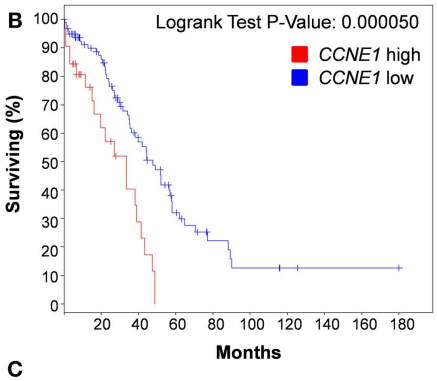
promoter activity in reporter assays, contains several E2F and ETS binding sites as well as a CREB binding site (**Figure 1A**). The human *BRCA2* promoter has been characterized to a lesser extent but also contains functionally relevant E2F and ETS binding sites within its proximal region (**Figure 1B**) (49). Traditionally, E2F transcription factors are considered as the main mediators of G_1–S progression. Activator E2Fs (E2F1–3) transcribe many of the genes involved in DNA replication, checkpoint control, and DNA repair (50). Importantly, E2F function couples proliferation and DNA repair by coordinating the induction of genes required for DNA synthesis, such as thymidine kinase (*TK1*) and dihydrofolatereductase (*DHFR*), and DNA repair, such as *BRCA1*, *BRCA2*, and *RAD51* (51). However, mouse models have demonstrated that while activator E2Fs are critical for cell cycle progression in some cell systems, E2F-independent proliferation occurs in others (52–54). There is mounting evidence that other classes of transcription factors can compensate for loss of E2F function in some cell types. For example, E2f1–3-null mouse retinal progenitor cells continued to divide possibly due to compensation by Mycn, a member of the MYC family of basic helix-loop-helix transcription factors (52). In breast cancer cells, MYC was found to directly regulate the *BRCA1* promoter via binding to distal regulatory regions (55) (**Figure 1A**). In addition, oncogenic ETS family transcription factors were shown to induce a subset of E2F target genes (56, 57), including *BRCA2* (58). Thus, a number of cancer-relevant transcription factors regulate *BRCA1* and *BRCA2*. In order to achieve effective downregulation of *BRCA* transcription, it is necessary to identify and target the main drivers of *BRCA* gene expression in different subgroups of HGSOC.

Pharmacologic targeting of oncogenic transcription factors driving BRCA expression

Some of the transcription factors driving *BRCA* gene expression are known oncogenes (*MYC*) or putative oncogenes in ovarian cancer, based on functional data and evidence of genetic activation in primary HGSOC: *MYC* is amplified in 30% of HGSOC (6), and *E2F3* is amplified in about 10% (60). While most transcription factors are not easily druggable with currently available agents, targeting of BET bromodomain proteins has been described as an effective means of inhibiting MYC-dependent transcription (61). Preclinical studies with the small molecule inhibitor JQ1 have yielded promising results in MYC-dependent hematologic malignancies, medulloblastoma (62), and *KRAS*-mutant lung cancer (63), but its compatibility with platinum-based chemotherapy, as well as its effect on *BRCA* gene expression, has yet to be established.

ETS family transcription factors have been implicated in platinum resistance. ETS1 was shown to be overexpressed in C13 cells, a cisplatin-resistant derivate of 2008 ovarian cancer cells (64), and ectopic expression of ETS1 in 2008 cells conferred platinum resistance. Similarly, ETV4 (PEA3) is overexpressed in cisplatin-resistant PEO1 ovarian cancer cells (65). Both the *BRCA1* and the *BRCA2* promoter are bound and activated by ETS transcription factors: GA-binding protein α/β (GABPα/β) binds the RIBS element in the *BRCA1* core promoter (**Figure 1A**), and overexpression of GABPα/β in breast cancer cells was able to stimulate *BRCA1* promoter activation (66). Similarly, *BRCA2* gene transcription may be activated by the binding of ELF1, another ETS family member (49) (**Figure 1B**).





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Gene Name	Median Z-score High	Median Z-score Low	p-value
CCNE1	3.1635	-0.2718	3.88E-12
BRCA1	0.0654	-0.5358	3.06E-08
BRCA2	0.4077	-0.5749	0.0069
RAD51	0.0443	-0.4135	0.0013
XRCC2	1.0171	-0.0302	0.0006
E2F1	0.8970	-0.0972	2.69E-06
BIRC5	0.1416	-0.5556	0.0001
CDK1	0.3572	-0.4719	1.20E-05
TK1	0.0494	-0.3606	0.0442

FIGURE 2 | Targeting cyclin E1-dependent ovarian cancers. (A) Cyclin E1 function is dependent on it interaction with CDK1 and CKD2. CDK1/2 inhibitors inhibit this interaction and cause E2F-mediated transcriptional downregulation of *BRCA1* and *BRCA2*. CDK1/2 inhibition may also prevent their phosphorylation and subsequent activation of the BRCA1 and BRCA2 proteins. Alternatively, use of Bortezomib, a proteasomal inhibitor, can inhibit HR function and potentially sensitize cyclin E1-dependent tumors to platinum-based chemotherapy. NLS, nuclear localization signal; BRCT, BRCA1 C-terminus domain. **(B)** Kaplan–Meier curve showing that *CCNE1* overexpression ($n = 32$ tumors, Z-score ≥ 2) in HGSOC was associated with significantly reduced overall survival (OS) as compared with tumors with normal or low expression of *CCNE1* ($n = 97$ tumors, Z-score < 2). Median OS for cancers with *CCNE1* high and normal/low expression were 33.44 and 47.47 months, respectively. A subset of 129 HGSOC tumors from the TCGA dataset was used for this analysis and was selected based on p53-mutant and *BRCA1*- and *BRCA2*-wildtype status as well as availability of RNAseq V2 data. Data were accessed using the cBioPortal for Cancer Genomics maintained by the Computational Biology Center at Memorial Sloan-Kettering Cancer Center. **(C)** Increased expression of HR and DNA repair-related genes in cyclin E1-overexpressing tumors. Known E2F targets, *E2F1*, *BIRC5* (survivin), *CDK1* (CDC2), and *TK1* are included for reference. Median Z-score values for each *CCNE1* expression subset are shown; P values were determined using Student's t-test.

Studies in prostate cancer suggest a targeting strategy for oncogenic ETS factors. Constitutive activation of ERG, ETV4, or ETV5 following gene fusion with the *TMPRSS2* promoter renders the fusion gene oncogenic in prostate cancer cells (67). ERG was shown to interact with PARP and require PARP activity for its transcriptional activity. Inhibition of PARP by Olaparib specifically sensitized ERG-driven prostate cancer xenograft to the alkylating agent, temozolomide (68). Similarly, ETS-dependent, BRCA-proficient ovarian cancers may be susceptible to PARPi. However, ETS gene fusions have not been detected in ovarian cancer, and biomarkers of ETS dependency have yet to be identified in HGSOC. Thus, direct targeting of transcription factors is an interesting therapeutic strategy, but requires additional studies prior to translation into the clinic.

REGULATION OF *BRCA1* AND *BRCA2* BY ONCOGENIC SIGNALING

A more immediate option may present itself in targeting the signaling pathways that lead to activation of transcription factors. ETS, MYC, and E2F transcription factors are downstream mediators of several oncogenic signaling pathways (**Figures 2A** and **3**). Inhibition of these pathways may result in loss of transcriptional activity and subsequent downregulation of *BRCA* gene expression. TCGA identified the retinoblastoma (RB) pathway, phosphatidylinositide 3-kinase (PI3K), and RAS signaling as the most frequently altered signaling pathways in HGSOC (6).

The RB pathway

The RB pathway governs G_1/S transition in mammalian cells. In proliferating cells, growth factor or oncogene-induced expression of cyclin D results in activation of the cyclin-dependent kinases, CDK4/6, and inactivation of the RB protein. RB forms complexes with E2F1–3 proteins and inhibits their transactivating activity. Phosphorylation of RB by CDK/cyclin complexes unleashes E2F activity, resulting in expression of E2F target genes, including cyclin E1 (*CCNE1*), *CDK2*, and *E2F1–3*, which amplify the signal in a positive feedback loop and transition the cell from G_1 to S phase. The CDK/cyclin–RB–E2F axis (RB pathway) is frequently

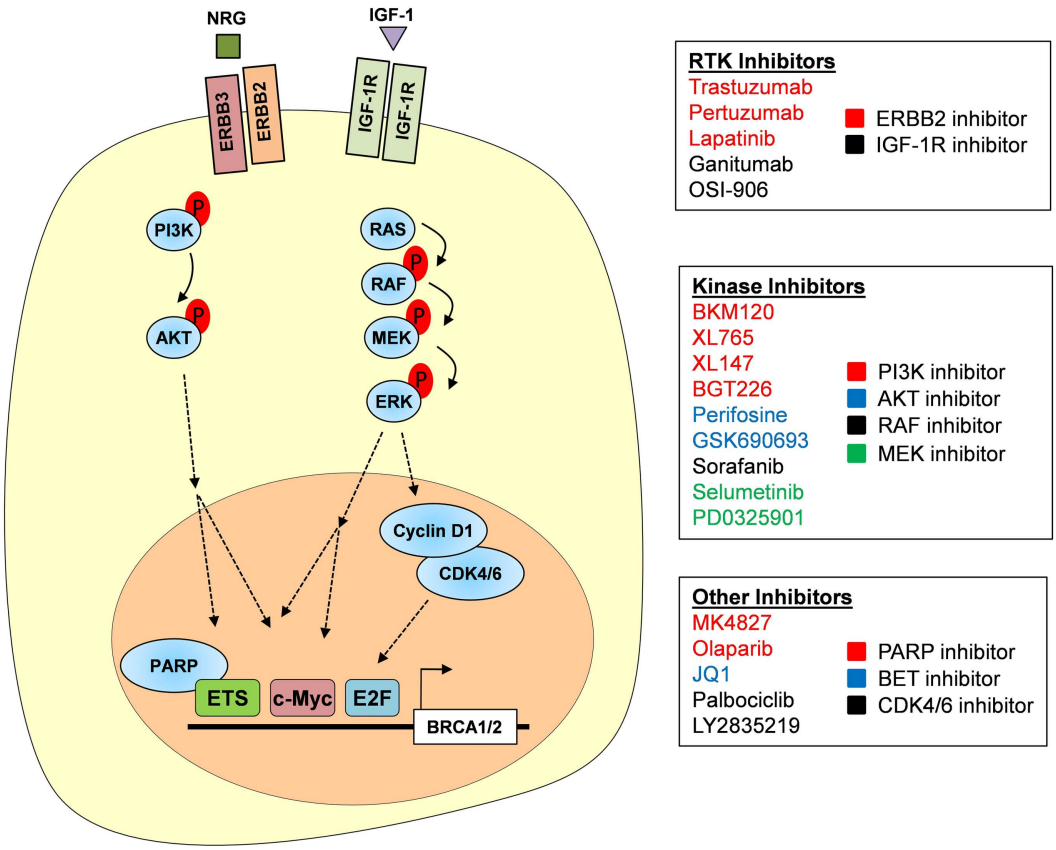

FIGURE 3 | Targeting the RTK–PI3K/MAPK axis. RTKs, including ERBB2/ERBB3 and IGF-1R, are activated by their respective growth signals and are capable of signaling through both the PI3K–AKT and MAPK pathways. Subsequent activation of AKT and ERK allows for the phosphorylation of multiple transcription factors including ETS and MYC that are important in *BRCA1* and *BRCA2* gene transcription. Activation of ERK also induces cyclin D1 production, which in complex with CDK4/6 promotes E2F-mediated gene transcription. We propose that *BRCA1* and *BRCA2* expression could be inhibited at each of these levels; inhibitors of RTKs, PI3K signaling, MAPK signaling, and CDK4/6 are listed. PARPi, such as Olaparib, may also downregulate *BRCA* gene expression as PARP1 is required as a co-factor for some ETS family members.

deregulated in cancer as a result of mutations or deletions in *RB1* (10% in HGSOC), amplification of cyclin genes (*CCNE1* is amplified in 20% of HGSOC, *CCND1* is amplified in 4%), or functional loss of endogenous CDK inhibitors (CDKi), such as $p16^{INK4A}$ (*CDKN2A*, downregulated in 30% of HGSOC) (6). The net result of RB pathway alterations in cancer is increased proliferation and elevated E2F activity compared to normal tissue. Wang et al. studied regulation of the murine *Brca1* promoter by the CDK/cyclin–RB–E2F axis. Using a luciferase *Brca1* promoter construct (+6 to −1003), ectopic cyclin D1 induced luciferase activity while RB suppressed activity. They also identified a conserved 5′-*GCGGGAAT*-3′ E2F binding site at −37 to −19 relative to the TSS (−23 to −5 in the human *BRCA1* promoter) and demonstrated physical binding of E2F protein to this region (69) within the *BRCA1* core promoter (**Figure 1A**). Hence, the RB pathway directly controls *BRCA* expression via regulation of E2F activity, and targeting of cancer-specific RB pathway lesions, such as *CCNE1*, may result in downregulation of *BRCA* gene expression.

TARGETING *CCNE1* DEPENDENCY

Direct targeting of CDK/cyclin signaling

CCNE1-amplified cancers make up a large subgroup (20%) of HGSOC that lack *BRCA* mutations (6) and are therefore less likely to respond to PARPi. For likely the same reason, *CCNE1*-amplified tumors were reported to be among the most chemoresistant HGSOC (70), and recurrent *CCNE1*-amplified HGSOC continue to be dependent on the presence of the *CCNE1* amplicon (71). The *CCNE1* gene product, cyclin E1, is a co-factor for cyclin-dependent kinases 1 and 2 (CDK1/2) and activates *BRCA1* and *BRCA2* transcription via E2F transcription factors (50) (**Figure 2A**). Therefore, *CCNE1*-amplified HGSOC often express high levels of wildtype *BRCA1* and exhibit an "anti-BRCAness" phenotype in terms of their relative resistance to platinum chemotherapy. Using the TCGA dataset, we show that cyclin E1-overexpressing, *BRCA*-wildtype HGSOC have significantly reduced OS compared to *BRCA*-wildtype cancers with lower cyclin E1 expression (**Figure 2B**). This suggests that high levels of cyclin E1 confer an added advantage to BRCA-proficient

cancers. We attribute this, at least in part, to increased expression of several HR genes, including *BRCA1*, *BRCA2*, *RAD51*, all of which have significantly higher RNA levels in cyclin E1-overexpressing cells (**Figure 2C**).

CDK inhibitors may be a therapeutic option for cyclin E1-dependent HGSOC, including *CCNE1*-amplified cancers. Cyclin E1 has the highest affinity for CDK2, its main binding partner in actively cycling cells (72–74), and CDK1 (CDC2) (75). Both CDK2 and CDK1 directly phosphorylate BRCA1 (76, 77) (**Figure 2A**). Furthermore, a functional link between CDK1 and BRCA1 has been established in lung cancer. Using an inducible shRNA targeting CDK1 in a human non-small cell lung cancer cell line, Johnson et al. showed that CDK1 contributes to S phase checkpoint control following DNA damage (77). CDK1 directly phosphorylated BRCA1 at several serine residues and is required for the formation of BRCA1 foci following DNA damage. Genetic depletion or pharmacological inhibition of CDK1 sensitized BRCA1-proficient cancer cell lines to cisplatin (77) and PARPi (78). Similarly, BRCA2 is phosphorylated by both CDK1 and CDK2 (79).

While there are still no selective inhibitors of CDK1 or CDK2, latest generation CDKi have increased specificity and potency compared to early CDKi, such as flavopiridol, which failed in the clinic. The compound Dinaciclib inhibits CDK1/2/5/9 at low nanomolar concentrations (IC_{50} values: CDK1: 3 nM, CDK2, 1 nM, CDK5: 1 nM, CDK9: 4 nM). It induces apoptosis in model systems and prevents tumor progression in A2780 ovarian cancer xenografts (80, 81). We hypothesize that of the currently available CDKi, Dinaciclib may have the best therapeutic potential in cyclin E1-dependent ovarian cancer. Our unpublished data show that Dinaciclib exposure results in downregulation of *BRCA1* and *BRCA2* and sensitized cyclin E1-dependent ovarian cancer cells to cisplatin. Hence, Dinaciclib may have a dual effect on BRCA1 and BRCA2 by causing E2F-mediated transcriptional downregulation and inhibiting CDK1/2-mediated activation of the BRCA proteins (**Figure 2A**).

Dinaciclib is currently being evaluated in a phase 3 clinical trial for chronic lymphocytic leukemia (CLL) and may have clinical potential in cyclin E1-dependent HGSOC as a chemosensitizing agent. In order to select eligible patients, *CCNE1*-amplified cancers can be easily identified by fluorescence *in situ* hybridization (FISH). Other available CDK2 inhibitors, including Roscovitine, SNS032 (82), and AZD5438 (83), may have similar chemosensitizing potential. CDK1, the only mammalian CDK required for proliferation in all cell types (84), is not inhibited by SNS032, rendering this compound potentially less potent but also less toxic to normal cells. In principle, other CDKi such as the CDK4/6 inhibitor Palbociclib (formerly PD0332991) could be used to reduce *BRCA* expression in cyclin D-dependent HGSOC. However, Palbociclib was shown to be cytostatic in most systems (85–87) and may thus interfere with the cytotoxic activity of platinum agents.

Targeting of HR components induces synthetic lethality in CCNE1-amplified HGSOC

An independent approach to target *CCNE1*-amplified HGSOC identified the proteasome inhibitor, Bortezomib (88). In contrast to CDKi, Bortezomib primarily affects the homologous repair system itself, resulting in synthetic lethality in *CCNE1*-amplified cancer cells: a genome-wide shRNA screen in 102 cancer cell lines revealed that BRCA1 was specifically required in *CCNE1*-amplified cell lines. Genetic depletion of *BRCA1* resulted in significant loss of viability in *CCNE1*-amplified cells, including the ovarian cancer cell line OVCAR3, whereas a lesser effect was observed in *CCNE1*-wildtype cells, such as the SKOV3 cell line (88). This suggests a synthetic lethal relationship between *CCNE1* amplification and loss of BRCA1 function and provides a potential explanation for the observed mutual exclusivity between *CCNE1* amplification and *BRCA* mutation (6, 89). In addition to *BRCA1*, the shRNA screen identified the DNA repair genes *ATR* and *XRCC2* as specific genetic hits in *CCNE1*-amplified cell lines, suggesting that inhibition of the DDR may be a therapeutic strategy in *CCNE1*-amplified ovarian cancer. To this end, the authors tested Bortezomib, a potent inhibitor of the HR system, in a panel of ovarian cancer cell lines and found that *CCNE1*-amplified lines were most sensitive. While Bortezomib had minimal activity as a single agent in recurrent ovarian cancer (90) combination with platinum resulted in clinical activity (91). As discussed for CDKi, specific selection of patients with *CCNE1*-amplified cancers for Bortezomib treatment may further increase the response rate.

RECEPTOR TYROSINE KINASE, PI3K, AND RAS SIGNALING

In addition to genetic aberrations within the RB pathway, several oncogenic signals contribute to cell cycle deregulation and E2F activity. Oncogenic *KRAS* (amplified in 11% of HGSOC), MYC, and receptor tyrosine kinases (RTK) all converge on cyclin D and require its function for their oncogenic activity (92–94). Moreover, a synthetic lethal relationship was described for KRAS and CDK4, the binding partner of cyclin D (94). KRAS and ERBB2 signal through the mitogen-activated kinase (MAPK) signaling pathway, which culminates in activation of ERK (**Figure 3**). Phosphorylation by ERK activates multiple cellular target proteins, including ETS, CREB, and MYC. The molecular link between oncogenic signaling pathways and expression of *BRCA1* and *BRCA2* offers additional opportunities for therapeutic intervention, including direct targeting of ERBB2 and other RTK, targeting of the MAPK pathway by specific MEK or ERK inhibitors, or targeting of the PI3K–AKT axis by specific inhibitors (**Figure 3**). However, due to the complex signaling events elicited by RTK and other oncogenic pathways, regulation of *BRCA* gene expression is only one of many downstream events, some of which may actually interfere with the intended chemosensitizing effect. Functional studies are needed to determine the compatibility of individual targeted agents with platinum and PARPi.

Targeting RTK signaling and downstream pathways

Although amplifications or mutations of individual RTK are rare events in primary HGSOC (*ERBB2* amplification: 3%, *ERBB3* amplification: <5%, *IGF1R* amplification: <4%, **Figure 3**), altered ERBB receptor signaling and overexpression of the downstream RTK mediator ETV4 were found in cisplatin-resistant PEO1 cells (65). RTK can be targeted directly by small molecule inhibitors (e.g., Lapatinib for ERBB2) or antagonistic antibodies (e.g., Trastuzumab or Pertuzumab for ERBB2). However in unselected patients, the addition of Pertuzumab to

carboplatin-based chemotherapy did not result in prolonged progression-free survival (95), highlighting the importance of patient selection and companion diagnostics to identify likely responders to RTK inhibitors. Activation of the MAPK and PI3K–AKT signaling pathways by RTK, or as a result of amplification, offers additional potential therapeutic targets [also reviewed in Ref. (96)]. Both *PIK3CA*, the gene encoding the catalytic subunit of PI3K, and the *AKT* genes are frequently amplified in HGSOC (*PIK3CA*: 28%, *AKT1*: 5%, *AKT2*: 8%, *AKT3*: 9%), indicating their oncogenic roles in ovarian cancer. Interestingly, AKT was shown to directly phosphorylate BRCA1 at serine 694 and promote its protein stability (97). A second AKT phosphorylation site at threonine 509 was described but its functional implications are unknown (98).

Functionally, a recent study in triple negative breast cancer demonstrated that PI3K signaling was required for BRCA1 function. Ibrahim et al. showed that inhibition of PI3K phenocopied loss of BRCA1 and induced synthetic lethality in combination with Olaparib (99). Loss of PI3K resulted in reduced *BRCA1* expression both in cell line models and in patient-derived tumor xenografts. Combined treatment with BKM120, a small molecule PI3K inhibitor, and Olaparib significantly delayed tumor progression in two out of three xenografts whereas single agent treatment had little effect on tumor progression. On the molecular level, the MAPK pathway mediated the transcriptional effect on *BRCA1* via ETS1-dependent downregulation of *BRCA1* (99). Interestingly, BKM120 treatment resulted in activation of the MAPK pathway and phosphorylation of ETS1 by ERK, indicating a repressor function for ETS1 in this system. This finding highlights the complexity of *BRCA* gene regulation and its dependency on genetic context. Due to feedback mechanisms and functional compensation among RTKs, downstream kinases, and transcription factors, specific pharmacological combinations may be limited in their effectiveness to small genetically defined subsets of HGSOC. Moreover, as a result of the genetic complexity and genomic instability that is a hallmark of HGSOC, individual cancers are likely to harbor or develop resistant cell clones to any given combination. Thus, it will be important to identify multiple agents targeting different pathways, all of which should be characterized with respect to: (1) their ability to induce BRCA loss-of-function, (2) their compatibility with platinum and/or PARPi, both in terms of toxicity and mechanism of action, and (3) accompanying biomarkers that allow for careful patient selection.

TARGETING BRCA PROTEIN STABILITY

A different approach to inhibit BRCA1 function involves targeting of the chaperone protein heat shock protein 90 (HSP90), required for BRCA1 protein stability (100). Interestingly, *HSP90AB1* is also amplified in a small subset of HGSOC (6). Specific inhibition of HSP90 in BRCA1-proficient breast cancer cells-induced BRCA1 ubiquitination and proteasomal degradation ultimately resulting in compromised DSB repair by HR. HSP90 inhibition sensitized BRCA1-proficient breast and ovarian cancer cells *in vitro* to carboplatin treatment at concentrations similar to BRCA1-mutant cells (100). In a subsequent study, treatment with the pan-histone deacetylase complex (HDAC) inhibitors vorinostat or panobinostat induced hyperacetylation of HSP90 gene (*HSP90AA1I0*), thereby inhibiting its chaperone function and leading to proteasomal degradation and depletion of BRCA1 (101). Of clinical significance, treatment of human triple negative breast cancer cell lines with vorinostat was able to induce BRCA1 degradation and a subsequent BRCAness phenotype, which synergistically induced cell death in combination with PARPi or cisplatin (102). Based on these preliminary studies, it is suggested that treatment of platinum-resistant HGSOC with HDAC inhibitors may also be able to induce a BRCAness phenotype and resensitize these cells to platinum or other targeted agents. Trials evaluating vorinostat as a single agent in recurrent epithelial ovarian cancer showed it had limited activity (103). Vorinostat may show better therapeutic efficacy as a biologic response modifier in combination with platinum-based chemotherapy and trials assessing this use in ovarian cancer are underway.

CONCLUDING REMARKS

In HGSOC cells, a functional HR system and intact BRCA1 and BRCA2 function are often associated with resistance to platinum-based chemotherapeutic agents and PARPi. A plethora of targeted agents are currently available to modulate BRCA function via transcriptional or post-translational intervention. With the advent of novel diagnostic tools, such as the use of deep sequencing to repeatedly profile cancers throughout their evolution, it should become possible to predict rational therapeutic combinations that prevent or at least delay the onset of platinum resistance and should ultimately result in improvements in OS.

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Platinum-sensitive recurrence in ovarian cancer: the role of tumor microenvironment

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Despite several advances in the understanding of ovarian cancer pathobiology, in terms of driver genetic alterations in high-grade serous cancer, histologic heterogeneity of epithelial ovarian cancer, cell-of-origin for ovarian cancer, the survival rate from ovarian cancer is disappointingly low when compared to that of breast or prostate cancer. One of the factors contributing to the poor survival rate from ovarian cancer is the development of chemotherapy resistance following several rounds of chemotherapy. Although unicellular drug resistance mechanisms contribute to chemotherapy resistance, tumor microenvironment and the extracellular matrix (ECM), in particular, is emerging as a significant determinant of a tumor's response to chemotherapy. In this review, we discuss the potential role of the tumor microenvironment in ovarian cancer recurrence and resistance to chemotherapy. Finally, we propose an alternative view of platinum-sensitive recurrence to describe a potential role of the ECM in the process.

Keywords: ovarian cancer, extracellular matrix, platinum-sensitive recurrence, platinum resistance, cancer stem cell

The majority of patients with advanced ovarian cancer develop recurrent disease within 3 years (1, 2) and die within 5 years because relapsed disease is almost always incurable (3). Although initial recurrences are frequently platinum-sensitive, patients eventually develop resistance to platinum-based chemotherapy (3). Accordingly, resistance to chemotherapy, whether intrinsic (primary) or acquired (secondary) resistance, is a major problem in the treatment of ovarian cancer and the main contributing factor in cancer-associated mortality.

The initial response to platinum-based chemotherapy in ovarian cancer can be broadly classified into three groups: platinum-refractory, platinum-resistant, and platinum-responsive. These classifications are based mainly on clinical evidence and useful in the clinical management of ovarian cancer. Among them, the platinum-refractory group is perhaps the easiest to conceptualize because these patients do not respond to platinum-based therapy and show progression during the course of the therapy. On the other hand, platinum resistance is defined by less than 6 months of remission following chemotherapy (3). Clinically, these patients will show initial response to chemotherapy but experience relapse within 6 months of the last round of chemotherapy, a time course often described as platinum-free interval or treatment-free interval. Treatment-free interval less than 6 months is often used as a clinical cutoff to define platinum-resistant disease because of empirical evidence (4). For patients who initially respond to platinum-based therapy, there is a spectrum of response that lasts from a little over 6 months to several years.

Although several genomic studies have been conducted to identify the underlying genetic basis of this tumor behavior in response to chemotherapy, major mechanisms or biological pathways that contribute to differential response to chemotherapy are not fully understood. It is generally accepted that multiple molecular mechanisms contribute to chemotherapy resistance and that a single mechanism is unlikely to account for tumor response to chemotherapy.

Recent review by Galluzzi et al. provides an excellent conceptual view of tumor intrinsic mechanisms associated with cisplatin resistance (5). Alterations in pre-targets (associated with drug metabolism and transport before it reaches to its intracellular targets), on-targets (associated with DNA damage signaling and repair), post-targets (associated with apoptosis and survival signaling), and off-targets (components not directly affected by cisplatin but counteract the lethal effect of cisplatin) are associated with cisplatin resistance (5). Although cisplatin-resistant mechanisms are well studied and reviewed, molecular mechanisms associated with platinum-sensitive recurrence is not well understood.

An interesting aspect of ovarian cancer is that the majority of patients who relapse long after chemotherapy can be rechallenged with the same chemotherapy (4). These patients are described as having platinum-sensitive recurrence. Therefore, the traditional view of intratumor heterogeneity and the clonal selection of resistant cancer cells by chemotherapy does not fit well with the clinical evidence because the selection of resistant cells from heterogeneous tumor cell population following chemotherapy would

have resulted in platinum-resistant recurrence and not platinum-sensitive recurrence. Platinum-sensitive recurrent ovarian cancer is a subject of numerous research and clinical studies because the majority of ovarian cancer patients fall into this category (2, 6). From a research point of view, platinum-sensitive recurrence is an enigma. In a traditional viewpoint, platinum-resistant or -refractory ovarian cancer can be explained by a simple model in which intrinsically resistant tumor cells from heterogeneous tumor population were selected for by chemotherapy resulting in emergence of chemotherapy-resistant or -refractory tumors (7, 8) (**Figures 1A,B**). It is difficult to apply this simplistic model to platinum-sensitive recurrent disease because not all tumor cells that persist through initial rounds of chemotherapy become resistant to chemotherapy. In fact, provided that patients experience long remission prior to relapse, these patients will likely respond to platinum-based chemotherapy again.

CANCER STEM CELLS AS A MECHANISM OF PLATINUM-SENSITIVE RECURRENCE

With the emergence of cancer stem cell hypothesis, platinum-sensitive recurrence could be explained by putative cancer stem cells. Agarwal and Kaye proposed that in patients with platinum-sensitive recurrence, heterogeneous chemo-naïve tumor may contain clonal population of chemotherapy-sensitive tumor cells, quiescent, or dormant tumor cells that are resistant to chemotherapy, and chemotherapy-resistant tumor cells (**Figures 1C,D**). The last two groups of tumor cells may constitute a small proportion in the tumor. Therefore, upon treatment, the bulk of tumor will respond to chemotherapy, and patient will experience long remission. However, upon the completion of chemotherapy, the last two populations of cells persist as residual tumor cells, and they begin to regrow and repopulate the tumor, resulting in recurrence (**Figures 1C,D**). In this model, quiescent or dormant tumor cells that persist after chemotherapy repopulate the tumor with rapidly proliferating chemotherapy-sensitive tumor cells, thus leading to platinum-sensitive recurrence. This model is supported by the observation of increased density of post-chemotherapy residual tumors having increased cancer stem cells, but recurrences remote from treatment having similar densities of cancer stem cells as the primary tumor (9). With subsequent rounds of platinum-based chemotherapy, the initially small fraction of intrinsically resistant, non-quiescent tumor cells continue to expand, thus leading to eventual platinum resistance. In this model, putative tumor stem cells fit the role of chemotherapy-resistant, quiescent tumor cells that persist after chemotherapy and repopulate the tumor with differentiated, chemotherapy-sensitive tumor cells. Several studies indicate the presence of putative cancer stem cells in ovarian cancer, thus supporting the plausibility of tumor stem cells in platinum-sensitive recurrent ovarian cancer (10, 11).

TUMOR DORMANCY AS A MECHANISM OF PLATINUM-SENSITIVE RECURRENCE

In addition to putative cancer stem cells that exist as quiescent, dormant, or intrinsically resistant tumor cells that persist through chemotherapy and repopulate the tumor after chemotherapy, some tumor cells may enter dormancy due to specific tumor microenvironment. It is suggested that cancer cells in transit and cancer cells in unfavorable microenvironment (such as hypoxia, nutrient stress, and lack of growth factors) may enter dormancy (12). For example, Kreso et al. studies indicate that dormant cells in colorectal cancer persist through chemotherapy though they retain potent tumor initiating potential (13). Therefore, after non-quiescent tumor cells are eliminated by chemotherapy and when favorable microenvironment is restored, these dormant cells have the potential to repopulate the tumor (13). In addition, recent studies have shed more light into autophagy as a player in inducing tumor dormancy. Elegant studies by Lu et al. (14), showed that although the tumor suppressor gene *ARHI* promoted autophagy-induced cell death *in vitro*, factors from the tumor microenvironment switched *ARHI*-induced autophagy to a tumor survival mechanism and caused tumor dormancy *in vivo*. Therefore, autophagy and tumor dormancy may constitute another mechanism by which tumor cells persist through chemotherapy and repopulate the tumor upon completion of chemotherapy, thereby resulting in recurrence. Interestingly, Lu et al also show that the inhibition of tumor microenvironment-induced autophagy with the autophagy inhibitor chloroquine results in cell death (14), and therefore autophagy may be therapeutically exploited to minimize tumor dormancy and enhance therapeutic effect of conventional chemotherapy.

MATRIX-DEPENDENT CHEMOTHERAPY RESISTANCE AS A POSSIBLE MECHANISM OF PLATINUM-SENSITIVE RECURRENCE

Here, we propose an alternate hypothesis for platinum-sensitive recurrence. In this view point, we propose that cancer cells can acquire extracellular matrix (ECM)-dependent platinum resistance (15). These matrix-associated cells persist after chemotherapy and repopulate the tumor after chemotherapy, resulting in recurrence (**Figure 1E**). Implicit in this hypothetical model is that these cancer cells are not intrinsically resistant to platinum-based therapy. Rather, they are resistant to chemotherapy due to their contact with particular components of ECM. Therefore, tumor repopulated by these persistent residual cells is likely to be sensitive to chemotherapy again if repopulated tumor cells are not in contact with the right components of ECM, thereby resulting in platinum sensitivity. It is important to note that our proposed model represents an alternative hypothesis that seeks to complement and not substitute previous hypothetical models involving tumor stem cells or cancer dormancy. In fact, our proposed hypothetical model may be related to tumor stem cells and cancer dormancy. It is suggested that tumor stem cells exist in particular niche (16, 17) and that specific components of ECM are involved in the establishment of stem cell niche (18). Therefore, it is conceivable that ECM, through its role in the maintenance of stem cell properties, may contribute to chemotherapy resistance. In addition, ECM has been shown to modulate tumor dormancy and serve as a "gatekeeper" in transition from quiescence to proliferation in cancer cells (19). Therefore, it is conceivable that ECM, through its regulation on tumor dormancy, may contribute to chemotherapy resistance.

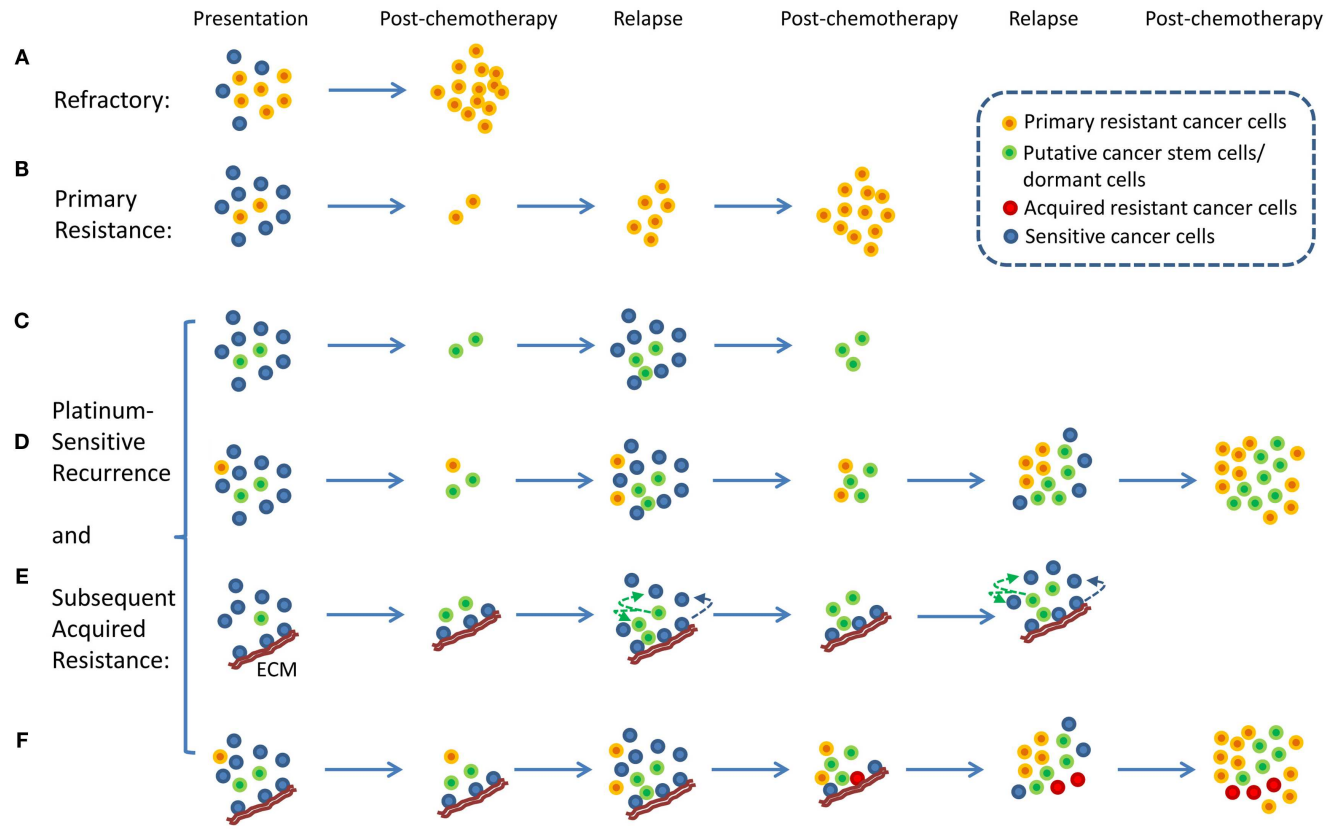

FIGURE 1 | Proposed models of platinum-resistant and platinum-sensitive recurrence. **(A)** Platinum-refractory tumor. In this model, tumor consists mainly of chemotherapy-resistant cells. Upon treatment, these resistant cells continue to expand thereby resulting in progression during the treatment. **(B)** Platinum resistance. In this model, tumor consists of a large proportion of chemotherapy-sensitive and a small proportion of chemotherapy-resistant tumor cells. Upon treatment, chemotherapy-sensitive cells are eliminated, resulting in measurable clinical response. However, resistant tumor cells repopulate the tumor, and recurrence is observed within 6 months from the last chemotherapy. **(C)** A model of platinum-sensitive recurrence. In this model, quiescence "cancer stem cells" persist following chemotherapy, and repopulate the tumor after chemotherapy resulting in recurrence. Because repopulated tumor is derived from "cancer stem cells," these tumor cells retain original phenotype in terms of their response to chemotherapy. Therefore, these tumors are expected to be sensitive to chemotherapy, thereby resulting in platinum-sensitive recurrence. **(D)** A model of acquired platinum resistance. In this model, original tumor is composed of heterogeneous population of tumor cells, consisting of a small population of chemotherapy-resistant tumor cells (orange colored cells), putative "cancer stem cells" (green colored cells), and chemotherapy-sensitive tumor cells (blue colored cells). Upon treatment, residual cells are composed of chemotherapy-resistant cells and cancer stem cells. Based on the cancer stem cell models, putative cancer stem cells are capable of re-initiating the tumor. Due to the high replicative potential of transit-amplifying cells that are derived from cancer stem cells, these cells may contribute the bulk of recurrent tumor. It is important to note that chemotherapy-resistant tumor cells (orange colored cells) will continue to expand and contribute to recurrence. However, due to the population expansion dynamics, these resistant cells may constitute a small proportion of tumor bulk. However, with subsequent rounds of chemotherapy, resistant tumor cell population will continue to expand to a point where they become the majority of tumor bulk. At this point, tumor will not display clinically measurable response, leading to clinical classification of acquired platinum resistance. **(E)** Alternative view of platinum-sensitive recurrence. In this model, tumor bulk is composed of heterogeneous population of tumor cells. Some tumor cells are associated with specific components of extracellular matrix (ECM), and this interact protect the cells from chemotherapy. These cells persist along with putative cancer stem cells, and both contribute to platinum-sensitive recurrence. It is important to note that this model posits that some tumor cells that grow out will lose their contact with particular components of the ECM, and they will become sensitive to chemotherapy. **(F)** Unified model of acquired resistance. In this model, the original tumor is composed of heterogeneous population of tumor cells consisting of cancer stem cells, quiescent or dormant tumor cells, chemotherapy-resistant cells, and tumor cells that are or are not associated with particular components of the ECM. Following chemotherapy, four types of tumor cells are posited to persist: chemotherapy-resistant tumor cells, cancer stem cells, quiescent or dormant tumor cells, and tumor cells associated with specific components of the ECM. All four cell types contribute to recurrent disease. After several rounds of chemotherapy, the model also posits the emergence of novel clones (red colored cells, derived from cells associated with the matrix, from dormant tumor cells, or from cancer stem cells) that acquired additional genetic alterations that allow *de novo* resistance to chemotherapy. All these cells may contribute to acquired resistance.

EVIDENCE SUPPORTING MATRIX-DEPENDENT CHEMOTHERAPY RESISTANCE

This proposed model is based on previous studies by various groups indicating that cancer cells grown on specific matrix proteins acquire resistance to chemotherapy (20, 21). For example, Pat Morin and his colleagues have shown that ovarian cancer cells grown on collagen VI are resistant to cisplatin (20). Moreover, cells grown on collagen VI are more resistant than cells grown

on collagen III (20), suggesting that acquired resistance is context specific. It is also interesting to note that the initial discovery of the potential role of ECM protein in cisplatin resistance was made from *in vitro* cell line models in which cisplatin-sensitive cell lines were made resistant to cisplatin by exposing the cells to increasing concentrations of cisplatin. Subsequent analysis of gene expression between the cisplatin-sensitive cells and the isogenic cisplatin-resistant cells indicates higher level of collagen VI expression in cancer cells that became resistant to cisplatin (20). These results highlight the dynamic nature of cellular response to cisplatin and suggest that chemotherapy treatment could affect the composition of the ECM by modulating gene expression within cancer cells as well as within stromal cells.

Recent studies that used gene expression profiling technologies also point to a particular group of tumors with pronounced stromal/mesenchymal gene signatures to have worse outcome compared to non-stromal gene signature groups (22, 23). In particular, Helleman et al. suggested that ECM signature is associated with chemotherapy resistance (22). Pathway analysis of gene expression data from tumors with differential response to chemotherapy showed enrichment of ECM signatures in tumors with chemotherapy resistance. In addition, Bowtell and his colleagues showed that two molecular subsets underlies platinum resistance in ovarian cancer: in one subset, cyclin E amplification is associated with platinum resistance, and in another subset without cyclin E amplification, enrichment of cell adhesion, and ECM pathways are associated with platinum resistance (23).

Recently, our own analysis of three datasets [the Cancer Genome Atlas Ovarian Cancer data set (24), Tothill et al. (25), and Bonome et al. (26)] resulted in the identification of several ECM proteins as candidate biomarkers for poor clinical outcomes with respect to recurrence and overall survival (27). In particular, we identified ECM protein fibrillin-1 as a central node in ECM network, and high levels of fibrillin-1 expression in primary tumor are associated with early recurrence in platinum-sensitive ovarian cancer (27). Moreover, another set of gene signature, that is identified from the same study to be associated with early recurrence and early death, consists of nuclear signaling mediated by Fos and Jun nuclear factors (27). These two factors are known to serve as downstream mediators of ECM signaling mediated through integrins (28). Collectively, these two sets of observations point to a potentially significant role of ECM-cell interaction in tumor cell's response to chemotherapy. It is important to note that expression levels of fibrillin-1 are not associated with platinum-resistant or -refractory ovarian cancer. Rather, it is associated with early recurrence of platinum-sensitive ovarian cancer. Based on these results, we propose a hypothetical model in which ECM, consisting of fibrillin-1 and other components, confers contact-dependent platinum resistance.

In this model, cancer cells not directly attached to specific components of ECM are sensitive to platinum and are eliminated during chemotherapy (**Figure 1E**). Cancer cells that are directly attached to specific components of ECM (such as Fibrillin-1) are resistant to chemotherapy and persist during chemotherapy. These cells repopulate the tumor giving rise to recurrent ovarian cancer. The amount of residual tumor cells that remain after chemotherapy will be dependent on the amount of ECM, and therefore determine the speed of recurrence. It is conceivable that tumors with a larger component of ECM will have larger amount of residual cells remaining after chemotherapy and quicker recurrence, whereas tumors with smaller component of ECM will have smaller amount of residual cells remaining after chemotherapy and slower recurrence. Results from our immunohistochemical analysis of fibrillin-1 support this view (27). Although ECM components in this model of acquired resistance are tumor extrinsic, it should be noted that the levels of ECM component within tumor microenvironment is a function of tumor intrinsic factors and host intrinsic factors, and therefore intrinsic gene expression within tumor cells may also contribute to differences in ECM deposition and resistance.

A UNIFIED VIEW

In this unified hypothetical model, in patients with platinum-refractory ovarian cancer, tumor contains intrinsically resistant tumor cells; thus tumor cells are refractory to treatment and progress through treatment (**Figure 1A**). In patients with platinum-resistant ovarian cancer, i.e., those that recur within 6 months from the last round of chemotherapy, chemo-naïve tumor initially contains heterogeneous populations of chemosensitive as well as intrinsically resistant tumor cells. Upon treatment, chemosensitive tumors were eliminated, thus producing partial treatment response. However, intrinsically resistant tumor cells persist and expand during the treatment, thus leading to early recurrence (**Figure 1B**). Another scenario might exist whereby these tumors contain a larger component of ECM, which allows a larger component of residual cells to persist after chemotherapy, thereby permitting quicker relapse. If this were true, these tumors that recur within 6 months from the last round of chemotherapy may still contain chemotherapy-sensitive cancer cells and may respond to chemotherapy. In fact, an objective clinical response can be obtained in small percentage of patients with less than 6 months of platinum-free interval, the so called platinum-resistant tumors. Finally, in the last component of the unified hypothesis, patients with platinum-sensitive ovarian cancer are expected to have heterogeneous populations of tumor cells, consisting of putative cancer stem cells, dormant or quiescent tumor cells, and tumor cells that are in contact with specific components of ECM. All these cells are expected to persist after chemotherapy and contribute to platinum-sensitive recurrence (**Figure 1F**). Eventually, after several rounds of chemotherapy, these cells may evolve to acquire additional genetic alterations leading to acquired resistance. It is also possible that intrinsically resistant tumor cells may exist as a small fraction of total initial tumor bulk. After multiple rounds of chemotherapy, their proportional representation may increase to a point that they eventually dominate the tumor behavior and produce a resistant phenotype.

If proven, the proposed model of matrix-dependent platinum resistance and disease recurrence has several clinical implications. First, although the majority of ovarian cancer cells are intrinsically sensitive to platinum-based chemotherapy, a small fraction of tumor cells acquire matrix-dependent platinum resistance and escape from chemotherapy, leading to recurrence. Second, it will be important to understand the role of ECM components in platinum resistance because enhanced understanding in this area

will allow us to design rational therapeutic approaches to eliminate residual cancer cells and provide more durable treatment options. Third, targeting the tumor microenvironment by disrupting cell-matrix interactions may be more "druggable" than targeting putative cancer stem cells because experimental compounds are already available to disrupt cell-matrix interactions (heparin, RGD peptides, integrin inhibitors, etc.) or block kinase signaling initiated by cell-matrix interactions (inhibitors of FAK, Src, PI3K, Akt, etc.). Therefore, small molecule inhibitors and peptides that block upstream ECM signaling or downstream intracellular signaling cascades initiated by ECM signaling should be tested in conjunction with conventional chemotherapy.

Finally, recent studies by Muranen et al. described matrix-dependent resistance to dual-specificity PI3K/mTOR inhibitor BEZ235 and other PI3K or mTOR inhibitors, such as Rapamycin, LY294002, GDC0941, and PIK-90, in ovarian cancer cell lines (29). Therefore, ECM may promote resistance to a broad spectrum of cancer drugs and targeting the ECM and tumor microenvironment may provide significant advances in improving the therapeutic efficacy of conventional as well as emerging novel therapeutics.

CONCLUSION

Tumors can be considered as developmental organs defined by abnormal signaling within tumor cells and between tumor cells and their microenvironment. Tumor microenvironment, consisting of (1) cellular components characterized by tumor cells, tumor-associated fibroblasts, immune cells, endothelial cells, and other resident cells, (2) physical components characterized by ECM, and (3) biochemical components characterized by oxygen tension, inflammatory cytokines, chemokines, and growth factors, have long been recognized as a critical determinant of tumor behavior. For example, the activation of v-*src* by Rous sarcoma virus in chick embryo did not produce abnormal growth (30, 31), but when these viral infected tissues were removed from the embryonic microenvironment, they produced a transformed phenotype (32). Similarly, melanoma cells injected into the embryonic microenvironment are reprogramed to remain indolent whereas those cells injected into other microenvironments are capable of inducing abnormal growth (33, 34). Finally, ECM attenuates chemotherapy-induced cytotoxicity in several cancer cell lines from various cancer types (20, 21) – a phenomenon referred to as cell adhesion-mediated drug resistance (35, 36). These studies and others indicate that tumor microenvironment can contribute to tumor dormancy, tumor progression, angiogenesis, metastasis, and chemotherapy resistance (15, 37).

Given the significance of the tumor microenvironment in regulating tumor behavior and, in particular, a tumor cell's response to chemotherapy, it is important that future drug discovery efforts should include strategies to disrupt cell-matrix interactions or downstream signaling cascades to determine the extent to which these approaches will synergize with conventional chemotherapy to enhance the effectiveness and durability of conventional chemotherapy. Synthetic lethal screens should be performed in more appropriate cellular context, such as 3D culture or matrix-coated cultures to identify drug target genes or drug candidates that synergize conventional chemotherapy.

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Targeted disruption of the JAK2/STAT3 pathway in combination with systemic administration of paclitaxel inhibits the priming of ovarian cancer stem cells leading to a reduced tumor burden

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Chemotherapy resistance associated with recurrent disease is the major cause of poor survival of ovarian cancer patients. We have recently demonstrated activation of the JAK2/STAT3 pathway and the enhancement of a cancer stem cell (CSC)-like phenotype in ovarian cancer cells treated *in vitro* with chemotherapeutic agents. To elucidate further these mechanisms *in vivo*, we used a two-tiered paclitaxel treatment approach in nude mice inoculated with ovarian cancer cells. In the first approach, we demonstrate that a single intraperitoneal administration of paclitaxel in mice 7 days after subcutaneous transplantation of the HEY ovarian cancer cell line resulted in a significant increase in the expression of CA125, Oct4, and CD117 in mice xenografts compared to control mice xenografts which did not receive paclitaxel. In the second approach, mice were administered once weekly with paclitaxel and/or a daily dose of the JAK2-specific inhibitor, CYT387, over 4 weeks. Mice receiving paclitaxel only demonstrated a significant decrease in tumor volume compared to control mice. At the molecular level, mouse tumors remaining after paclitaxel administration showed a significant increase in the expression of Oct4 and CD117 coinciding with a significant activation of the JAK2/STAT3 pathway compared to control tumors. The addition of CYT387 with paclitaxel resulted in the suppression of JAK2/STAT3 activation and abrogation of Oct4 and CD117 expression in mouse xenografts. This coincided with significantly smaller tumors in mice administered CYT387 in addition to paclitaxel, compared to the control group and the group of mice receiving paclitaxel only. These data suggest that the systemic administration of paclitaxel enhances Oct4- and CD117-associated CSC-like marker expression in surviving cancer cells *in vivo*, which can be suppressed by the addition of the JAK2-specific inhibitor CYT387, leading to a significantly smaller tumor burden. These novel findings have the potential for the development of CSC-targeted therapy to improve the treatment outcomes of ovarian cancer patients.

Keywords: ovarian carcinoma, cancer stem cells, metastasis, chemoresistance, recurrence, JAK2/STAT3 pathway

INTRODUCTION

The gold standard for the management of ovarian cancer patients after debulking surgery is the systemic administration of platinum (cisplatin/carboplatin) and taxane-based (paclitaxel) drugs. This treatment regimen results in a significant reduction of tumor burden due to substantial cancer cell death via DNA and cytoskeletal damage response pathways (1). Most of the ovarian cancer patients (~80%) respond well to the standard treatment regimen and enjoy a short-lived period of remission with asymptomatic minimal disease. However, this asymptomatic microscopic residual disease persisting after the first line chemotherapy leads to consecutive episodes of recurrent disease and eventual death. Hence, the 5-year survival period of ovarian cancer patients is as low as ~30% (2, 3). Thus, to increase the survival rate of ovarian cancer patients, there is an urgent need to identify the mechanisms that allow residual tumor cells to overcome first line chemotherapy and propagate within the changed tumor microenvironment.

Chemoresistant tumor cells that have the ability to resist the cytotoxic effects of chemotherapy have high expression of multidrug resistance transporters, enhanced ability to repair damaged DNA, and proliferate slowly (4). Recent studies have shown these phenotypes to be commonly displayed by cancer stem cells (CSCs)

(5, 6). A few recent studies have also demonstrated enrichment in CSCs and stem cell mediator pathways in chemoresistant and recurrent ovarian tumors, suggesting that CSCs and their associated pathways may be important intermediaries in the emergence of disease recurrence (7–10).

Several cell signaling pathways have been associated with self-renewal and the tumorigenic phenotype of CSCs. The Wnt, Sonic Hedgehog (Shh), and the Notch signaling pathways have been shown to be the drivers for the progression of cancers, including ovarian cancer (11–13). Another signaling pathway that is implicated in ovarian as well as other solid cancers is the signal transducer and activator of transcription protein 3 (STAT3) (14–16). In normal cells, STAT3 is transiently activated in response to specific growth factors and cytokines [interleukin-6 (IL-6), granulocyte colony stimulating factor (G-CSF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), etc]. However, in cancers, including breast, ovarian, and prostate, STAT3 is constitutively active in some cancer cells (17), and is believed to be responsible for several key points in tumor progression, starting from uncontrolled cellular proliferation to the promotion of angiogenesis and importantly facilitating resistance to apoptosis induced by conventional chemotherapy (18, 19). STAT3 is also involved in integrating the signals received from a variety of external agents such as growth factors or cytokines or genotoxic stressors and mediates the response of such agents by regulating downstream gene expression linked with cell survival and other cellular functions (20–22). Moreover, the STAT3 pathway has been shown to be a requisite for the proliferation and maintenance of glioblastoma stem cells (23), as well as rapidly cycling intestinal stem cells (24).

Recent studies have shown a link between the activation of STAT3 and CSCs. Coupling of the stem cell marker CD44 with the embryonic stem cell marker Nanog has been shown to be associated with the activation of STAT3 in ovarian cancer cells (25). The activation of STAT3 in these cells resulted in multidrug resistance gene expression and concomitant chemoresistance. Furthermore, we have previously demonstrated sustained activation of the STAT3 pathway in advanced-stage ovarian tumors and in cisplatin-treated ovarian cancer cell lines (15, 26). A recent study has shown significantly enhanced activation of STAT3 sustained by infiltrating macrophages in drug-resistant recurrent ovarian tumors compared to the matched primary tumors (27). Hence, the JAK2/STAT3 pathway is a potential target for the development of novel drugs aimed at suppressing its constitutive as well as ligand-induced activation. In the last decade, several anti-STAT3 small molecule inhibitors have shown promising potential by counteracting cancer cell-associated proliferation, inflammation, and importantly chemoresistance (17, 28). However, none of these compounds have been shown to have an effect on CSCs, which theoretically have been suggested to drive chemoresistance.

We have previously demonstrated that the *in vitro* treatment of OVCA 433 and HEY cell lines with cisplatin or paclitaxel resulted in the activation of the JAK2/STAT3 pathway (7, 26). We have also shown that intraperitoneal transplantation of chemotherapy-treated cells in nude mice resulted in a significantly higher tumor burden associated with enhanced CSC-like expression compared to control untreated cells (29). In the study reported here, we aimed to determine the effect of a novel small molecule inhibitor of the JAK2/STAT3 pathway, CYT387, in combination with systemic administration of paclitaxel and assess the molecular phenotype of the resultant xenografts. We demonstrate that irrespective of the length of paclitaxel treatment, systemic administration of paclitaxel enhanced the expression of Oct4 and CD117 in residual tumors. However, administration of CYT387 (by daily oral gavages) in combination with weekly systemic paclitaxel administration resulted in a significantly reduced tumor volume compared to control and paclitaxel alone treatment mice. These tumors displayed diminished JAK2/STAT3 activation as well as diminished Oct4 and CD117 expression compared to tumors generated during systemic administration of paclitaxel only. These novel data suggest that the inclusion of a JAK2/STAT3 inhibitor such as CYT387 with paclitaxel has the potential of reducing the tumor volume further than that achieved by using chemotherapy alone. Such observations in animal models provide "proof of concept" demonstrating the potential of CYT387 in reducing the intraperitoneal tumor burden in ovarian cancer patients further than that achieved by paclitaxel on its own. This may provide the patients with a lower or/zero incidence of tumor recurrence or longer disease-free survival period, and better quality of life so lacking following the current treatment options in these patients.

MATERIALS AND METHODS

CELL LINES

The human ovarian HEY cell line was derived from a peritoneal deposit of a patient diagnosed with papillary cystadenocarcinoma of the ovary (30). The cell line was grown as described previously (29, 31).

ANTIBODIES AND REAGENTS

Polyclonal antibody against phosphorylated (Tyr-705) STAT3 (P-STAT3), total STAT3 (T-STAT3), phosphorylated (Tyr-1007/1008) JAK2 (P-JAK2), and total JAK2 (T-JAK2) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against cytokeratin 7 (cyt7), Ki67, CA125, E-cadherin, vimentin, CD34, Oct4, and CD117 (c-Kit) used for immunohistochemistry were obtained from Ventana (Roche, AZ, USA). CYT387 [Momelotinib (GS-0387/CYT-0387)] was obtained from Gilead Sciences (CA, USA).

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Tumors obtained from mice were homogenized and cells lysed prior to RNA extractions and cDNA synthesis as described previously (29). For quantitative real-time PCR (q-PCR), four tumors in each group [control, paclitaxel-treated, CYT387-treated, and paclitaxel+CYT387-treated] were analyzed in triplicate as described previously (32). Each gene was validated by using an amplified, purified, and sequenced PCR fragment (originating from HEY cell line) as a positive control. The primers used for 18S, Oct-4A, IL-6, interleukin-6 receptor (IL-6R), glycoprotein 130 (gp130), C-X-C chemokine receptor type 4 (CXCR4), matrix metalloproteinase 2 (MMP-2), and matrix metalloproteinase 9 (MMP-9) are described in **Table 1**. q-PCR was carried out using ViiA 7 real-time PCR system (Applied Biosystems). Relative gene expression was calculated as $2^{-\Delta\Delta C_t}$ using 18S as the endogenous reference gene and the average of the controls as the calibrator.

Table 1 | Human oligonucleotide primer sequences for quantitative real-time PCR.

Gene symbol	Accession no.	Primer sequences from 5′ to 3′	Size (bp)
RNA18S	NR 003286.2	Forward GTAACCCGTTGAACCCCATT	153
		Reverse CCATCCAATCGGTAGTAGCG	
POU5F1 (OCT4A)	NM 002701.4	Forward CTCCTGGAGGGCCAGGAATC	381
		Reverse CCACATCGGCCTGTGTATAT	
IL6	NM 000600.3	Forward TACCCCCAGGAGAAGATTCC	175
		Reverse TTTTCTGCCAGTGCCTCTTT	
IL6R	NM 000565.3	Forward CTCCTGCCAGTTAGCAGTCC	198
		Reverse TCTTGCCAGGTGACACTGAG	
IL6ST (gp130)	NM 002184.3	Forward TGTAGATGGCGGTGATGGTA	246
		Reverse CCCTCAGTACCTGGACCAAA	
CXCR4	NM 001008540.1	Forward GAAGCTGTTGGCTGAAAAGG	94
		Reverse CTCACTGACGTTGGCAAAGA	
MMP2	NM 004530.4	Forward TTGACGGTAAGGACGGACTC	153
		Reverse ACTTGCAGTACTCCCCATCG	
MMP9	NM 004994.2	Forward TTGACAGCGACAAGAAGTGG	179
		Reverse GCCATTCACGTCGTCCTTAT	

ANIMAL STUDIES

Animal ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of the Laboratory Animals of the National Health and Medical Research Council of Australia. The experimental protocol was approved by the Ludwig/Department of Surgery, Royal Melbourne Hospital, and University of Melbourne's Animal Ethics Committee (Project-006/11), and was endorsed by the Research and Ethics Committee of Royal Women's Hospital Melbourne, VIC, Australia.

Animal experiments

Female Balb/c *nu/nu* mice (age, 6–8 weeks) were obtained from the Animal Resources Centre, Western Australia. Animals were housed in a standard pathogen-free environment with access to food and water.

HEY cells (5×10^6) were inoculated subcutaneously in each flank of nude mice as described previously (33). Mice were inspected weekly and tumor progression was monitored based on overall health and body weight until one of the pre-determined endpoints was reached. Endpoint criteria included exceeding 20% loss of initial body weight and a general pattern of diminished well-being such as reduced movement due to tumor burden, ulceration of tumors due to constant irritation of the protruding tumors with the mouse bedding, and lethargy resulting from lack of interest in daily activities. As such, the endpoint of the experiment was noted at day 7 (first approach) or day 28 (second approach) after the start of paclitaxel and/or CYT387 treatments. The 7- or 28-day duration was chosen as the tumor volume in the control group reached the defined volume of end point as specified in the Animal Ethics application.

In the first approach, mice were divided into two groups with $n = 3$ mice in each group and HEY cells were inoculated subcutaneously in each flank of nude mice. The first group of mice was treated as a control. After 7 days, the second group of mice was treated once intraperitoneally with 15 mg/kg of body weight of paclitaxel. These mice were followed for 7 days, after which the experiment was terminated.

In the second approach, mice were divided into four groups with $n = 5$ in each group and HEY cells were inoculated subcutaneously in each flank of nude mice. The first group of mice was designated as control. The second group was treated once a week with an intraperitoneal injection of paclitaxel at 15 mg/kg of body weight, 2 days post inoculation of the HEY cells. The third group was treated with the same dose of paclitaxel weekly in addition to daily doses of CYT387 at 5 mg/kg of body weight by oral gavages. The weekly intraperitoneal injection of paclitaxel and oral gavages of CYT387 was continued for 28 days. The fourth group of mice was treated with a daily dose of 5 mg/kg of body weight of CYT387 by oral gavages for 28 days. Mice were euthanized at the experimental endpoint and the tumors were excised for further examination.

Tumor volume measurements were performed with calipers at day 0 and days 7 and 28 (the experimental endpoint). Measurement of tumor volume in cubic millimeter was determined using the formula (length $\times$ width^2)/2; where length was the longest axis and width was the measurement at right angles to the length (33). Fold change in tumor volume was calculated from the ratio of tumor volume at day 0 to day 7 or day 28.

Immunohistochemistry

For immunohistochemistry, formalin-fixed, paraffin-embedded 4 μm sections of the xenografts were stained using a Ventana Benchmark Immunostainer (Ventana Medical Systems, Inc., AZ, USA) described previously (29). Detection was performed using Ventana's ultra view diaminobenzidine (DAB) detection kit (Roche/Ventana, AZ, USA). Tumor sections were dewaxed with Ventana EZ Prep and endogenous peroxidase activity was blocked using the Ventana's universal DAB inhibitor. Primary antibodies against Oct4, Ki67, cancer antigen 125 (CA125), CD117 (c-Kit), total JAK2 (T-JAK2), phospho-JAK2 (P-JAK2), total STAT3 (T-STAT3), and phospho-STAT3 (P-STAT3) were diluted according to the instruction provided by the manufacturer as described in **Table 2**. For nuclear staining, the sections were counter stained with Ventana hematoxylin and bluing solution. For each antigen, a parallel paraffin-embedded section was prepared without the primary antibody as a negative control. High-grade serous ovarian tumor sections were used as positive controls.

Immunohistochemistry images were created using an Axioskop 2 microscope, captured using a Nikon DXM1200C digital camera and processed using NIS-elements F3.0 software. Slides were scored independently by four blind reviewers, as described previously (34). For each slide, the extent of positive staining was scored as five grades, namely, 0 ($\leq$10%), 1 (>10–25%), 2 (>25–40%), 3 ($\geq$40–50%), 4 ($\geq$50–75%), and 5 (>75%). The intensity of staining (IS) was classified into four grades: no staining (−), pale brown (1), moderate brown (2) and dark brown (3). Scoring was determined by using the Allred method of visual quantification as per the following formula: percentage of staining (PS) + IS = total score (range 0–8) (35).

Table 2 | Antibody information.

Antibodies	Concentrations used	Incubation time (minutes)	Supplier	Catalog number
Mouse anti-human Ki67	2 μg/ml	12	Ventana	790-4286
Mouse anti-human Oct4 (we use Oct3/4)	1.2 μg/ml	32	Novocastra	NCL-L-OCT3/4
Mouse anti-human CD34	0.8 μg/ml	32	Ventana	790-2927
Mouse Anti-Human CD117	5 μg/ml	32	Ventana	790-2951
Rabbit anti-human JAK2	10 μg/ml	40	Cell Signaling	3230
Rabbit anti-human Phospho-JAK2 (Tyr1007/1008) (C80C3)	10 μg/ml	40	Cell Signaling	3776
Mouse anti-human STAT3 (124H6)	2.5 μg/ml	40	Cell Signaling	9139
Mouse anti-human phospho-STAT3 (Tyr705) (E2)	10 μg/ml	40	Cell Signaling	9138
Mouse anti-human CA125	0.23 μg/ml	32	Ventana	760-2610
HRP-conjugated secondary	Unknown, proprietary reagent	8	Ventana	760-500

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. A probability level of $p < 0.05$ was adopted throughout to determine statistical significance. Treatment groups were compared with the control group using one-way ANOVA followed by Bonferroni or Dunnett's multiple comparison post-tests.

RESULTS

EFFECT OF SINGLE DOSE OF PACLITAXEL ON HEY TUMORS

Subcutaneous injection of mice with HEY cells resulted in the formation of tumors within 5 days. Intraperitoneal injection of paclitaxel (15 mg/kg of body weight) given 7 days post inoculation of HEY cells was well tolerated by the mice. Treatment with a single dose of paclitaxel did not result in any significant change in the tumor volume compared to the control untreated mice (Figures S1A–C in Supplementary Material).

Mouse tumors were excised and assessed by immunohistochemistry. Xenografts that received paclitaxel treatment demonstrated significantly enhanced staining for the CSC-like marker CD117 and the embryonic stem cell maker Oct4 compared to the control group (Figure S2 in Supplementary Material). These results coincided with a significantly enhanced staining for CA125 in the mice treated with paclitaxel compared to the control group (Figure S2 in Supplementary Material). The expression of CD117 and CA125 in untreated and paclitaxel-treated groups was confined to the cytoplasm and cell membranes. The staining of Oct4 was observed in the cytoplasm as well as in the nucleus in both control and paclitaxel-treated mouse tumors (Figure S2 in Supplementary Material).

VOLUME OF XENOGRAFTS GENERATED THROUGH ADMINISTRATION OF A COMBINATION OF DAILY ORAL GAVAGES OF CYT387 WITH WEEKLY INTRAPERITONEAL PACLITAXEL INJECTIONS

In the second approach, mice were treated with either paclitaxel weekly, or CYT387 daily or treated with a combination of both or observed without intervention (control group) 2 days after subcutaneous inoculation of HEY cells in both flanks of each mouse. Mice were followed for a 28-day treatment period and tumor volumes were analyzed at day 0 (before the start of treatment regimens) versus day 28. A single tumor localized at the site of inoculation (each flank) was obtained from each mouse. Mice in the control group had an approximately 90-fold increase in tumor volume at day 28 compared to day 0 (**Figure 1**). On the other hand, tumors in mice treated with weekly doses of paclitaxel demonstrated an approximately 30-fold increase in volume at day 28 compared to day 0. However, tumors in mice receiving daily doses of CYT387 demonstrated only an approximately 20-fold increase in tumor volume at day 28 compared to day 0. Remarkably, mice that were treated with a combination of weekly paclitaxel and daily CYT387 developed significantly smaller tumors when compared to paclitaxel-treated and control groups, with a tumor fold change of less than 10-fold at day 28 compared to day 0 (**Figure 1**). This group had the smallest tumor volume of all the groups. The volume of the tumors produced in the CYT387 treatment group was not significantly different than the paclitaxel treatment group.

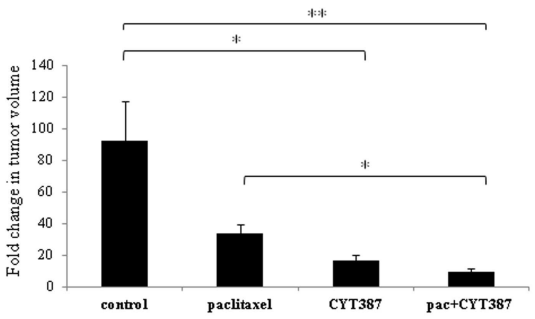

FIGURE 1 | HEY xenograft volume in mice administered with or without paclitaxel, CYT387 or a combination of paclitaxel and CYT387 (pac + CYT387). Average fold change in subcutaneous tumor volume. Tumors were measured at day 0 (prior to treatment) and at the end of the experiment (day 28). Data have been obtained from $n = 5$ mice in each group. Significant intergroup variations are indicated by $*p < 0.05$, $**p < 0.01$.

PHENOTYPE OF XENOGRAFTS GENERATED THROUGH WEEKLY SYSTEMIC PACLITAXEL AND DAILY ORAL GAVAGES OF CYT387

Xenografts were collected and analyzed using immunohistochemistry. The expression of phosphorylated (P) and total (T)-JAK2 and STAT3 was mostly confined to the cytoplasm. However, some scattered nuclear staining was also evident. Staining for

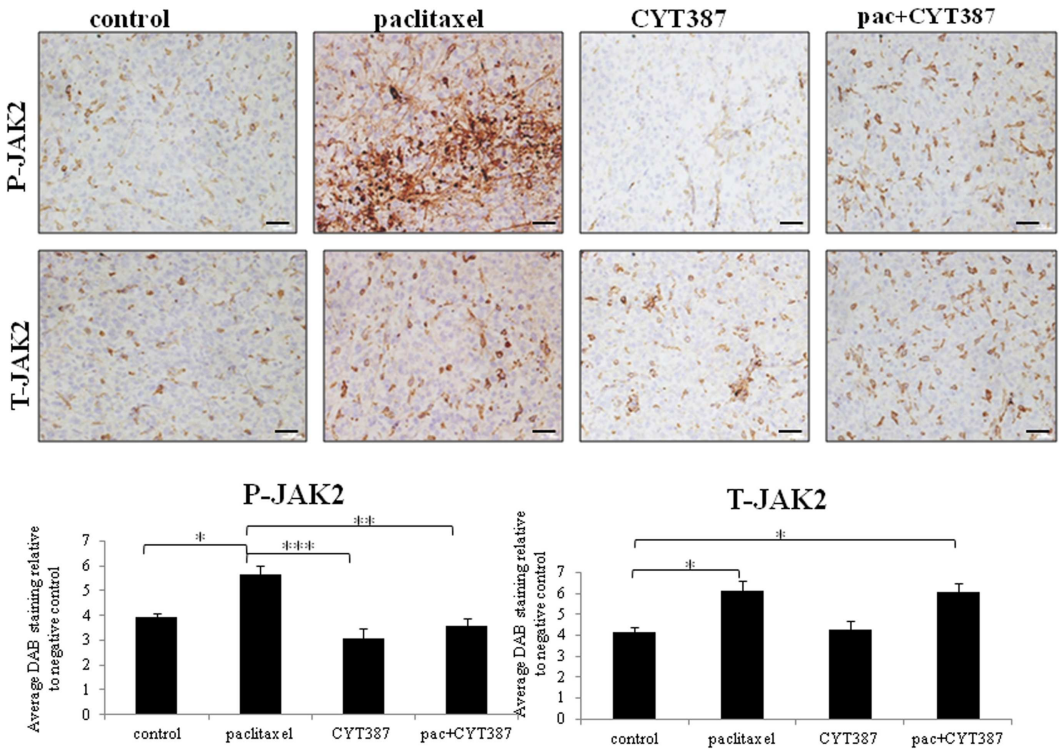

FIGURE 2 | Immunohistochemistry images of phosphorylated P-JAK2 and T-JAK2 staining in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT387. Tumor sections were stained with antibodies specific for P-JAK2 and T-JAK2 as described in the Section "Materials and Methods." Magnification 200×, scale bar = 10 μM. Average DAB intensity and proportion of staining of P-JAK2 or T-JAK2 in xenografts was standardized to a negative control. The quantification was derived from the staining of five independent xenografts in each group. Significant intergroup variations are indicted by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

phosphorylated (P) and total (T) JAK2 and STAT3 was significantly increased in tumors derived from mice receiving weekly paclitaxel treatment compared to the control group (**Figures 2** and **3**). Daily administration of CYT387 on its own had no effect on the phosphorylation of JAK2 or STAT3 compared to the control group, however it significantly decreased the endogenous expression of T-STAT3 without any effect on the expression of T-JAK2. Administration of daily CYT387 in conjunction with paclitaxel treatment resulted in tumors that displayed a significantly decreased staining of P-JAK2 and P-STAT3, compared to paclitaxel only treated group. However, no effect on T-JAK2 or T-STAT3 in the paclitaxel+CYT387 group compared to the paclitaxel only treated group could be observed. This suggests that daily doses of CYT387 abolished the paclitaxel-induced activation of the JAK2/STAT3 pathway without having a significant effect on the expression of the total proteins (**Figures 2** and **3**).

Mice receiving paclitaxel developed tumors that displayed significantly enhanced staining of the cell proliferation maker Ki67 when compared to the control group (**Figure 4**). The staining of Ki67 both in the control and treated groups was confined to the nucleus. However, this enhanced nuclear staining of Ki67 in response to paclitaxel administration was significantly reduced when CYT387 was added in combination with paclitaxel (**Figure 4**). Moreover, mice that received daily CYT387 alone developed tumors that displayed a significantly reduced Ki67 staining when compared to the paclitaxel only treatment group. However, it had no effect on basal Ki67 staining (**Figure 4**).

Similar to that demonstrated in the first approach, tumors derived from mice treated with paclitaxel alone displayed significantly enhanced staining for CA125, the CSC-like marker CD117, and the embryonic stem cell marker Oct4, when compared to the control group (**Figures 4** and **5**). However, tumors derived from mice treated with a combination of paclitaxel and CYT387 demonstrated significantly reduced staining of CD117, Oct4, and CA125 compared to the paclitaxel-treated group (**Figures 4** and **5**). CYT387 on its own had no effect on the basal expression of CA125, CD117, and Oct4. These results suggest that the addition of CYT387 abrogates the paclitaxel-induced CA125, CD117, and Oct4 expression. The expression of CD117, Oct4, and CA125 was present mostly in the cytoplasm and cell–cell membrane junctions. Very little nuclear staining of Oct4 was also evident in xenografts generated upon paclitaxel treatment.

Mice treated with paclitaxel developed tumors with significantly enhanced expression of $CD34^+$ cells when compared to the control as well as the CYT387 treatment groups (**Figure 6**). The addition of CYT387 to paclitaxel did not reduce the paclitaxel-induced enhanced expression of CD34 which was mostly cytoplasmic.

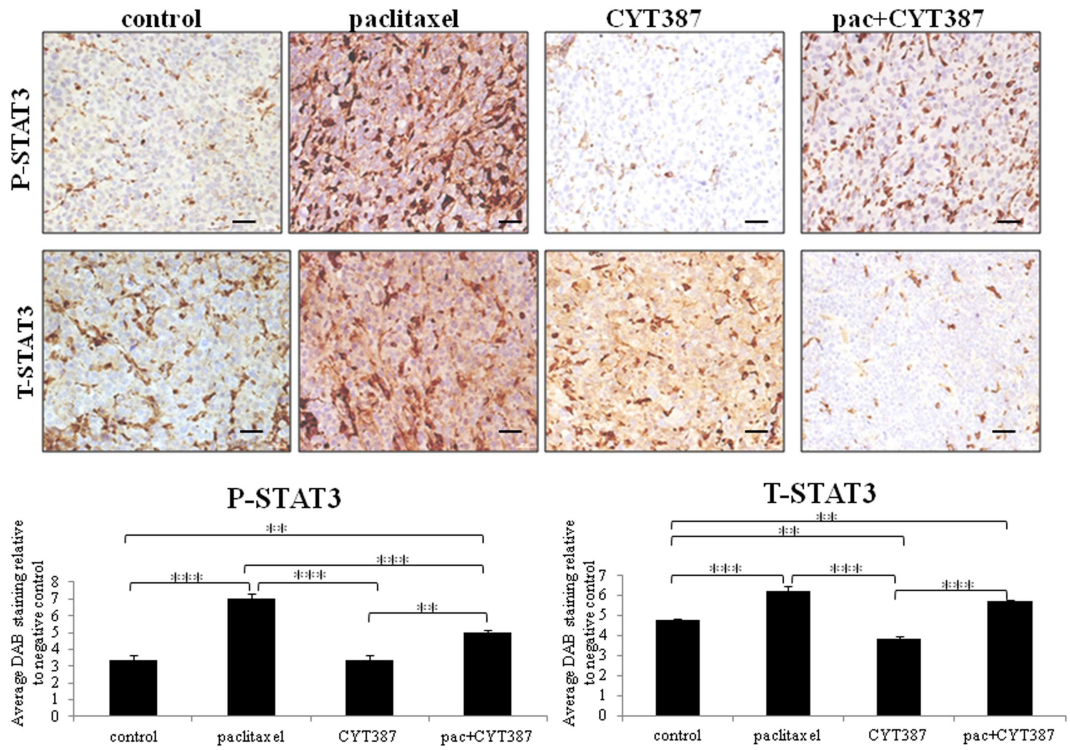

FIGURE 3 | Immunohistochemistry images of P-STAT3 and T-STAT3 staining in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT387. Tumor sections were stained with antibodies specific for P-STAT3 and T-STAT3 as described in the Section "Materials and Methods." Magnification 200×, scale bar = 10 μM. Average DAB intensity and proportion of staining of P-STAT3 or T-STAT3 in xenografts was standardized to a negative control. The quantification was derived from the staining of five independent xenografts in each group. Significant intergroup variations are indicted by $^{**}p < 0.01$ and $^{***}p < 0.001$.

To determine if changes in the embryonic stem cell marker Oct4 seen at the protein level in mouse xenografts were consistent at the mRNA level, q-PCR on cDNA prepared from RNA extracted from mouse tumors was performed. An analysis of the embryonic stem cell marker Oct4 revealed significantly enhanced mRNA expression in tumors derived from mice treated with paclitaxel when compared to the control group (**Figure 7**). Consistent with the Oct4 immunohistochemistry staining, the addition of daily CYT387 treatment resulted in a significant reduction of Oct4 mRNA in tumors compared to mice treated with paclitaxel (**Figure 7**). Treatment with CYT387 alone did not result in any significant change in the mRNA expression of Oct4 compared to the control untreated group (**Figure 7**).

EFFECT OF ORAL GAVAGES OF CYT387 IN COMBINATION WITH SYSTEMIC ADMINISTRATION OF PACLITAXEL ON INTERLEUKIN-6-MEDIATED RESPONSES IN MOUSE XENOGRAFTS

We investigated whether the mRNA expression of IL-6 in the control tumor xenografts had any correlation with the mRNA levels of invasion-associated genes such as CXCR4, MMP-2, and MMP-9 in response to systemic administration of paclitaxel and oral gavages of CYT387. Untreated control and paclitaxel administered mouse tumors expressed human IL-6R as well as gp130 mRNA (**Figure 8**). There were increased trends in the expression of IL-6, CXCR4, MMP-2, and MMP-9 in the paclitaxel-treated group compared to the control group. This increased trend however, did not receive statistical significance between the two groups (**Figure 8**). When the combination of paclitaxel and CYT387 was administered, the mRNA expression of IL-6R, gp130, IL-6, CXCR4, MMP-2, and MMP-9 did not change relative to the house keeping gene 18S (**Figure 8**). On the other hand, CYT387 administration on its own significantly increased the mRNA expression of MMP-2 compared to the control group. An increased trend in the expression of MMP-9 in the CYT387 treatment group compared to control was also observed but it did not receive statistical significance (**Figure 8**). No change in the mRNA expression of 18S was observed under the same conditions (Figure S3 in Supplementary Material).

DISCUSSION

CYT387 is an orally available, potent small molecule inhibitor of the JAK1/2 pathway currently undergoing Phase I/II clinical trials for the treatment of myelofibrosis, a frequently diagnosed fatal myeloproliferative disorder (36). CYT387 has so far been the best candidate among the JAK inhibitors for the management of myelofibrosis with the preliminary data showing significant responses with a low level of toxicity (http://www.gilead.com/research/pipeline). CYT387 has demonstrated efficacy in a JAK2V617F mutation-associated animal model where it inhibited STAT3 functions associated with constitutively activated JAK2, by normalizing inflammatory cytokines (37). In the murine

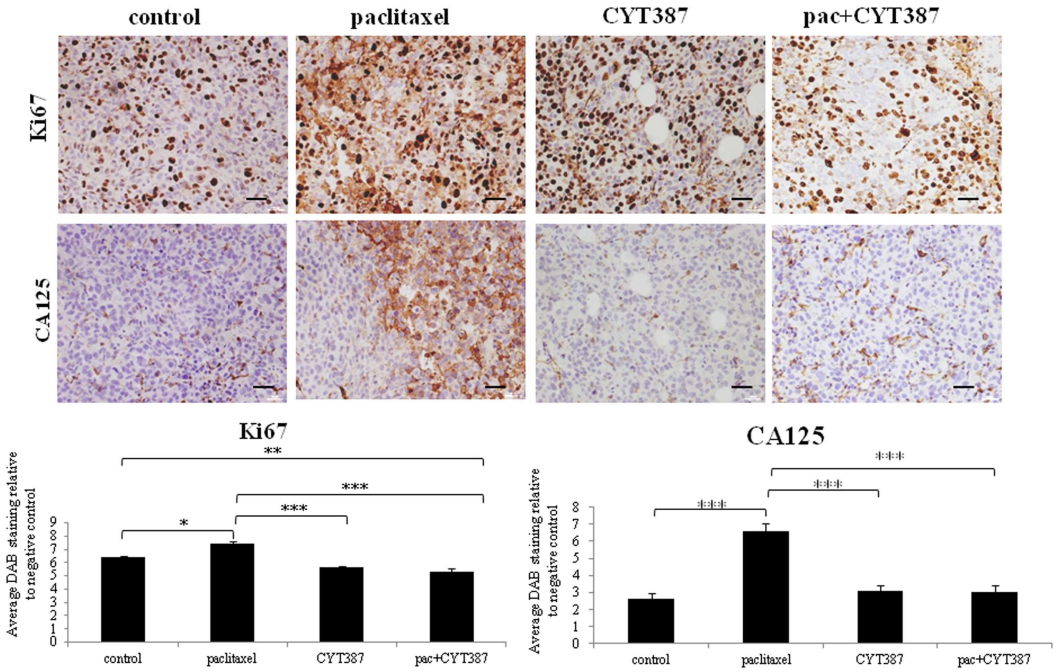

FIGURE 4 | Immunohistochemistry images of Ki67 and CA125 staining in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT387. Tumor sections were stained with antibodies specific for Ki67 and CA125 as described in the Section "Materials and Methods." Magnification 200×, scale bar = 10 μM. Average DAB intensity and proportion of staining of Ki67 or CA125 in xenografts was standardized to negative control. The experiments were performed using five independent samples in each group. Significant intergroup variations are indicted by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

JAK2V617F mutation-associated animal model, CYT387 also normalized white cell counts, the hematocrit, and also restored the normal spleen size (37). Preclinical analysis has shown CYT387 to be well tolerated in mice when administered orally at doses up to 50 mg/kg of body weight, with no sign of overt toxicity (37). Besides myeloproliferative disorders, CYT387 has the potential for the treatment of solid and hematological malignancies and inflammatory conditions (36). In this proof of principle study, we demonstrate the novel effect of CYT387 in combination with paclitaxel in significantly suppressing the tumor growth greater than that achieved by paclitaxel on its own. We also show suppression of the expression of CA125, Oct4, and CD117 by CYT387, induced by the activation of JAK2/STAT3 pathway in response to systemic paclitaxel administration in an ovarian xenograft model.

We have recently demonstrated that human ovarian cancer cell lines as well as primary- and ascites-derived ovarian cancer cells treated with cisplatin or paclitaxel generate a surviving residual population of cells which display enhanced expression of the chemoresistant-associated markers ERCC1 and β-tubulin as well as enhanced expression of CSC-like markers CD44, CD24, CD133, CD117, and EpCAM, compared to parental untreated ovarian cancer cells (6, 9, 29). In addition, xenotransplantation studies showed that chemotherapy-treated ovarian cancer cells generate significantly larger tumor burden compared to untreated cells and retain an enhanced stemness profile (29). This suggests that some CSC-like and chemoresistant characteristics may be synchronously regulated in the residual cells that survived chemotherapy (7). In this study, we provide further novel data which demonstrates that Oct4 and CD117 expression are enhanced in a mouse xenograft model by intraperitoneal administration of chemotherapy (paclitaxel) after the subcutaneous implantation of an ovarian cancer cell line. We also demonstrate that the expression of Oct4 and CD117 in tumors generated in response to multiple doses of chemotherapy can be suppressed by the administration of a novel small molecule JAK2-specific inhibitor CYT387. The advantage of using a subcutaneous instead of the intraperitoneal model of ovarian tumor is that it allows for the accurate measurement of tumor volume, thus permitting the monitoring of tumor growth in response to treatments.

In the first part of this study, we demonstrate that a single systemic administration of paclitaxel 1 week after subcutaneous implantation of ovarian cancer cells led to a tumor which had a significant enhancement in the expression of Oct4 and CD117 within the 7 days post treatment. This enhancement in the Oct4 and CD117 expression coincided with a significant enhancement of CA125 staining. Such dramatic changes in Oct4, CD117, and CA125 staining had no bearing on tumor volume within the 7 days after paclitaxel administration. This suggests that although no reduction in tumor volume was observed, a single dose of paclitaxel treatment had imposed certain molecular changes in the paclitaxel surviving residual tumor populations. This process likely occurs consecutively in chemoresistant populations while the chemosensitive populations undergo cell death in response to paclitaxel treatment, a process that results in the eradication of

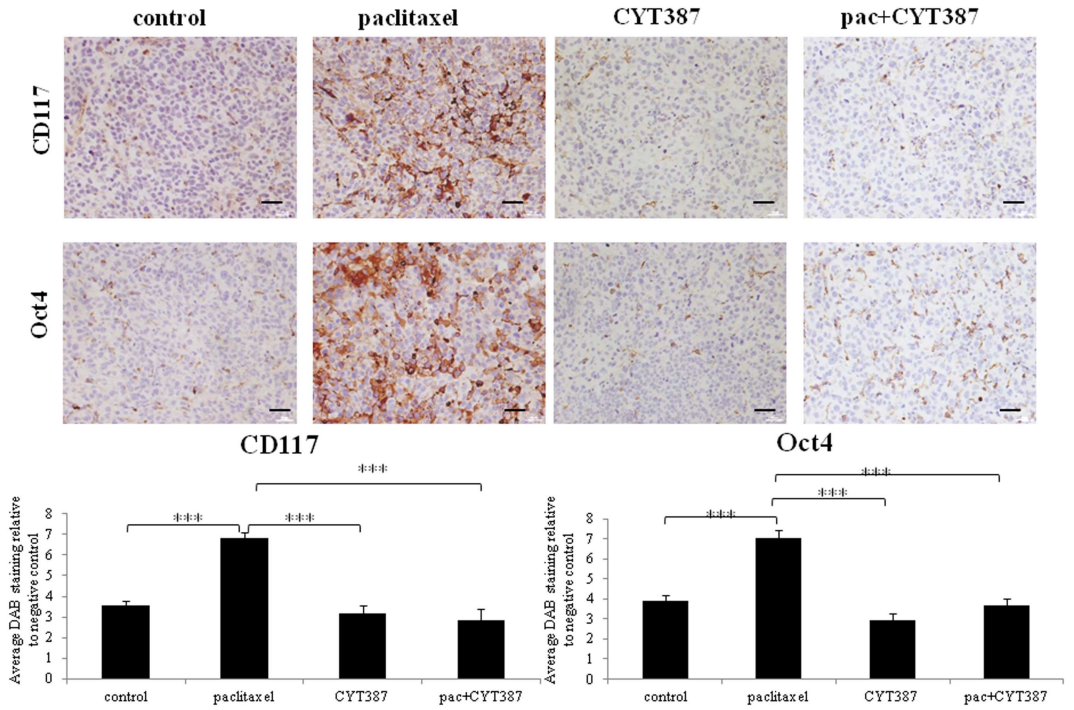

FIGURE 5 | Immunohistochemistry images of CD117 and Oct4 staining in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT387. Tumor sections were stained with antibodies specific for CD117 and Oct4 as described in the Section "Materials and Methods." Magnification 200×, scale bar = 10 μM. Average DAB intensity and proportion of staining of CD117 or Oct4 in xenografts was standardized to a negative control. The experiments were performed using five independent samples in each group. Significant intergroup variations are indicted by $***p < 0.001$.

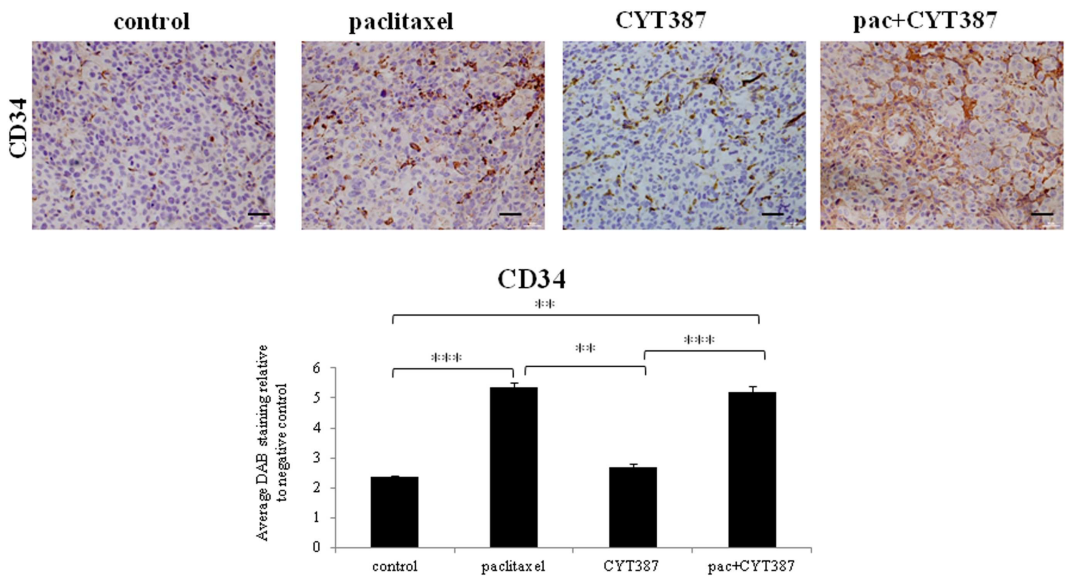

FIGURE 6 | Immunohistochemistry images of CD34 staining in mice xenografts from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT387. Tumor sections were stained with antibodies specific for CD34 as described in the Section "Materials and Methods." Magnification 200×, scale bar = 10 μM. Average DAB intensity and proportion of staining of CD34 in xenografts was standardized to a negative control. The experiments were performed using five independent samples in each group. Significant intergroup variations are indicted by $**p < 0.01$ and $***p < 0.001$.

the majority of the tumor mass leaving behind theoretically the CSC-enriched tumor initiating residual disease (38).

In the second approach, we demonstrate that weekly treatment of paclitaxel over 28 days resulted in a dramatic reduction of the mouse tumor volume as evidenced by a 30-fold increase in the tumor volume in mice treated with paclitaxel when compared to a ~90-fold increase in the volume in mice not receiving the treatment. These results suggest that systemic weekly administration of paclitaxel-induced cytotoxic and anti-proliferative effects on the tumor population restricting the growth of the tumors compared to the control untreated tumors. However, CYT387 in combination with paclitaxel was able to significantly reduce the tumor volume greater than that can be achieved with paclitaxel alone. This was achieved by using a concentration of CYT387 (5 mg/kg of body weight) that was one-tenth of that used in the JAK2V617F mutation-associated animal model (50 mg/kg of body weight) (37). In addition, CYT387 was administered twice a day in the JAK2V617F mutation-associated animal model compared to a single administration of the drug each day in our study. Hence, it can be anticipated that increasing the concentration of CYT387 toward the same level as that used for the JAK2V617F mutation-associated animal model would be well tolerated in our mouse model and therefore has the potential of reducing tumor volume further.

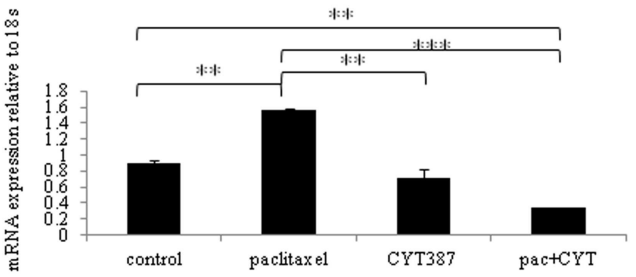

FIGURE 7 | The mRNA expression of embryonic stem cell marker Oct4 in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or a combination of paclitaxel and CYT387 (pac + CYT). mRNA from xenografts generated from the control group and treatment groups was extracted, cDNA was prepared, and q-PCR for Oct4 was performed as described in the Section "Material and Methods." The resultant mRNA levels were normalized to 18S mRNA. The experiments were performed using five independent samples in triplicate. Significant intergroup variations are indicated by $**p < 0.01$ and $***p < 0.001$.

Tumor cells from mice which survived the paclitaxel treatment were found to have an activated JAK2/STAT3 pathway and to have significantly enhanced staining of embryonic stem cell transcription factor Oct4 and CSC-like marker CD117. In addition, tumors derived from mice treated with paclitaxel showed significantly enhanced CA125 staining. These novel findings suggest that while the tumor volume was smaller in paclitaxel-treated mice, these tumor cells underwent specific molecular changes. As elevated level of CA125 is the hallmark of ovarian cancer diagnosis and frequently observed in recurrent disease, enhanced expression of CA125 in paclitaxel-treated tumor cells may suggest priming of the residual cells for recurrence.

The above *in vivo* mice data are consistent with the data obtained after analyzing several stem cell markers in ovarian tumor specimens collected at diagnosis (before treatment), after chemotherapy treatment and at first recurrence (10). It has been reported that CD133, CD44, and ALDH1A1 were present at low numbers in primary tumors, however, this was found to

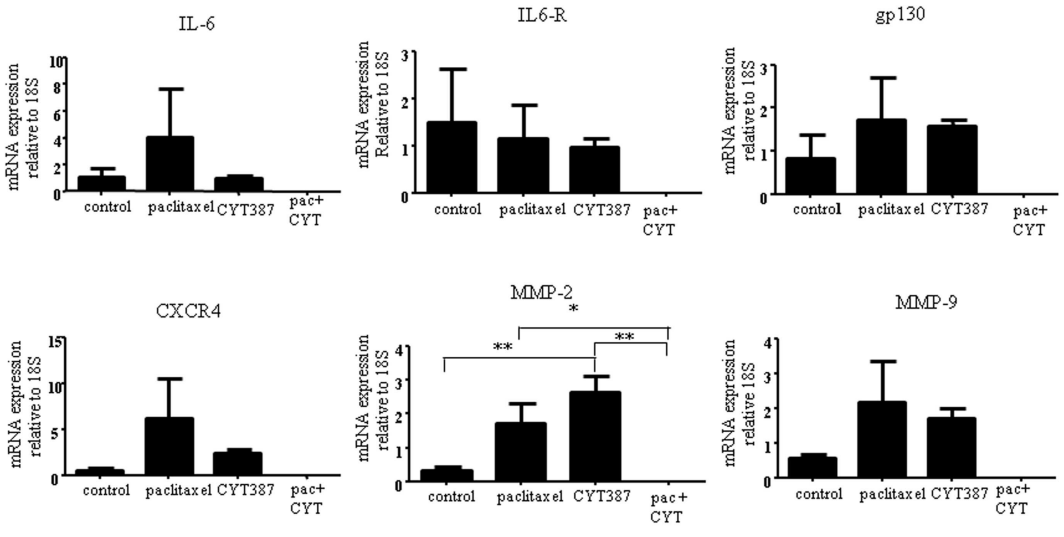

FIGURE 8 | The mRNA expression of IL-6, IL-6R, gp130, CXCR4, MMP-2, and MMP-9 in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT. mRNA from xenografts generated from the control group and treatment groups was extracted, cDNA was prepared, and q-PCR for IL-6, IL-6R, gp130, MMP-2, MMP-9, and CXCR4 was performed as described in the Section "Material and Methods." The resultant mRNA levels were normalized to 18S mRNA. The experiments were performed using four independent samples in triplicate. Significant intergroup variations are indicated by $*p < 0.05$ and $**p < 0.01$.

increase in tumor specimens taken immediately after chemotherapy treatment and then reduced to initial numbers in recurrent tumors, suggesting that these so-called "CSC markers" identify "chemoresistant cells." Such observations in animal models and clinical specimens suggest that chemotherapy treatment may induce a "chemoresistant niche," which protects residual chemoresistant cells from cell death by promoting a microenvironment appropriate for the survival of CSCs. In that context, CD133 positive colon CSCs have been shown to protect themselves *in vivo* from apoptosis by autocrine secretion of interleukin-4 (IL-4) (39). Paclitaxel treatment has been shown to promote angiogenesis in tumors through the mobilization of bone marrow-derived endothelial cells to tumors by an acute drug-mediated release of stromal-derived factor-1 (SDF-1) and G-CSF (40).

We also demonstrate that combining a daily dose of CYT387 with weekly paclitaxel treatment resulted in the development of mouse tumors which had a significantly reduced activation of the JAK2/STAT3 pathway compared to the group which received only paclitaxel. This correlated with the significantly reduced expression of the paclitaxel-induced Oct4, CD117, CA125, and Ki67 expression. In addition, the tumor volume in the mice group that received daily doses of CYT387 in combination with weekly paclitaxel treatment was significantly smaller compared to the treatment group that received only paclitaxel.

Our data also demonstrate a significantly enhanced accumulation of $CD34^+$ cells in tumors treated with paclitaxel compared to control untreated tumors. $CD34^+$ cells are a well-characterized population of mesenchymal stem cells derived from bone marrow or adipose tissue that have been used clinically to reconstitute the hematopoietic system after radiation or chemotherapy (41). More recently, $CD34^+$ cells have also been shown to induce therapeutic angiogenesis in animal models of myocardial, peripheral, and cerebral ischemia by direct incorporation of cells into the expanding vasculature and/or paracrine secretion of angiogenic growth factors that supports the developing microvasculature (42). The fact that CYT387 had no effect on the accumulation of $CD34^+$ cells in response to paclitaxel treatment indicates that CYT387 may not have an effect on angiogenesis.

We have recently shown enhanced secretion of interleukin-6 (IL-6) and G-CSF and activation of associated downstream STAT3 pathway in several ovarian cancer cell lines in response to cisplatin or paclitaxel treatments *in vitro* (7, 43). This suggests that an "acute" drug-induced secretory response is promoted in the tumor microenvironment following therapeutic administration, which may have a negative impact on the therapeutic response and act in favor of tumor cells by protecting them from the cytotoxic effects of the chemotherapy. In addition, ovarian cancer-related inflammation has recently been shown to be associated with autocrine cytokine network mediated by tumor necrosis factor (TNF), CXCL12 (also known as SDF-1, ligand for CXCR4 receptor), and IL-6 (44). Autocrine secretion of IL-6 by tumor cells or the associated infiltrated cells, not only promotes tumor growth and invasion (45) but also facilitates chemoresistance (43, 46). A recent study has demonstrated metastatic and drug-resistant recurrent ovarian tumors to have a significantly higher IL-6 expression compared to the matched primary tumors. In that study, the use of a monoclonal IL-6 antibody was shown to suppress IL-6 induced STAT3 phosphorylation and nuclear translocation. This resulted in the decreased expression of STAT3 downstream targets such as Mcl-1 and sensitization of paclitaxel-resistant ovarian cancer cell lines to chemotherapy (27). Our study on the other hand, showed no significant increase in human IL-6 mRNA expression and its downstream metastasis-associated genes MMP-2, MMP-9, and CXCR4 (receptor for CXCL12) in mouse tumors generated during systemic administration of paclitaxel. This suggests that the activation of STAT3 observed in the paclitaxel-treated mouse xenografts may have been triggered by stimulatory agent(s) other than IL-6.

The results from this study reflect the poorer outcomes for patients receiving paclitaxel on its own as a first line chemotherapy. However, these results also provide fresh hope for the potential of a new combination therapy involving CYT387. For the first time, we demonstrate that while the tumor volumes are kept small as a result of paclitaxel treatment, populations of tumor cells within the residual tumors retain the activated JAK2/STAT3 pathway and are enriched in markers such as Oct4 and CD117. We propose that treatment of patients by first line chemotherapy is in fact a process that enables chemotherapy surviving cells to undergo molecular activation of the JAK2/STAT3 pathway. Our data suggests that the inhibition of JAK2/STAT3 pathway by CYT387 at a very low concentration in combination with paclitaxel can suppress the molecular changes induced by chemotherapy in the residual tumors. This can result in a smaller tumor volume than that achieved by the chemotherapy alone. These preliminary "proof of concept" data warrant further investigation of CYT387 in preclinical and clinical models. One of the potential positive outcomes of combining CYT387 with the first line chemotherapy may be a longer disease-free survival period or a decreased incidence of recurrence or perhaps even prevention of the inevitable emergence of fatal recurrent disease.

AUTHORS CONTRIBUTION

Khalid Abubaker designed the study, performed the experiments, and contributed to the writing of the manuscript; Rodney B. Luwor helped with the animal experiments; Ruth Escalona designed primers and contributed to the PCR experiments; Orla McNally, Michael A. Quinn, Erik W. Thompson, and Jock K. Findlay edited the manuscript; Nuzhat Ahmed conceived the idea, designed the study, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2014.00075/abstract

Figure S1 | Tumor volume in mice treated with a single dose of paclitaxel. **(A)** Representative image of subcutaneous tumors in control and paclitaxel-treated mice. **(B)** Fold change in tumor volume (mm^3) at the end of the study (7 days post treatment) was standardized to initial tumor volume prior to receiving paclitaxel treatment. Data were obtained from $n = 3$ mice in each group. No significant difference between treatment groups was observed.

Figure S2 | Immunohistochemistry images of CD117, Oct4, and CA125 staining in HEY xenografts derived from mice treated with or without a single dose of paclitaxel. Tumor sections were stained with antibodies specific for CD117, Oct4, and CA125 as described in the Section "Materials and Methods." Average DAB intensity and proportion of staining for CD117, Oct4, and CA125 in mouse tumors was standardized to a negative control. The experiment was performed on three independent xenografts from each group. Significant intergroup variations are indicated by $***p < 0.001$. Magnification 200×, scale bar = 10 μM.

Figure S3 | The mRNA expression of housekeeping gene 18S in xenografts generated from subcutaneous transplantation of HEY cells into mice administered with or without paclitaxel, CYT387, or pac + CYT. mRNA from xenografts generated from the control group and treatment groups was extracted, cDNA was prepared, and q-PCR for 18S was performed as described in the Section "Materials and Methods." The experiments were performed using four independent samples in triplicate.

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Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research

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More than one third of ovarian cancer patients present with ascites at diagnosis, and almost all have ascites at recurrence. The presence of ascites correlates with the peritoneal spread of ovarian cancer and is associated with poor disease prognosis. Malignant ascites acts as a reservoir of a complex mixture of soluble factors and cellular components which provide a pro-inflammatory and tumor-promoting microenvironment for the tumor cells. Subpopulations of these tumor cells exhibit cancer stem-like phenotypes, possess enhanced resistance to therapies and the capacity for distal metastatic spread and recurrent disease. Thus, ascites-derived malignant cells and the ascites microenvironment represent a major source of morbidity and mortality for ovarian cancer patients. This review focuses on recent advances in our understanding of the molecular, cellular, and functional characteristics of the cellular populations within ascites and discusses their contributions to ovarian cancer metastasis, chemoresistance, and recurrence. We highlight in particular recent translational findings which have used primary ascites-derived tumor cells as a tool to understand the pathogenesis of the disease, yielding new insights and targets for therapeutic manipulation.

Keywords: ovarian carcinoma, ascites, chemoresistance, recurrence, metastasis, cytokines

INTRODUCTION

Ovarian cancer has the highest mortality rate of all gynecological cancers worldwide and is frequently (>75%) diagnosed at an advanced-stage (1). As the disease is asymptomatic, early detection is difficult so that at the time of diagnosis the tumor has metastasized (FIGO stages III–IV). Even with optimal debulking surgery followed by aggressive front-line chemotherapy, which results in an 80% initial cure rate, advanced-stage disease in the majority of cases is incurable. This is due to the development of a chemoresistant disease which results in recurrence within 16–22 months and a 5-year survival rate of only ∼27% (2). More than one third of ovarian cancer patients present with malignant ascites at diagnosis; additionally, development of ascites is a fundamental part of chemoresistant and recurrent disease (2, 3). The onset and progression of ascites is associated with poor prognosis and deterioration in the quality of life of patients, as ascites can cause debilitating symptoms such as abdominal pain, early *satiety* and compromised respiratory, gastrointestinal, and urinary systems (2). In newly diagnosed ovarian cancer patients, ascites is treated by using standard treatment for the underlying disease, that is, intravenous treatment of combination of platinum and taxol-based chemotherapy. However, once the chemoresistant and recurrent features of the disease develop, management of large volumes of ascites can be a major problem, and the majority of patients are subjected to frequent *paracentesis* to temporarily relieve the symptoms. This in turn can lead to visceral and vascular injury resulting in septic complications, further complicating the treatment of the patients. In addition, ascites contains a rich tumor-friendly microenvironment which not only promotes tumor cell growth and motility (4, 5) but also results in inhibiting the response of chemotherapy (6). In short, ascites plays a major role in the progression of the advanced-stage disease, emphasizing the necessity to understand its pathophysiology and its impact on the biology of ovarian tumor cells, including its role in chemoresistance and mechanisms of tumor progression.

MECHANISM OF INTRAPERITONEAL DISSEMINATION OF OVARIAN CANCER

Ovarian cancer is characterized by rapid growth and spread of intraperitoneal tumors and accumulation of ascites (1). Early metastasis in ovarian cancer occurs by direct extension of cancer growth to sites proximal to primary tumors, through a series of complex processes which involves cellular proliferation, epithelial-to-mesenchymal transition (EMT) which results in tumor cells migration to distant sites, and mesenchymal-to-epithelial transition (MET) for colonization (7, 8). The early steps of cancer progression also involve disruption of the ovarian tumor capsules and shedding of malignant cells from the primary tumors into the peritoneum where they survive as single cells or free-floating multicellular aggregates, commonly known as spheroids, in the ascites. Under this scenario, attachment and disaggregation of spheroids on mesothelial extracellular matrix (ECM) allows them to anchor as secondary lesions on pelvic organs and at a later stage, metastasize to distant organs (9, 10). Dissemination to distant sites, which

carries a poor prognosis for ovarian cancer patients, has been suggested to occur via transcoelomic, lymphatic, or hematogenous routes (11, 12). Among these, metastasis through transcoelomic route is commonly observed in advanced-stage patients and is frequently associated with the production of ascites (11). The term "malignant ascites" is commonly used when the tumor fluid is tested positive for malignant cells and has a high level of lactate dehydrogenase (13, 14), suggesting that the ascites may contain tumor cells with rapid proliferative rates indicative of rapid progression of the disease. The fact that Stage 1A ovarian cancers (disease is confined to the ovary) have fewer relapses (29%) than Stage 1C (59%) (capsule has ruptured and peritoneal washings are positive for malignant cells), suggests that if the tumor can be removed before it is exposed to ascites in the peritoneum, subsequent metastatic spread, and relapses can be reduced (11, 15).

ORIGIN OF ASCITES

Under normal physiological conditions, capillary membranes of the peritoneal cavity continuously produce free fluid to keep the serosal surfaces of the peritoneal lining lubricated so that there is an easy passage of solutes between the peritoneum and the adjacent organs. Two thirds of this peritoneal fluid is reabsorbed into the lymphatic channels of the diaphragm and is propelled into the right subclavian vein by the negative intrathoracic pressure (16). In cases of disseminated intra-abdominal cancer, further increased production of peritoneal fluid is induced by the tumors due to the increased leakiness of tumor microvasculature and obstruction of the lymphatic vessels (17, 18). As a result, fluid accumulation in the peritoneal cavity exceeds fluid reabsorption, resulting in the build-up of ascites. It has been suggested that the flow of ascites currents within the peritoneal cavity dictate the routes of dissemination of ovarian cancer (11, 19). The physiological factors that drive this process are gravity, diaphragmatic pressure, organ mobility, and recesses formed by key anatomical structures (20). The three most common intra-abdominal sites of ovarian cancer metastasis are the greater omentum, right subphrenic region, and pouch of Douglas, areas which have easy access to ascites (21). Detached ovarian tumor cells either singly or in the form of multicellular spheroids primarily colonize to these distant sites under the influence of ascites flow; however, little is known about the impact of ascites flow on the heterogeneity of metastatic ovarian tumors that colonize to distant sites (20).

SOLUBLE COMPONENTS OF ASCITES

Accumulation of ascites is a combined result of lymphatic obstruction, increased vascular permeability and secretions of resident tumor, and associated stromal and immune cells (11). As a result, malignant ascites constitutes a dynamic reservoir of survival factors, including cytokines, chemokines, growth factors, and ECM fragments, which individually and in a combined fashion affect tumor cell growth and progression through different cellular mechanisms (4, 5, 22, 23). A recent multiplex profiling of cytokines in the ascites obtained from 10 epithelial ovarian cancer patients has demonstrated enhanced expression of several factors including angiogenin, angiopoietin, GRO, ICAM-1, IL-6, IL-6R, IL-8, IL-10, leptin, MCP-1, MIF, NAP-2, osteoprotegerin (OPG), RANTES, TIMP-2, and urokinase plasminogen activator receptor (uPAR) (24). Among these OPG, IL-10, and leptin in the ascites of ovarian cancer patients were shown to be associated with shorter progression-free survival (24). OPG, a secreted member of tumor necrosis factor receptor (TNFR) superfamily, has been shown to bind and inhibit TRAIL-induced apoptosis of ovarian cancer cells, suggesting that ovarian tumor cells in the ascites with high expression of OPG may be able to evade TRAIL-induced cell death (25). Leptin is an adipokine produced predominantly by adipocytes and leptin-mediated signaling has been shown to promote ovarian cancer cell growth *in vitro* (26). On the other hand, IL-10 is known to inhibit T helper cell proliferation, hamper dendritic cell maturation, and inhibit T cells co-stimulatory molecules suggesting that IL-10 in ascites may help tumor cells to evade host immunological surveillance (27–29). Consistent with that, ascites-derived ovarian tumor cells have been shown to constitutively release CD95 ligand (also known as Fas ligand), which can induce apoptosis in immune cells expressing CD95 (30).

Exosomes derived from the ascites of ovarian cancer patients have been shown to impair the cytotoxic activity of peripheral blood mononuclear cells (31). Malignant ascites has been shown to also contain GD3 ganglioside, which inhibits the innate natural killer T (NKT) cell activity (32), while MUC16 expressed on the surface of ovarian cancer cells has been shown to inhibit the interaction of ovarian cancer cells with natural killer cells thus providing protection to ovarian cancer cells from host immunity (33). Additionally, correlations between the occurrence of regulatory T cells (Treg) (which inhibit tumor-specific T-cell immunity) in the ascites and reduced survival in ovarian cancer patients have been noted (11). These findings suggest that ascites contain the amenities to help tumor cells evade host immunosurveillance so that the tumor cells can avail unrestricted growth characteristics.

The concentration of inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10 was shown to be significantly higher in the ascites of ovarian cancer patients compared to that present in the serum, and correlated with poor prognosis and response to therapy (34, 35). The expression of IL-8 has been associated with increased tumorigenicity and ascites formation in animal models (36). IL-6, on the other hand, not only promotes tumor growth, migration, and invasion (34, 37, 38) but also facilitate chemoresistance (39, 40) and angiogenesis (41). In addition, high level of IL-6 in ovarian cancer ascites has been associated with shorter progression-free survival (42–44). Moreover, patients who responded to chemotherapy tended to have lower ascites IL-6 levels, compared with patients who did not respond to chemotherapy (45), suggesting that level of IL-6 in the ascites of ovarian cancer patients is an independent predictor of patient's response to therapy.

Hepatocyte growth factor present in malignant ascites of ovarian cancer patients has been shown to stimulate the migration of ovarian cancer cells (46). Finally, lysophosphatidic acid (LPA), a bioactive phospholipid present in high levels in the ascites of ovarian cancer patients and produced by ovarian cancer cells, signals through cell surface bound G-protein dependent receptors and impose diverse affects on ovarian cancer cells which includes increased transcriptional regulation of vascular endothelial growth factor (VEGF), uPA, IL-6, and IL-8 (47, 48). Among many other functions, LPA has been shown to increase *de novo* lipid synthesis in ovarian cancer cells crucial for LPA-induced

proliferation of ovarian cancer cells (49). LPA also disrupts the junctional integrity of epithelial ovarian cancer cells (50) which not only results in the metastatic dissemination of ovarian cancer cells but also results in increased membrane permeability which leads to enhanced ascites accumulation (2).

Vascular endothelial growth factor is found in abundance in the ascites of ovarian cancer patients and plays a central role in modulating the tumorigenic characteristics of ovarian cancer cells. VEGF is over expressed in ovarian tumor cells and is associated with poor prognosis (51, 52). High VEGF production from primary tumors has been reported to correlate with increased metastatic spread and worse prognosis compared to low VEGF secreting tumors (53). Retroviral enforced expression of VEGF in ovarian cancer cells has been shown to dramatically reduce the time of onset of ascites formation (54). One of the mechanisms by which VEGF modulates permeability of peritoneal membranes is by down regulation of tight junction protein claudin 5 in the peritoneal endothelial cells (55). In addition, VEGF has been shown to induce tyrosine phosphorylation of cadherin-catenin complex which results in decreased endothelial junctional strength and increased permeability (56). Several factors have been shown to influence the production of VEGF by ovarian cancer cells. These included hypoxia, LPA, tumor necrosis factor, matrix metalloproteinases, insulin-like growth factor, epidermal growth factor, platelet derived growth factor, and transforming growth factor beta (2). In line with these studies, systemic administration of the VEGF-Trap have been shown to prevent ascites accumulation and inhibit the growth of disseminated cancer in mouse models (54), suggesting that VEGF expression is crucial for ascites accumulation and ovarian cancer progression. Several agents that target VEGF have been evaluated in Phase II trials in women with recurrent ovarian cancer (57). Bevacizumab, a humanized monoclonal antibody against VEGF is currently in several Phase III studies with encouraging results (58).

CELLULAR COMPONENTS OF ASCITES

The origin and phenotype of the cells in the ascites is poorly understood. Similar to other tumor microenvironments, ascites contains a complex heterogeneous mixture of "resident" and "non-resident" cell populations, each having a defined role and connected with each other through soluble mediators, some of which have been described above. Belonging to the resident components of the ascites are tumor cells and cancer-associated fibroblasts (CAFs), to be distinguished from the non-resident populations, i.e., cells recruited from the outside the tumor microenvironment such as infiltrating macrophages/monocytes, bone marrow-derived mesenchymal stem cells (MSCs), and cytotoxic or Treg (59). Tumor cells within the ascites of ovarian cancer patients are either present as single cells or, more commonly, as aggregates of non-adherent cells, also known as spheroids (60). In this scenario, multiple (a few hundred) tumor spheroids can be seen either floating or embedded in the peritoneal cavity during primary debulking surgery (61). Some of these tumor spheroids are loosely attached to the underlying mesothelium and are detached during debulking surgery, while others are tightly attached to the peritoneum as individual small adherent tumors having independent vasculatures (61).

Neoplastic progression of ovarian carcinomas in the ascites occurs as differentiated epithelial tumors floating as tumor spheroids (62). However, it has been suggested that primary ovarian tumor cells may undergo an EMT-like process during localized invasion in the peritoneum and retain mesenchymal features in advanced tumors (8, 63). Even though the mesenchymal phenotype is central to EMT, ovarian cancer cells in ascites retain epithelial features and cell–cell contacts and are able to invade (60). Although enhanced E-cadherin expression, indicative of an epithelial cell type, has been demonstrated in the tumor cells of the ascites, especially those obtained from chemoresistant recurrent ovarian tumors (60), its expression is most commonly lost in metastasis (62). E-cadherin expressing ovarian carcinoma spheroids have been shown to adhere to and invade the surrounding mesothelium (9). Spheroids undergo reduced proliferation and have limited drug penetration resulting in decreased susceptibility to chemotherapy (64) and thereby mimic traits of cancer stem cells (CSCs)-like cells (62). Contributing to the heterogeneity of the resident ascites cells, CSCs are a population of cells that resists chemotherapy and is the source of proliferating tumor cells with progressive differentiating potential (65). These CSCs, when purified by sorting and xenografted into nude mice, have been shown to generate a significantly greater tumor burden compared to unsorted tumor cells (66, 67). On the other hand, non-resident cells within the ascites include non-cancer cells such as inflammatory cells, immature myeloid cells and activated mesothelial cells, and MSCs (which can be resident or non-resident) (68), all of which influence tumor cell behavior and response to chemotherapy (69). The resident and non-resident elements of the ascites microenvironment constantly interact with each other forming a unique tumor microenvironment (69). We discuss the non-resident cell populations within the ascites in detail below.

IMMUNE CELLS INFILTRATING THE ASCITES MICROENVIRONMENT

Recent studies have demonstrated that immune system influences the clinical outcome of high-grade serous ovarian cancer patients (70, 71). The presence of tumor-infiltrating $CD8^+$ T cells in primary tumors is associated with prolonged disease-free and overall survival of ovarian cancer patients (70, 71). In this context, the polyfunctional T-cell response of ovarian cancer patients has been shown to be disrupted by the factors in the ascites (72). Some of these factors have been discussed above, while additional factors include T cell co-stimulatory ligands B7-H4, stromal derived factor (SDF)-1, Fas ligand, and soluble IL-2 receptor (70, 71). A recent study has demonstrated that ovarian tumor T cell suppression can be alleviated by leukocyte depletion, suggesting that soluble factors secreted by leukocytes may also contribute to the suppression of T cells (73). Furthermore, a high CD4/CD8 T cell ratio in ascites was shown to be an indicator of the presence of Treg, which was associated with poor survival outcome (74). It has been reported that a high T cell/Treg ratio independently predicts increased survival (75). However, it was suggested that it is not so much the presence of Treg but in general the presence of immune responsive T cells which was observed to exert survival effects (75). In addition, reduced accumulation of $CD3^+CD56^+$ cells (natural killer or natural killer-like T cells) in

the ascites was also correlated with increased platinum resistance (76). Furthermore, ascites from ovarian cancer patients containing elevated levels of IL-17 (a cytokine predominately produced by Th17 and other effector T cells) was correlated with increased overall survival (77).

In addition to above, malignant ascites contains significant numbers of activated $CD163^+$ M2 type of macrophages the presence of which correlates with enhanced levels of IL-6 and IL-10 and inversely correlates with relapse-free survival period in ovarian cancer patients (78). Ascites also contains rare plasmacytoid dendritic cells (PDCs) (<0.1% of blood monocytes) (79). Activated macrophages and PDCs cells secrete CCL22 which is present in high levels in the ascites of ovarian cancer patients (80). *In vivo* treatment with monoclonal antibody to CCL22 resulted in significantly decreased Treg cell migration into tumors, suggesting that CCL22 may be contributing to the presence of Treg in ascites (80). In this context, tumor-associated PDC have been shown to induce angiogenesis *in vivo* by secreting TNF-α and IL-8 (81). In contrast, myeloid dendritic cells (MDCs) were absent from malignant ascites. MDCs derived *in vitro* suppressed angiogenesis *in vivo* through production of interleukin 12. Thus, the tumor may attract PDCs to augment angiogenesis while excluding MDCs to prevent angiogenesis inhibition, demonstrating a novel mechanism for modulating tumor neovascularization (81). In addition, myeloid-derived suppression cells (MDSCs) have been found in ovarian cancers transplanted in immune-compromised mouse models (82). These are a heterogeneous population of cells derived from immature granulocytes or monocytes released from bone marrow in response to stress induced by the tumor (83). The common functional feature of these cells is the repression of infiltrating functional T lymphocytes and natural killer cells (83). Hence, these cells critically control tumor progression but its role is yet to be identified in ovarian cancer. The above studies suggest that several factors and concerted mechanisms in the ascites create a microenvironment where cancer cells can grow unhampered.

STROMAL AND MESOTHELIAL CELLS IN THE ASCITES MICROENVIRONMENT

The pro-metastatic role of inflammatory stroma has been described in the literature (84). A significantly enhanced number of CAFs has been associated with high-grade ovarian tumors compared to benign and borderline tumors (85). Abundant CAFs were associated with the occurrence of lymph node and omental metastases and increased lymphatic and microvessel densities (85). CAFs isolated from high-grade ovarian tumors facilitated more migration and invasion in ovarian cancer cell lines than those isolated from normal tissues (85). In another study, CAFs isolated from omentum were shown to be activated by ovarian tumor cells to promote ovarian cancer growth, adhesion, and invasiveness through the TGFβ1 pathway (86). Interleukin-1β secreted by ovarian tumor cells was shown to induce a p53/NFκB-mediated stromal inflammatory response to support ovarian tumorigenesis (87). A recent study has provided evidence of the inter-conversion of CAFs into MSCs required for promoting tumor growth by paracrine production of inflammatory cytokines (88). Ovarian cancer-associated MSCs have also been shown to have a greater ability to promote tumor growth compared to normal MSCs (68). This was shown to be mediated through abnormal production of BMP2. Treatment *in vitro* of ovarian cancer cell lines with recombinant BMP2 was shown to enhance the production of $ALDH^+CD133^+$ ovarian CSCs (68). In another study, the expression of HOXA9, a Müllerian-patterning gene, was shown to promote ovarian cancer growth by converting normal peritoneal fibroblasts into ovarian CAFs (89). In the same study, the expression of HOXA9 was also shown to induce normal adipose and bone marrow-derived MSCs to acquire features of CAFs by transcriptional activation of TGFβ2 mediated by the expression of CXCL12, IL-6, and VEGFA. These studies, even though not directly related to CAFs in the ascites of ovarian cancer patients implicate CAFs as an important modulator of promoting ovarian tumor growth.

In addition to CAFs, ascites contains a significant proportion of activated mesothelial cells which remain as single cells or are embedded with floating spheroids. These mesothelial cells are a major source of VEGF and LPA in ascites which have demonstrated enhanced adhesion, migration, and invasion of ovarian cancer cells *in vitro* (90). Peritoneal mesothelial cells also have an enhanced expression of SDF-1/CXCR4-dipeptidyl peptidase IV (DPPIV) which has been suggested to be involved with the re-epithelization of discarded peritoneal basement membranes after the attachment of secondary tumors on the peritoneum (91).

CANCER STEM CELLS IN THE ASCITES MICROENVIRONMENT

In recent years, many reports have described the CSC characteristics of ovarian cancer (66, 69, 92). In these models, resident cells in the ascites or primary tumors have been demonstrated to have the features of self-renewal, multi-lineage differentiation, and tumor initiation characteristics *in vivo* (93, 94). CSCs in these reports have also been demonstrated to have the ability to colonize to distant sites and to survive chemotherapy. Genetic and epigenetic mechanisms appear to be the main factors in this scenario (69). *In vitro* enrichment and propagation of CSCs are achieved by growing cells in an unattached condition in the form of "spheroids" (94–96). As one of the features of ascites-derived ovarian cancer cells is to survive in a free-floating anchorage independent condition, the highest concentration of CSCs in ovarian cancer has been proposed to reside within the free-floating tumor spheroids contained in the ascites (60, 62). In support of this notion, it has recently been demonstrated that cells within the ascites have CSC characteristics (60, 93). It has also been shown that the abundance of CSCs is more in the ascites-derived spheroids of chemoresistant and recurrent patients compared to that in the chemonaive patients (60). This may be due to the chemoresistant phenotype of ovarian CSCs in ascites which remains undetected as residual tumor cells after treatment and gradually increase in number with consecutive cycle of treatments.

Wintzell et al. (97), also reported high levels of CSCs in freshly derived ascites, in both spheroids as well as in cells existing as single-cell population, but these authors concluded that the single-cell population was more enriched in CSCs than the spheroids. Both Wintzell et al. (97), and Latifi et al. (60), showed that ascites spheroids were high expressers of E-cadherin and EpCAM and low/negative expressers of vimentin, CD44 and MMPs (MMP2 and MMP9) compared to single-cell population. In addition, Latifi

et al. (60), showed that the single-cell population from ascites also have high expression of MSC markers such as CD73, CD90, CD105 as well as fibroblast surface protein (FSP), indicative of the CAF-like phenotype of single cells described by Wintzell et al. (97). However, Latifi et al. (60) found high expression of Oct4, STAT3, and CA125 in spheres and lack of expression of CA125 in the single-cell population. These observations were consistent with the lack of tumor forming ability of single cells in nude mice for as long as 20 weeks while the same number of cells collected from spheres formed tumors in nude mice within 12–14 weeks (60). These observations suggest that the tumorigenic component of ascites may exist within the spheres while single cells (potentially CAFs) may be the supporting entity, which is contrary to the conclusions of Wintzell et al. (97).

Distinct pattern of CSC marker co-expression may exist in spheres and single cells of the ascites and this needs to be explored further in future studies. High expression of Oct4 in single cells as described in Wintzell et al. (97), in contrast to high expression of Oct4 in spheres shown in Latifi et al. (60) may occur due to the differences in the separation techniques used by the two studies which may impact on the phenotypic changes in the cells. In addition, differences in the recruitment of patients in two studies may also contribute to the differences in the findings. While in Latifi et al. (60), only high-grade primary serous patients were recruited, the patient cohort in Wintzell et al. (97) contained different histologic subtypes of ovarian, Fallopian tube, and peritoneal cancers. Moreover, the expression of Oct4 was deduced at the mRNA level in Latifi et al. (60), while Western blot was used to detect the protein expression of Oct4 in Wintzell et al. (97). These differences in the approaches may contribute to the ambiguity of the Oct4 status in the spheres or single cells in the two studies. Hence, future studies on bigger cohorts of ovarian cancer patients are needed to determine if ascites spheres or single cells are the main repository of CSCs. Nevertheless, existing evidence indicates that the ascites microenvironment is a CSC-niche which facilitates processes such as EMT, inflammation, hypoxia, and angiogenesis in the resident cells which ultimately determine the function and fate of CSCs (69, 98).

EXPERIMENTAL AND TRANSLATIONAL APPROACHES TO THE STUDY OF ASCITES-DERIVED CELLS

Ascites is an indicator of poor prognosis in ovarian cancer patients, with the tumor cells within the ascites postulated to play dominant roles in metastatic spread, chemoresistance, and ultimately, the recurrence of the cancer (2, 60). Hence, a thorough understanding of the biology of the ascites microenvironment is essential for developing effective therapeutic intervention for metastatic ovarian cancer. Established ovarian cancer cell lines, often originally isolated from ascites, are readily available, immortalized, and low-cost options to assess tumor cell behavior. However, the distinct disadvantage of cell lines is their accumulation of numerous genetic and phenotypic abnormalities over years of culture which no longer accurately reflect the clinical disease (99). Ascites isolated from ovarian cancer patients represents a readily accessible source of primary cancer cells and cancer-associated cells with the potential to provide direct insights into the molecular and cellular pathophysiology of ovarian cancers as they metastasize within the peritoneal cavity. Reviewed below are some of the clinically relevant model systems which have provided novel insights into the contribution of ascites-derived cells and the ascites microenvironment to ovarian cancer tumorigenicity and the metastatic progression of the disease.

ISOLATION AND CHARACTERIZATION OF ASCITES-DERIVED CELL POPULATIONS

As reviewed above, ascites contains a complex heterogeneous mixture of malignant and non-malignant cell types. Tumor cells can be isolated from ascites without mechanical or enzymatic digestion (100) and, if cultured under non-adherent conditions, retain their molecular and phenotypic profiles long-term (60). Most methods devised for the isolation and primary culture of ascites-derived cells incorporate a step to remove contaminating red blood cells, with some methods further separating cell populations based on their molecular and/or phenotypic profiles (60, 61, 93, 97, 101, 102). Notably, there have been several studies which isolated presumptive CSC populations from ascites using clonal selection (93) or FACS sorting for particular cell surface markers (101) or Hoechst dye 33342 exclusion (103). Isolated cells are characterized for their expression of stem cell markers, such as Oct4, Nanog, Bmi1, ABCG2, and then tested *in vitro* and *in vivo* for self-renewal and differentiation capabilities (104). These studies resulted in the paradigm-shifting identification of ovarian CSC populations within the ascites and the recognition of the roles CSCs play in the pathophysiology of epithelial ovarian cancer. CSCs are capable of asymmetric division which enables their own self-renewal as well as the generation of the heterogeneous differentiated cell populations that comprise the majority of the tumor mass (66, 67). When transplanted into immunodeficient mice, CSCs isolated from tumors can recapitulate the primary disease (93). Furthermore, the high rate of cancer recurrence following platinum and taxol-based chemotherapeutics is thought to be due to a failure to eradicate CSCs, which exhibit heightened chemoresistance compared to the rest of the tumor (62, 67, 105, 106). These data underscore the need to understand the central regulatory pathways critical to CSC survival in order to effectively target recurrent disease therapeutically (69).

Distinct subpopulations of ascites-derived cells have also been separated during culture on the basis of their differing phenotypes. For example, mesenchymal-like cells can be separated from epithelial tumor cells on the basis of their relative adherence to low-attachment plates (60, 107). In this method, the bulk of the ascites-derived tumor cells float as aggregates while non-tumorigenic mesenchymal cells attach to the plates (60). This method has been used to understand how the biology and molecular profile of the ascites microenvironment in patients with chemonaive and chemoresistant disease differs and how these differences relate to tumor behavior in *in vitro* and *in vivo* assays (60). Specifically, these studies demonstrated that chemotherapy treatment induces a CSC-like phenotype *in vitro* (107) which is recapitulated in primary ascites-derived ovarian cancer cells from chemoresistant patients with recurrent disease (60). These findings are supported by an independent study of ascites-derived cells, which characterized stromal progenitor cells within the ascites (101). These researchers noted that ascites from patients with

recurrent and late-stage epithelial ovarian cancers contained more cells with a higher expression of stem cell markers than ascites from patients with early-stage tumors (101). Various cell isolation and culture methods have been used to access ascites cell populations, and the current data present a picture of significant intra- and inter-patient heterogeneity. Nevertheless, subpopulations of cells from ascites with CSC-like cell surface protein expression profiles and self-renewal capabilities consistently display a more aggressive metastatic, chemoresistant phenotype than cell populations lacking CSC-like features in both *in vitro* and *in vivo* xenograft models of ovarian cancer metastasis (102, 105).

These novel findings suggest the need for a thorough evaluation of the subpopulations of ascites-derived cells in association with cancer stage, patient response to chemotherapy, and overall patient survival in order to identify molecular or protein signatures within ascites subpopulations with prognostic and diagnostic significance. To this end, comprehensive gene expression assessment methods such as RNA and microRNA screens, proteomics strategies, and NextGen sequencing are being applied to the analysis of ascites-derived cells (2). A recent study demonstrated the clinical potential of one such a high-throughput, integrative approach. Using microarray and clinical data from over 1000 patients with high-grade serous epithelial ovarian cancer a novel prognostic model was developed which was based on the altered profiles of family members of lethal-7 (let-7) microRNAs (108). This study identified let-7b as the master regulator of a network of genes, with higher levels of let-7b predictive of poorer outcomes after primary chemotherapy (108). Notably, patients could be stratified on the basis of their let-7b profiles into low, intermediate, and high-risk groups which corresponded to response to front-line chemotherapy and 5-year survival rates (108). While this method was developed using publicly available gene array data sets derived from advanced ovarian cancers, adaptation of this method to the study of freshly isolated ascites-derived tumor cells from chemonaive and chemoresistant patients would represent a means for improved prediction and monitoring of patients' response to chemotherapies.

FUNCTIONAL ANALYSES OF ASCITES-DERIVED CELL POPULATIONS

As spheroid formation within ascites is postulated to directly contribute to disease spread and to the development of chemoresistance (see above), several methods have been developed for the functional assessment of ascites-derived cells *in vitro* and *in vivo*, with the aim of mirroring various *in vivo* microenvironments as accurately as possible. The overarching aim of these studies is the development of new therapeutic approaches which specifically target particular stages of ovarian cancer metastasis, e.g., the formation or stability of spheroids within the ascites to enhance sensitivity to chemotherapeutics or the attachment and invasion of spheroids into the peritoneal lining to block colonization at distal sites.

In vitro modeling of spheroid formation, survival, and metastasis

Cancers which spread through the blood and lymphatic vasculature undergo repeated intravasation and extravasation through vessel walls. In contrast, during ovarian cancer metastasis, cancer cells are shed from the primary tumor into the peritoneal cavity and must survive suspended within the ascites (8, 10). To model this stage of ovarian cancer metastasis, ovarian cancer cell lines or primary ascites-derived cells are maintained under non-adherent conditions, such as in hanging-drops, in a liquid overlay, or in low-attachment culture dishes (109, 110). Under non-adherent conditions, cancer cells inherently aggregate together to form multicellular spheroids, which exhibit enhanced abilities to avoid anoikis (111). The main advantage that spheroid cultures have over monolayer cultures is that spheroid cultures more accurately model the complex three-dimensional structures assumed by ovarian cancers metastasizing within the peritoneum and recapitulate the molecular (e.g., oxygen, nutrient, metabolite) gradients found *in vivo*. Thus, multicellular spheroids cultured under non-adherent conditions which mimic the ascites more accurately predict *in vivo* behaviors and responses to therapies. For example, cancer cells grown as spheroids can be up to 100 times less sensitive to chemotherapies than the same cells cultured as monolayers, reflecting the inherent chemoresistance exhibited by metastasizing ovarian cancer spheroids in a clinical setting (109). The enhanced survival capabilities of spheroids were recently demonstrated using primary ascites-derived epithelial ovarian cancer cells (112). In this study, when grown as spheroids in non-adherent culture, ascites-derived tumor cells exhibited resistance to Myxoma-virus-mediated death despite the virus entering and replicating within the spheroids. In contrast, if the tumor cells were grown as monolayers in adherent culture or if spheroids were replated onto adherent surfaces, they exhibited sensitivity to Myxoma-virus-mediated death (112). This study has important implications for the development of treatments for advanced, metastatic ovarian cancers, underscoring the need to study the non-adherent spheroid stage of ovarian cancer metastasis in the development of new therapeutic regimens in order to ensure that new treatment options are effective against tumor spheroids floating within the ascites.

For experimental study, spheroids can be harvested freshly from ascites using centrifugation or low-attachment plates (60, 97). Harvested spheroids can be replated onto different solid surfaces or co-cultured with other peritoneal cell populations to model later stages of ovarian cancer metastasis, when multicellular spheroids attach to and invade the peritoneal lining to form a secondary tumor (113–115). In particular, co-cultures of ovarian cancer cells with specific subpopulations of the peritoneal lining and omentum, such as fibroblasts (86, 116), adipocytes (117), and mesothelial cells (115) have provided particular insights into the physical, biomechanical, and chemical interactions between invading tumor cells and the peritoneal environment in the establishment of metastatic nodules within the peritoneum. These models are growing increasingly sophisticated, with the use of primary omental and peritoneal tissue for three-dimensional organotypic models (116, 118). These studies demonstrate that peritoneal and omental fibroblasts, adipocytes, and mesothelial cells directly contribute to the pro-metastatic environment of the peritoneal cavity, releasing soluble factors into the ascites, secreting ECM components, and supplying energy reserves for the invading cancer cells.

As over 75% of ovarian cancers have already metastasized at the time of diagnosis (1), the information gained from these approaches is urgently needed in order to derive novel strategies which specifically disrupt the interactions between ovarian

cancer spheroids and the peritoneal microenvironment, thereby preventing the establishment of secondary tumors. In recent years, three-dimensional spheroid culture methods have been adapted to a variety of high-throughput systems, with the aim to expediting the screening the effectiveness of therapeutic compounds and identifying the key factors underlying metastatic growth and dissemination (109, 110). Of note, a recent study has used a microfluidic platform to study the effects of the hydrodynamic forces of ascites on tumor phenotype (20). This study used several on-chip analyses [immunofluorescence for epidermal growth factor receptor (EGFR); mRNA isolation for RT-PCR; and protein isolation for biomarker quantification] to show that continuous flow induced EMT in an ovarian cancer cell line, which contributed to a more aggressively invasive phenotype. These data demonstrate yet another facet of the ascites microenvironment which contributes to the ovarian cancer metastatic process (i.e., biochemical), furthermore, this experimental approach represents a high-throughput modality in which to study the efficacy of various targeted therapies in the prevention of the establishment and growth of secondary tumors.

In vivo modeling of the intraperitoneal environment

A number of studies have studied the role of vascularization in ovarian cancer metastasis or verified their *in vitro* results using either subcutaneous or intraperitoneal injection of ascites-derived tumor cells into nude mice, e.g., the validation of the tumor-repopulating abilities of isolated ascites-derived tumor cells or putative ovarian cancer CSCs *in vivo* (60, 93, 102, 105). These models provide an *in vivo* microenvironment for testing established and novel chemotherapeutic approaches and are a necessary preclinical model system. However, these models lack the true metastatic features of ovarian cancer which occurs in the peritoneum and involves the ovaries, adjacent organs (extra-ovarian pelvic organs, e.g., colon, bladder, liver) as well as spheroids carried around in the ascites to distal organs of the peritoneal cavity (1). Moreover, the xenotransplantation immunocompromized mouse model currently used may select populations of tumor cells that can override the weak immunogenic response of nude mice which is entirely different from the immune response in patients against their own tumors (119) In recognition of this latter problem, recently a refined mouse xenograft model has been developed using human embryonic stem cells to generate a "human" microenvironment within immunocompromized mice. Using malignant cells freshly isolated from the ascites of an ovarian cancer patient, six derivative cell subpopulations were developed, and it was found that the human microenvironment permitted some patient-derived ascites cells to generate tumors which failed to grow in a conventional nude mouse model (120). This improved method may enable the study of the *in vivo* behaviors of previously unstudied cell subpopulations and also provides insights into the role of the human microenvironment in the tumorigenicity and metastatic capabilities of ovarian cancers.

ASCITES AS A PLATFORM FOR TRANSLATIONAL RESEARCH

As discussed above, ascites is a source of tumor material from which valuable information can be extracted not only to understand the pathophysiology of ovarian cancer progression but also for the development of markers which will predict prognosis and monitor the progression of the disease. The frequent presence of ascites at first presentation, and subsequent relapses, provides an accessible pool of tumor material that can be studied to determine the molecular characteristics of cells as the disease progresses. With the establishment of methods which can separate the different soluble and cellular components of the ascites (60), it may now be possible to identify and differentiate the true molecular perturbations that exist between the chemonaive, chemoresistant, and recurrent status of the disease. Isolated cellular components of the ascites can be preserved as paraffin embedded blocks for immunohistochemical analysis (121, 122), or can be frozen for molecular analysis at the RNA and protein levels (60, 122). Moreover, ascites provides a substantial amount of biological material which can be obtained to design studies which require relatively larger amounts of tumor material, which previously were only limited to genome-based studies due to the scarce availability of primary and metastatic tumors leftover after pathological diagnosis. These studies include methods to elucidate the protein profile of ascites-derived tumor and associated cells by proteomic methods such as matrix-assisted laser desorption and ionization (MALDI), surface enhanced laser desorption and ionization (SELDI), and liquid chromatography followed by mass spectroscopy (MS) (2, 123), all of which require larger amounts of samples than that used by genomic methods. In addition, high-throughput automated array-based proteomics techniques such as reverse phase protein arrays (RPAs) can be used to understand the differential expression of proteins in the isolated ascites cellular components from chemonaive and chemoresistant patients. A recent study which used the RPA analysis on ascites samples and pleural effusions obtained from ovarian cancer patients showed significantly higher expression of AKT, cAMP-responsive element binding protein (CREB), and Jun-N-terminal kinase (JNK) in malignant ascites compared to benign effusions (124). Given that deregulation of PI3 kinase and the downstream AKT pathway has been demonstrated in ovarian cancer (125, 126), and high levels of p38 and an increase in the ratio of phosphorylated EGFR and phosphorylated JNK were associated with bad prognosis in ovarian cancer patients (124), it seems that the proteomic profile of the ascites environment may imitate the protein expression profile of the original tumors (2). These observations suggest the enormous potential of using ascites samples for diagnostic, prognostic, and therapeutic endpoints.

Accessibility to ascites also provides a means of comparing the secretory components of the chemonaive and chemoresistant patients. A recent study has determined the cytokine expression profile of the ascites of ovarian cancer patients. Out of 120 cytokines analyzed OPG, IL-10, and leptin was found to be associated with worst prognosis in ovarian cancer patients (24). The concept that the damage of tumor cells in response to chemotherapy treatment can activate autocrine and paracrine secretory responses of residual tumor cells (69, 127, 128), suggest that the soluble component of the ascites microenvironment of chemonaive and chemoresistant patients may be significantly different. In addition, the tumor growth promoting effect of exosomes released by ovarian tumors has been reported (129). Malignant ascites-derived

exosomes of ovarian carcinoma patients have been shown to contain CD24 and EpCAM (130). The exosome-associated proteolytic activity in the tumor vicinity has been suggested to augment tumor invasion into the stroma (130). Exosomes released by ovarian cancer cells have been shown to induce apoptosis of mature dendritic cells and peripheral blood nuclear cells suggesting they have a negative effect on host immunity (31). In addition, ascites have been shown to contain pro-survival factors which compromised the therapeutic effects of TRAIL and were shown to be associated with shorter disease-free intervals in ovarian cancer patients (131). These data suggest that the signals derived from the soluble ascites microenvironment plays a crucial role in regulating ovarian tumor cells and targeting the survival promoting activity of the soluble component of ascites may be mandatory for the development of efficient therapies for ovarian cancer patients.

FUTURE DIRECTIONS

From a clinical perspective, our understanding of ascites and its associated cellular and soluble components are of utmost importance to understand the advanced-stage disease. The central component of such investigations would be ascites obtained from patients pre- and post-chemotherapy and understanding both the soluble and cellular components individually and/or in association with each other. These studies can be performed using microfluidic systems to investigate the impact of ascites on resident and non-resident cell systems either individually or in combination (20). Microfluidic platforms have been used to investigate the morphological parameters and migratory potentials of immune cells in response to external stimulus (132). Other studies have used cell-on-chip based platforms to investigate the interaction of tumor cells with endothelial cells (133). Recently, a simple cell-on-chip platform was developed to investigate the crosstalk between immune cells and cancer (89). Using this approach, which consisted of three wide parallel chambers interconnected via an array of short and narrow capillary migration channels, it was possible to visualize under the microscope the interaction between the immune and cancer cells (89). Hence, customized microfluidic platforms may be helpful to study and mimic the events of ascites-derived microenvironment. This can also provide helpful clinical information as understanding the crosstalk between cancer cells with associated surrounding cells in the native ascites microenvironment will result in the improvement of therapies for ovarian cancer.

CONCLUSION

The accessibility of ascites undeniably provides a rich source of tumor samples to monitor the course of chemotherapy treatment in patients. In addition, it also provides an opportunity for the identification of prognostic and treatment-monitor markers, as well as options for molecular profiling of both the cellular and soluble components. The cellular and molecular profile of individual ascites is a subject of inter-patient variations which will differ not only with the treatment protocol but also how each patient responds to a particular therapy. Hence, to provide a molecular characterization which would fit into a defined pattern to design appropriate targeted therapies would be challenging. Hence, long-term, longitudinal studies within the same patient cohorts, starting with chemonaive status and periodic evaluations of molecular and cellular characterization of the ascites components as the disease progresses would be useful to develop an individualized predictive profile which will be crucial for designing targeted therapies. The interrogation of soluble and cellular variations in ascites during the treatment regimen in patients may guide clinical decision making for patient management (134). This may form a basis for informed and effective personalized treatment approaches. Hence, with the advances in our understanding of the pathophysiology of ascites and the development of new methods which can delineate the cross talk between the different cellular components it is anticipated that more effective and targeted strategies for the management of ascites and ovarian cancer patients will be available in near future.

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A new spontaneously transformed syngeneic model of high-grade serous ovarian cancer with a tumor-initiating cell population

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Improving screening and treatment options for patients with epithelial ovarian cancer has been a major challenge in cancer research. Development of novel diagnostic and therapeutic approaches, particularly for the most common subtype, high-grade serous ovarian cancer (HGSC), has been hampered by controversies over the origin of the disease and a lack of spontaneous HGSC models to resolve this controversy. Over long-term culture in our laboratory, an ovarian surface epithelial (OSE) cell line spontaneously transformed OSE (STOSE). The objective of this study was to determine if the STOSE cell line is a good model of HGSC. STOSE cells grow faster than early passage parental M0505 cells with a doubling time of 13 and 48 h, respectively. STOSE cells form colonies in soft agar, an activity for which M0505 cells have negligible capacity. Microarray analysis identified 1755 down-regulated genes and 1203 up-regulated genes in STOSE compared to M0505 cells, many associated with aberrant Wnt/β-catenin and Nf-κB signaling. Upregulation of *Ccnd1* and loss of *Cdkn2a* in STOSE tumors is consistent with changes identified in human ovarian cancers by The Cancer Genome Atlas. Intraperitoneal injection of STOSE cells into severe combined immunodeficient and syngeneic FVB/N mice produced cytokeratin+, WT1+, inhibin−, and PAX8+ tumors, a histotype resembling human HGSC. Based on evidence that a SCA1+ stem cell-like population exists in M0505 cells, we examined a subpopulation of SCA1+ cells that is present in STOSE cells. Compared to SCA1− cells, SCA1+ STOSE cells have increased colony-forming capacity and form palpable tumors 8 days faster after intrabursal injection into FVB/N mice. This study has identified the STOSE cells as the first spontaneous murine model of HGSC and provides evidence for the OSE as a possible origin of HGSC. Furthermore, this model provides a novel opportunity to study how normal stem-like OSE cells may transform into tumor-initiating cells.

Keywords: high-grade serous cancer, stem cell, tumor-initiating cell, syngeneic, ovarian cancer, ovarian surface epithelium, mouse model of ovarian cancer

INTRODUCTION

Ovarian cancer is the most lethal gynecological malignancy with an estimated incidence of 238,719 cases in 2012, making it the eighth most common cancer in women worldwide (1). Epithelial ovarian cancer (EOC) is the most common type, which is further divided into endometrioid, clear cell, mucinous, low-grade serous, and high-grade serous (HGSC). HGSC is the most common and aggressive subtype of EOC, accounting for the majority of new cases (2). With a 5-year survival rate of only 40%, a greater understanding of HGSC is essential to improve patient outcome (1). The high mortality rate is due, at least in part, to a lack of screening methods to detect the disease before it metastasizes within the peritoneal cavity (3). The main reason for this inability to detect and diagnose early stage ovarian cancer is a lack of understanding of disease initiation, made even more challenging due to the current debate over the origin of HGSC. HGSC was long thought to arise from the ovarian surface epithelium (OSE) or inclusion cysts derived from them (2, 4, 5), but recent evidence has identified the distal fimbrial epithelium of the fallopian tube as the source for at least a subset of HGSC (2, 6–8).

To establish experimental models for the study of the initiation of EOC, much effort has been dedicated to the genetic modification of cells from an OSE or fimbrial origin, either in tissue culture or *in vivo*. Attempts to model HGSC have been particularly challenging and have yielded inconsistent results (5, 9, 10). Transgenic approaches have generally involved targeting specific genes known to be associated with human HGSC. This targeted approach to tumorigenesis may not be fully reflective of human

disease for a number of reasons. First, it is unclear, in human disease, whether commonly mutated genes are normally involved in disease initiation and/or progression. In addition, the expression of the designed genetic changes using developmentally regulated promoters may introduce founder effects that are not reflective of human disease (9). Furthermore, it has been shown that murine cells require fewer genetic alterations than human cells to undergo transformation, again making it difficult to draw conclusions on the origin of cancer in humans from transgenic murine models (11, 12) For this reason, spontaneous models of EOC would be helpful to better understand the origins of this disease, but these models are rare and limited to the spontaneous development of ovarian cancer in hens (13, 14). New spontaneous models of HGSC are clearly needed to provide opportunities to determine the molecular basis of ovarian and fallopian tube epithelial transformation.

There is growing evidence to support the contribution of cancer stem cells (CSC) to the initiation and recurrence of cancer. The CSC theory posits that tumors arise from cells with stem-like characteristics and these cells underlie tumor heterogeneity and recurrence (15–17). Stem cells are slowly dividing cells with drug efflux mechanisms that allow them to escape the effects of chemotherapeutics that commonly target rapidly dividing cells. Another characteristic of a stem cell is the ability to generate multi-lineage progeny. Recurrent cases of HGSC maintain the heterogeneity of the original tumor suggesting that a cell with multi-lineage potential underlies tumorigenesis, instead of a single clone with a survival advantage (15). A cell with all the characteristics of CSCs is still elusive in ovarian cancer but cells with some of these CSC characteristics, identified by their expression of CD44, CD133, CD117, CD24, and ALDH1 (3), have been reported. These CSC-like cells are referred to as tumor-initiating cells (TICs) due to their increased tumorigenic capacity. The role and identification of TICs in ovarian cancer is a rapidly growing area of study.

We recently reported the first stem cell marker that identifies a subpopulation of mouse OSE cells with progenitor cell characteristics. A population of cells expressing stem cell antigen 1 [SCA1; aka lymphocyte antigen 6 complex, locus A (LY6A)] is regulated by ovulation-associated factors present in the follicular fluid and possesses a number of features of stem cells, including slow growth and capacity for self-renewal (18). After several years of establishing and growing cultures of mouse OSE cells, one cell line that was grown for a prolonged period appeared to spontaneously transform. The following body of work describes the characterization of this spontaneously transformed OSE (STOSE) cell line, demonstrating that it reliably forms syngeneic HGSC tumors. Testing of the SCA1+ cells in the parental and transformed cell lines enabled us to compare the characteristics of these stem cell-like populations, as well as determine the relative malignant potential of SCA1+ vs. SCA1− STOSE cells.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Severe combined immunodeficient (SCID) and FVB/N mice were obtained from The Jackson Laboratory and housed with a 12 h light:12 h dark photoperiod. The animals had free access to food and water and experiments were done in accordance with the Canadian Council on Animal Care's *Guidelines for the Care and Use of Animals*. Protocols were approved by the University of Ottawa Animal Care Committee.

MOUSE OSE CELL ISOLATION AND CULTURE

The M0505 OSE cell line was isolated and established in 2005 according to the protocol described in Gamwell et al. (18). Upon long-term passage of the cells in adherent cultures on tissue culture plates (Becton Dickinson) using MOSE media (18), the M0505 cell line spontaneously transformed and were from that point on labeled STOSE cells, which were also maintained in MOSE medium. The M1107 OSE cell line was established and maintained using the same methods as the M0505 cell line and is used as an independent control for mouse OSE cells.

PROLIFERATION ASSAY

M0505 and STOSE cell proliferation was assessed from 1 to 3 days after seeding 2×10^4 cells in 24-well tissue culture dishes (Becton Dickinson) in MOSE medium. The number of viable cells was determined using the Vi-CELL XR cell viability analyzer (Beckman Coulter).

CHROMOSOMAL ANALYSIS

G-band karyotyping of 5-metaphase spreads each of M0505 and STOSE cells was carried out by the Cytogenomics and Genome Resource Facility at SickKids Hospital, Toronto, ON, Canada. Briefly, cells were harvested and colcemid (10 μg/mL) was added for 30 min and incubated at 37°C. Cells were washed, trypsinized, and a single-cell suspension was made. Following washing, a 0.075 M KCl hypotonic solution was added for 15 min and incubated at 37°C, and banding patterns were visualized.

CELL CYCLE ANALYSIS

The percentages of cells in G1/G0, S-phase, and G2/M phases as well as the percentage of apoptotic cells were determined for M0505 and STOSE cell lines using flow cytometry. Cells were trypsinized (0.05% trypsin/0.53 mM EDTA, Corning Cellgro), washed in phosphate-buffered saline (PBS), and 1×10^6 cells from three independent isolations of each cell line were resuspended in 300 μL of cold PBS. Cells were fixed in 70% ethanol for 2 h, washed, and resuspended in 250 μL of PBS and 5 μL of RNAse A (Sigma Aldrich) for 1 h. The cell suspension was then incubated for 30 min with 10 μL of propidium iodide (Sigma Aldrich) and the cell cycle was assessed by flow cytometry using a Beckman Coulter Epics XL and analyzed by ModFit LT software (Verity Software Inc.). Cell doublets were identified using fluorescence pulse height vs. area measurements and excluded from cell cycle analysis.

MICROARRAY ANALYSIS

RNA was extracted from M0505 and STOSE cells ($n = 3$) using RNeasy Mini Kit (Qiagen) and cDNA was made using the OneStep RT-PCR kit (Qiagen). Whole genome expression was determined using Affymetrix GeneChip Mouse Gene 1.0 ST arrays. Genes were annotated using T4-MEV software (Dana Farber Cancer Institute, Boston) and linear fold change was determined from robust multi-array average (RMA) normalized expression values. Ingenuity pathway analysis software (Ingenuity Systems, Qiagen) was

used to determine functionally relevant clusters of differential gene expression. Microarray data are publicly accessible from the GEO database at record GSE54633.

QUANTITATIVE RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA was made using the OneStep RT-PCR Kit (Qiagen). Quantitative-PCR was then performed on an ABI 7500 FAST qRT-PCR machine (Applied Biosystems) using the Taqman gene expression assay (Life Technologies) and SsoFast gene expression assay (Bio-rad). Probe (2.5 nmol) and primer (5 nmol) sequences are listed in **Table 1**. The level of *Tbp* was used as an endogenous control in the Taqman assay and *Ppia* was used as an endogenous control in the SsoFast assay.

Table 1 | Quantitative RT-PCR probe and primer sequences.

Gene	Assay	Probe/primer sequence
Cdkn2a	Taqman	Probe: 5′-/56-FAM/AGCAGAGCT/ZEN/AAATCCGG CCTCAG/3IABkFQ/-3′ Primers: forward, 5′-GCTTCAATCTGTTCCTGGCA-3′, reverse, 5′-CAACAACTTCCTCTCCTGCTAC-3′
Sfrp1	SsoFast	Primers: forward, 5′-CAGTTGTGGCTTTTGCATTG-3′, reverse, 5′-GAGGGAAGGGAGAGGGTTC-3′
Frzb	SsoFast	Primers: forward, 5′-GGACGGAGCGGATTTTCCTAT-3′, reverse, 5′-TGACAGGCTTACATTTGCAACG-3′
Sfrp4	SsoFast	Primers: forward, 5′-TGGAGAGATCAACTCAGTAGA AGG-3′, reverse, 5′-GGCTGGCTATCTGCTTCTTG-3′
Ccnd1	Taqman	Probe: 5′-/56-FAM/ATCAAGTGT/ZEN/GACCCGGA CTGCC/3IABkFQ/-3′ Primers: forward, 5′-CGCTAGAAGTGAAGCTAAG AAGA-3′, reverse, 5′-CTTTGTGTACCGCTGGGAA-3′
Ikbkε	SsoFast	Primers: forward, 5′-GGGAGAGTCTTTGCCTGATTC-3′, reverse, 5′-ATCTCCTGGGCTTGGCTATC-3′
S100a4	SsoFast	Primers: forward, 5′-GGAGCTGCCTAGCTTCCTG-3′, reverse, 5′-TCCTGGAAGTCAACTTCATTGTC-3′
Spp1	SsoFast	Primers: forward, 5′-GGAGGAAACCAGCCAAGG-3′, reverse, 5′-TGCCAGAATCAGTCACTTTCAC-3′
Ppia	SsoFast	Primers: forward, 5′-AGGGTGGTGACTTTACACGC-3′, reverse, 5′-GATGCCAGGACCTGTATGCT-3′
Tbp	Taqman	Probe: 5′-/56-FAM/ACTTGACCT/ZEN/AAAGACCATTGC ACTTCGT/3IABkFQ/-3′ Primers: forward, 5′-CCAGAACTGAAAATCAACG CAG-3′, reverse, 5′-TGTATCTACCGTGAATCTTGGC-3′

INTRAPERITONEAL (IP) AND INTRABURSAL (IB) INJECTIONS OF STOSE CELLS

M0505 and STOSE cells were released from adherent cultures using trypsin (0.05% trypsin/0.53 mM EDTA), washed with PBS, and resuspended in PBS. 1×10^7 M0505 cells in 500 μL of PBS were injected into the peritoneal cavity of FVB/N mice. 1×10^7 STOSE cells in 500 μL of PBS were injected into the peritoneal cavity of both SCID and FVB/N mice using a 25-gauge needle (Becton Dickinson). Disease progression was monitored until humane endpoint was reached, which included 15% weight gain and/or abdominal distension. Necropsies were performed at endpoint and tumors were fixed in 10% buffered formalin for 24 h and then paraffin embedded and sectioned at 5 μm for immunohistochemical analysis.

To perform intrabursal injections of STOSE cells, FVB/N mice were anesthetized using 3% isoflurane gas and 1% oxygen. A dorsal incision was made and ovaries were externalized. STOSE cells (4×10^4) were resuspended in 2 μL of PBS and injected under the bursal membrane using a 33-gauge needle and dispensing repeater (Hamilton). Tumor initiation was monitored every 2 days by palpation of the ovaries by someone blinded to the experimental groups. Disease progression was monitored until humane endpoint was reached, at which point tumors were fixed, embedded in paraffin blocks, and 5 μm sections were made for immunohistochemistry.

IMMUNOHISTOCHEMISTRY

Assessment of the histopathology of IP and IB STOSE tumors was performed by staining sections with hematoxylin and eosin (H and E) and by immunohistochemical analysis. Following deparaffinization in xylenes and rehydration in an ethanol gradient, antigen unmasking (antigen unmasking solution, Dako) was performed, followed by blocking endogenous peroxidase activity using 3% hydrogen peroxide in dH_2O. Sections were then rinsed in PBS. Immunostaining for mouse cytokeratin (pan-CK; pre-diluted, Abcam), mouse WT1 (1:100, Dako), and mouse inhibin (1:100, Dako) was performed according to the mouse-on-mouse kit (Vector). Immunostaining for rabbit PAX8 (1:400, Santa Cruz Biotechnology) was done by incubating sections with the PAX8 antibody overnight at 4°C, followed by anti-rabbit horseradish peroxidase-labeled polymer (Dako) for 30 min at room temperature. All sections were counterstained using hematoxylin and developed using diaminobenzidine. Following dehydration in an ethanol gradient, sections were mounted using Permount (Fisher Scientific). Images were acquired using the ScanScope CS2 (Aperio).

DNA SEQUENCING

Genomic DNA was extracted from STOSE cells using QIAamp DNA Blood Mini Kit (Qiagen) and PCR amplified using custom primers designed to cover each of the 11 exons in the mouse p53 gene. Following electrophoresis on a 1% agarose gel, bands pertaining to each exon were individually excised under UV light. DNA was extracted from the agarose gel pieces using the QIAquick Gel Extraction Kit (Qiagen). Extracted DNA was then diluted to a concentration of 1 ng/μL and mixed with the appropriate custom primer (2 μM) mapping to each exon. Individual exons were sequenced using the 3730 DNA analyzer (Applied Biosystems). Sequences were aligned using the DNA Dynamo program (BlueTractorSoftware).

FLOW CYTOMETRY FOR SCA1 EXPRESSION

M0505 and STOSE cells were trypsinized and a single-cell suspension was made using a 40 μm cell strainer. Cells were resuspended

in a flow buffer (4% fetal calf serum in 1× PBS) and incubated with anti-SCA1 allophycocyanin fluorophore-conjugated antibody (Miltenyi Biotec) for 15 min at 4°C. Following washing and resuspension in flow buffer, cells were sorted for SCA1 expression using the MoFlo cell sorter (Beckman Coulter).

COLONY FORMATION IN SOFT AGAR

Cells were released from adherent cultures using trypsin, washed with PBS, and a single-cell suspension was achieved by passing cells through a 40-μm cell strainer. A base layer 1:1 mix of 2× Ham's F-12:MOSE medium (Sigma Aldrich) and ultrapure LMP agarose (Life Technologies) was solidified at 4°C for 30 min and then warmed to 37°C prior to the addition of the top layer. The top layer consisting of a 1:1:1 mix of 2.5×10^4 cells from single-cell suspension, 2× Ham's F-12:MOSE medium, and ultrapure LMP agarose was added. The top layer was solidified at 4°C for 30 min and then incubated at 37°C for 7 days. Colonies were visualized using the EVOS XL imaging system (Life Technologies) and counted using ImageJ software.

WESTERN BLOT ANALYSIS

Protein was extracted from M0505 and STOSE cells using M-PER mammalian protein extraction reagent (GE Healthcare). Tumor tissue from SCA1+ and SCA1− tumors was homogenized and protein was extracted using M-PER mammalian protein extraction reagent. Protein extracts were run on a precast Nupage 4–12% bis–tris gradient gel (Life Technologies) and transferred to a nitrocellulose membrane. Following 1 h blocking in 5% non-fat milk, membranes were incubated with mouse monoclonal PAX8 (1:500, Santa Cruz Biotechnology) or mouse monoclonal P53 (1:1000, Cell Signaling) overnight at 4°C. Following washing, the membranes were incubated with rabbit anti-mouse IgG–HRP (1:5000, Abcam) for 1 h and developed using Select™ western blotting detection reagent (GE Healthcare). The same protocol was used for β-actin using mouse monoclonal anti-β-actin (1:40,000, Sigma Aldrich) and rabbit anti-mouse IgG-HRP (1:15,000, Abcam).

STATISTICAL ANALYSIS

All experiments were performed at least three times. A Student's *t*-test was used to determine significant differences between two experimental conditions. Analysis of variance (ANOVA) with Tukey's post-test was used to identify significant differences between more than two experimental groups. Statistical significance was assumed at $p < 0.05$.

RESULTS

CHARACTERIZATION OF M0505 AND STOSE CELL LINES

Early passage M0505 cells grow slowly, having a doubling time of 48 h. The growth rate increases as M0505 cells reach >35 passages and cells begin to lose the epithelial "cobblestone" morphology that is characteristic of early passage M0505 cells (data not shown), and has been reported by others studying spontaneous transformation of epithelial cells (19). Continual passage of late passage M0505 cells led to the establishment of the STOSE cell line. STOSE cells have lost the epithelial "cobblestone" morphology and have transitioned to a more mesenchymal morphology (**Figure 1A**). To determine the malignant potential of STOSE cells *in vitro*, STOSE cells were assessed for colony forming efficiency in soft agar, a measure of anchorage independent growth that is characteristic of transformed cells (20). STOSE cells formed colonies while early passage M0505 cells did not (**Figure 1B**). Another characteristic of transformed cells is rapid growth (20). STOSE cells have a doubling time of 13 h, almost four times faster than their untransformed M0505 counterpart. The growth rate of STOSE cells in comparison to early passage (passage 18–20) M0505 cells over 72 h is shown in **Figure 1C**. Since a greatly increased growth rate might be explained by aberrant cell cycle regulation, cell cycle analysis was used to determine if there were differences in the percentage of M0505 and STOSE cells in each phase of the cell cycle. Cell cycle analysis of the M0505 cells (monomers) revealed a large G1 peak ($59.6 \pm 1.0\%$), a minor S-phase population ($10.1 \pm 0.3\%$), and a surprisingly prominent, putative G2/M peak ($28.8 \pm 0.8\%$) (**Figure 1D**). Interestingly, the presence of a small percentage (1.5%) of hyperploid cells was detected in the analysis by the ModFit program. The presence of a small population of cells with abnormal DNA content was then confirmed by karyotype analysis that identified near-tetraploid M0505 cells (**Figure 2B**). In addition, the small number of diploid cells in S-phase was consistent with the observed slow proliferation of this cell line. In contrast, STOSE cells have a significantly increased proportion of cells in S-phase ($45.2 \pm 0.7\%$) and, a reduced proportion in the G1 phase ($46.7 \pm 0.7\%$). The small G2/M population and greatly increased S-phase population suggests that STOSE cell cycle checkpoints may be compromised, which could lead to the observed acceleration in the rate of proliferation.

Due to the role of aneuploidy in transformation and cancer and the abnormalities found in the cell cycle analysis, chromosomal analysis was performed on M0505 and STOSE cells to determine if aneuploidy is present. G-band karyotyping of five metaphase spreads revealed aneuploidy in both M0505 and STOSE cell lines; two representative karyotypes are shown for each cell line (**Figure 2**). STOSE cells have a high degree of aneuploidy with the majority of the population near-triploid (**Figure 2C**) and a smaller polyploid population (**Figure 2D**). All STOSE cells analyzed have an addition at the terminal end of chromosome 4. All near-triploid cases have a loss of chromosome 3, 5, and 8, and all polyploid cases are also hypoploid for chromosomes 3, 5, and 8 (**Figures 2C,D**). Surprisingly, chromosomal analysis of early passage (passage 15) M0505 cells also revealed some degree of aneuploidy with 2/5 near-tetraploid M0505 cells (**Figure 2B**), while 3/5 M0505 cells were near-diploid (**Figure 2A**). This presence of a near-tetraploid subset of M0505 cells is in agreement with the presence of M0505 cells with increased DNA content seen in the cell cycle analysis (**Figure 1D**). All M0505 cells analyzed have terminal deletions in chromosomes 1 and 4. All near-diploid cases have a loss of one chromosome 3, 8, and 12, and all near-tetraploid M0505 cells are hypoploid for chromosomes 3, 8, and 12 (**Figures 2A,B**).

MICROARRAY ANALYSIS OF STOSE CELLS

To determine the molecular mechanisms by which M0505 cells transformed into the STOSE cells, whole genome microarray analysis was performed on M0505 and STOSE cells and linear fold changes were calculated for STOSE cells relative to M0505 cells. The top 10 up- and down-regulated genes in STOSE compared

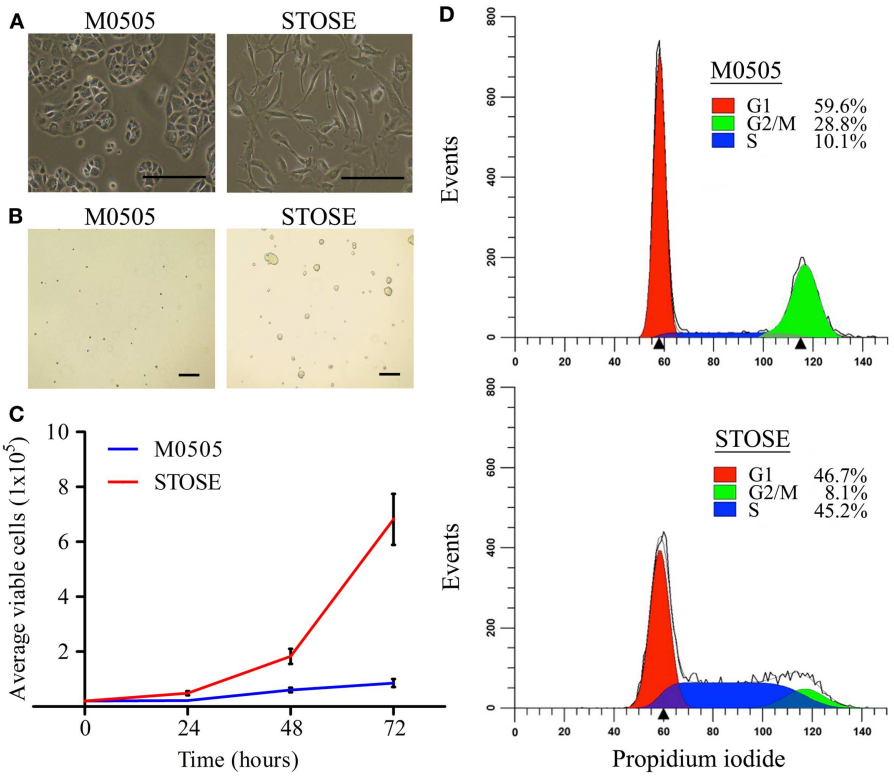

FIGURE 1 | STOSE cells exhibit classic characteristics of transformed cells. (A) Bright-field microscopy of M0505 and STOSE cells. Scale bar = 200 μm. **(B)** Colony forming assay in soft agar comparing M0505 and STOSE cells. Colonies were visualized after 7 days using bright-field microscopy. Scale bar = 200 μm. **(C)** Growth curve of M0505 and STOSE cells over 3 days. Error bars represent standard error of the mean (SEM). $p < 0.001$, two-way analysis of variance. **(D)** Cell cycle analysis of M0505 and STOSE cells. Cells were incubated with the fluorescent dye propidium iodide and analyzed by flow cytometry. The average percentage of cells in G1, S, and G2/M for STOSE cells and M0505 cells is shown ($n = 6$).

to M0505 cells are presented in **Table 2**. Interestingly, *Ddr2*, *Ereg*, *Glipr1*, *Calcr*, and *Ankrd1*, all up-regulated in STOSE cells, have been shown to be up-regulated in primary tumors and ovarian cancer cells (21–24). *Igfbp4* has been shown to be down-regulated in primary tumors (25, 26). The other up-regulated genes in STOSE cells: *Serpinb8*, *Epb41l4a*, *Aif1l*, and *Mgll* have no known links to ovarian cancer. Five of the 10 most down-regulated genes, *Aldh1a2*, *Enpp2*, *Lgfbp5*, *Thbd*, and *Uchl1*, have been previously implicated in ovarian cancer (25, 27–34). The remaining genes among these down-regulated candidates have no previous association with ovarian cancer: *Gpr64*, *Gpr126*, *Cybrd1*, *Star*, *Ncf2*. In accord with the more rapid proliferation of STOSE cells, two negative regulators of Cdk4, *Cdkn2b* and *Cdkn2a*, are down-regulated in STOSE cells 13.4- and 5.8-fold, respectively, and both *Ccna2* and *Ccnd1* are up-regulated (2.02- and 6.2-fold).

The Cancer Genome Atlas (TCGA) ovarian carcinoma array is a whole genome array database with analysis of 570 human HGSC tumors. The TCGA array dataset was analyzed by the Cancer Genome Research Analysis Network and two of the top gene changes in the STOSE cell microarray were among those reported in the pathways most frequently altered in ovarian carcinomas (35): downregulation of *Cdkn2a* (−5.8) and overexpression of *Ccnd1* (+6.2). Overexpression of *Ccnd1* is strongly correlated to decreased progression free survival (36) and loss of *Cdkn2a* through mutation or hypermethylation has also been shown in human ovarian carcinomas (35, 37–39). Ingenuity pathway analysis (IPA) was used to identify functionally related clusters of gene expression differences from the microarray data. IPA analysis revealed possible aberrant Wnt/β-catenin and Nf-κB signaling in STOSE cells. The expression of multiple genes associated with Wnt signaling are significantly altered including *Cdkn2a* and downregulation of Wnt signaling inhibitors *Sfrp1* and *Frzb*. Genes differentially expressed in the Nf-κB pathway include *Spp1*, *S100a4*, *IkBkε*, and *Ccnd1*. Interestingly, *Ccdn1* is associated with both Wnt/β-catenin and Nf-κB signaling. Validations of *Cdkn2a* and *Ccnd1*, as well as Wnt/β-catenin and Nf-κB-related genes were performed by quantitative RT-PCR on three microarray-independent samples of M0505 and STOSE cells (**Figure 3**).

STOSE CELLS PRODUCE HGSC TUMORS IN BOTH SCID AND SYNGENEIC FVB/N MICE

Given that STOSE cells exhibit transformed characteristics *in vitro*, their *in vivo* tumorigenicity was assessed using immunocompromised SCID mice and the syngeneic strain of mice, FBV/N. When STOSE cells (1×10^7) were injected IP into four SCID mice, tumors formed in all mice (4/4) with a median endpoint of 47 days. Tumors were collected from most organs within the peritoneal cavity and the average total tumor burden was 2.22 ± 0.21 g

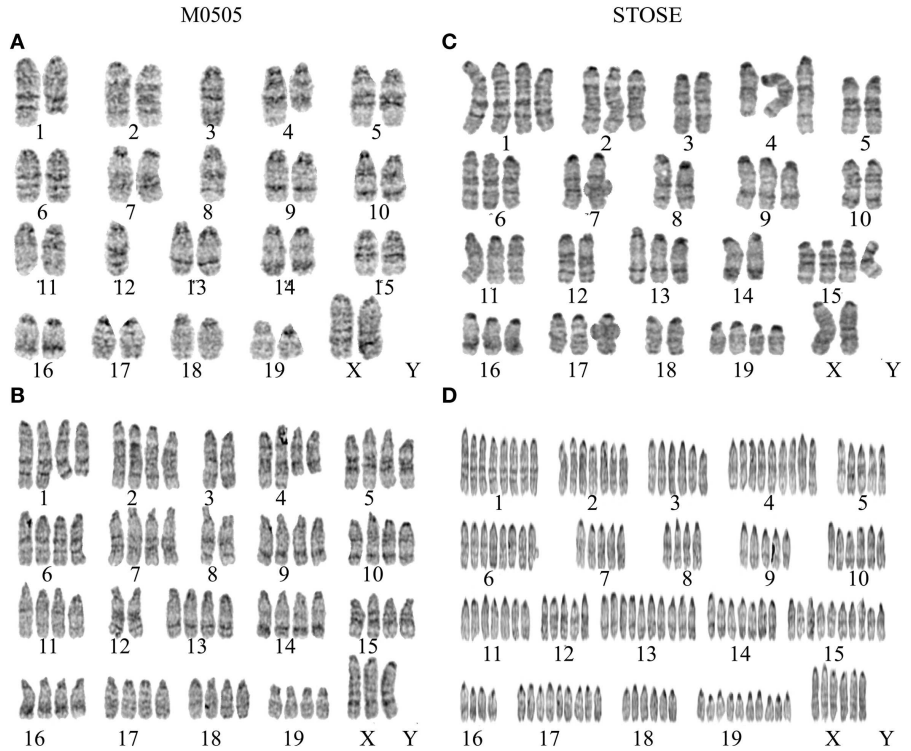

FIGURE 2 | Chromosomal analysis of M0505 and STOSE cell lines. G-band karyotyping of five metaphase spreads was performed for both M0505 and STOSE cell lines and representative karyotypes are presented. **(A)** Near-diploid M0505 cell with 37 chromosomes. **(B)** Near-tetraploid M0505 cell with 75 chromosomes. **(A,B)** Terminal deletion of chromosomes 1 and 4 as well as loss of a chromosome 3, 8, and 12 was evident in all M0505 cells analyzed. **(C)** Near-triploid STOSE cell with 54 chromosomes. **(D)** Polyploid STOSE cell with 143 chromosomes. **(C,D)** An addition at the terminal end of chromosome 4 as well as a loss of chromosome 3, 5, and 8 were evident in all STOSE cells analyzed.

per mouse. All SCID mice had ascites with an average volume of 5.25 ± 0.63 mL. Following IP injection of STOSE cells into immunocompetent syngeneic hosts, STOSE cells were tumorigenic in all FVB/N mice (4/4) with a median endpoint of 48 days. Necropsy revealed tumors throughout the peritoneal cavity and an average total tumor burden of 3.06 ± 0.21 g per mouse, not different from the tumors in SCID mice. All STOSE-injected FVB/N mice had ascites with an average volume of 3.08 ± 0.92 mL, also not significantly different from SCID mice ($n = 4$, $p = 0.98$). Intraperitoneal injection of 1×10^7 M0505 cells into FVB/N mice did not result in tumor formation in 107 days (0/6 mice).

Spontaneously transformed OSE-derived tumors from both SCID and FVB/N mice were analyzed by H and E staining for morphological classification (**Figure 4A**) and immunohistochemistry for expression of markers commonly found in human ovarian cancers (**Figure 4B**). Tumor morphology was mixed including regions of mucinous, undifferentiated, and papillary serous structures. The most common morphologies are presented in **Figure 4A**. To confirm an epithelial origin, tumors were stained for epithelial cytokeratins using a pan-CK antibody. Both SCID and FVB/N tumors have strong positive pan-CK staining. Wilms tumor-1 (WT1) positivity is a hallmark of HGSC (40), and all STOSE tumors stained strongly for WT1. Given the WT1 positivity, the tumors were examined for expression of another marker of HGSC, PAX8. All STOSE tumors had strong PAX8 expression. To exclude a granulosa cell origin of STOSE tumors, the expression of the granulosa cell marker inhibin was determined. No tumors expressed inhibin. Thus, STOSE-derived tumors have a pan-CK+, WT1+, inhibin−, PAX8+ profile, indicating that the STOSE tumors resemble HGSC. Since almost 100% of HGSC cases present with p53 mutations (9), DNA sequencing was performed on all 11 exons of the p53 gene in STOSE cells and no mutations were found (data not shown).

STOSE CELLS RETAINED A POPULATION OF SCA1+ CELLS THAT EXHIBIT GREATER MALIGNANT POTENTIAL

We have recently identified SCA1 as a marker of a defined stem-like population in the OSE (18). Flow cytometry confirmed that the parental M0505 cell line contains an average SCA1+ population of $14.5 \pm 1.4\%$ ($n = 6$). Interestingly, STOSE cells have retained a smaller SCA1+ population, on average $5.8 \pm 0.8\%$ ($n = 11$, **Figure 5A**). To determine if SCA1+ and SCA1− cells exhibit a difference in malignant potential *in vitro*, M0505 and STOSE cells were sorted for SCA1 expression and assayed for colony forming efficiency in soft agar. SCA1+ STOSE cells formed significantly more colonies than SCA1− STOSE cells ($p < 0.05$, **Figure 5B**).

Since SCA1+ STOSE cells exhibit a more malignant phenotype *in vitro*, SCA1+ STOSE cell malignancy was tested *in vivo*. To determine if SCA1 marks cells with enhanced ability to initiate

Table 2 | Differential gene expression in STOSE cells as compared to early passage M0505 cells.

Gene symbol	Gene name	Linear fold change	Publications relating these genes to ovarian cancer
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	90.7	Unknown
Epb4.1l4a	Erythrocyte protein band 4.1-like 4a	64.7	Unknown
Ddr2	Discoidin domain receptor family, member 2	46.4	(22)
Aif1l	Allograft inflammatory factor 1-like	37.8	Unknown
Ereg	Epiregulin	35.1	(21)
Glipr1	GLI pathogenesis-related 1 (glioma)	34.6	(23)
Igfbp4	Insulin-like growth factor binding protein 4	33.6	(25)
Calcrl	Calcitonin receptor-like	33.1	(26)
Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	30.4	(24)
Mgll	Monoglyceride lipase	29.8	Unknown
Ncf2	Neutrophil cytosolic factor 2	−61.7	Unknown
Star	Steroidogenic acute regulatory protein	−62.7	Unknown
Uchl1	Ubiquitin carboxy-terminal hydrolase L1	−70.5	(28, 32)
Thbd	Thrombomodulin	−76.2	(27)
Cybrd1	Cytochrome *b* reductase 1	−83.0	Unknown
Igfbp5	Insulin-like growth factor binding protein 5	−96.1	(25, 32–34)
Gpr126	G protein-coupled receptor 126	−96.6	Unknown
Gpr64	G protein-coupled receptor 64	−101.3	Unknown
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	−147.3	(30, 31)
Aldh1a2	Aldehyde dehydrogenase family 1, subfamily A2	−170.6	(29)

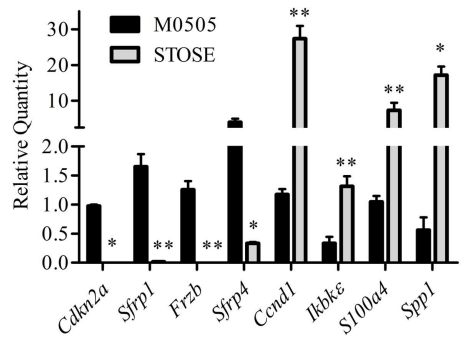

FIGURE 3 | Validation of genes differentially expressed in STOSE cells related to Wnt/β-catenin and Nf-κB signaling or in common with TCGA ovarian carcinoma arrays. Quantitative RT-PCR analysis for *Cdkn2a, Sfrp1, Frzb, Sfrp4, Ccnd1, Ikbkε, S100a4, and Spp1* expression is presented for M0505 and STOSE cells ($n = 3$). Samples used for validation are independent of those used for microarray analysis. Error bars represent SEM and $*p < 0.05$, $**p < 0.01$ by Student's *t*-test.

tumors, SCA1+ and SCA1− STOSE cells (4×10^4) were injected IB into 29 FVB/N mouse ovaries, 15 with SCA1− cells and 14 with SCA1+ cells. SCA1+ STOSE cells initiated tumorigenesis faster than SCA1− STOSE cells with the median times to a palpable tumor of 19 ($n = 15$) and 27 days ($n = 14$), respectively ($p < 0.01$, **Figure 6A**). There was no difference in total tumor burden between the two groups when the mice were euthanized 116 days after STOSE cell injection, with mice having a tumor burden of 2.70 ± 0.53 g ($n = 7$) for SCA1− tumors vs. 2.72 ± 0.32 g ($n = 6$) for SCA1+ tumors. At that time point, SCA1+ and SCA1− STOSE tumors also showed a similar degree of tumor dissemination, metastasizing consistently to the uterus, stomach, diaphragm, small and large intestines, spleen, and pancreas.

To determine if the increased initiation rate in SCA1+ compared to SCA1− STOSE tumors resulted in different histological presentation, immunohistochemistry was performed using markers of HGSC. Both SCA1+ and SCA1− STOSE tumors are pan-CK+, WT1+, inhibin−, and PAX8+ (**Figure 6B**), with no gross histological differences evident between SCA1+ and SCA1− tumors. Western blot analysis confirmed strong PAX8 positivity in both SCA1+ and SCA1− STOSE tumors (**Figure 6C**), relative to the positive control, normal uterine tissue, and to the low level of expression seen in M0505 and STOSE cells cultured *in vitro*. An independent non-tumorigenic normal OSE cell line, M1102, was used as a negative control. Expression of p53 in SCA1+ and SCA1− STOSE-derived tumors was determined using western blot analysis. SCA1+ and SCA1− tumors were positive for p53 expression (**Figure 6D**).

DISCUSSION

There is substantial need for new models of HGSC that have similar expression profiles, chromosomal aberrations, and histological features characteristic of human HGSC. These models should also account for the multiple origins of HGSC in order to effectively narrow down screening targets based on the tissue of origin. The body of work presented here describes the production and characterization of a STOSE cell line. STOSE cells have lost characteristic epithelial "cobblestone" morphology, have a greatly increased proliferation rate, and form colonies in soft agar. Interestingly, there is aneuploidy in both M0505 and STOSE cells, suggesting that aneuploidy may have preceded transformation. Linear fold

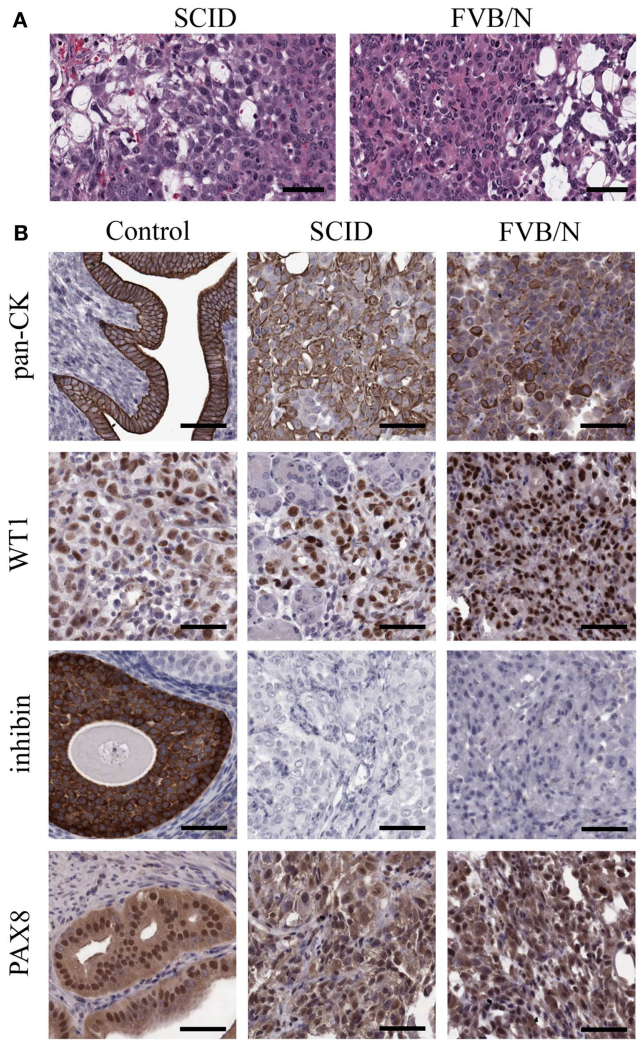

FIGURE 4 | STOSE produce high-grade serous epithelial tumors in both SCID and syngeneic FVB/N mice. Tumors were fixed in formalin and set in paraffin blocks; 5 μm sections were used for immunohistochemistry on SCID and FVB/N STOSE cell-derived tumors. **(A)** Hematoxylin and eosin staining of STOSE cell-derived tumors. Scale bars = 100 μm. **(B)** Detection of the epithelial tumor marker, cytokeratin is presented with uterus as a positive control. Wilms tumor-1 (WT1) is a marker of HGSC and is shown with a human high-grade serous ovarian carcinoma as a control. Detection of the granulosa cell and sex-cord stromal tumor marker, inhibin, is shown with granulosa cells as a positive control. PAX8 expression is shown with a fallopian tube (oviduct) positive control. Scale bars = 50 μm.

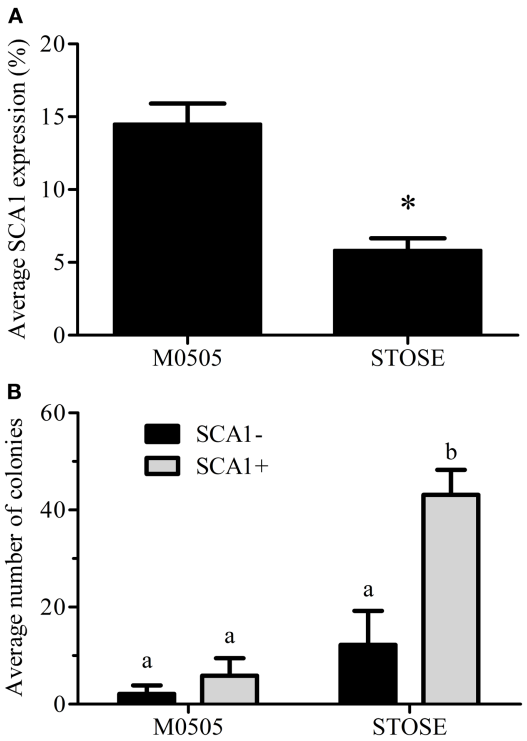

FIGURE 5 | A SCA1+ population is present in STOSE cells. **(A)** Percentage of SCA1+ cells in M0505 ($n = 6$) and STOSE ($n = 11$) cells as assessed by flow cytometry. *$p < 0.01$, Student's t-test. **(B)** Quantification of colony formation in soft agar by SCA1+ and SCA1− M0505 and STOSE cells. Colonies were counted using ImageJ software 7 days after seeding 2.5×10^4 cells in soft agar. The average number of colonies in five fields of view is presented ($n = 3$). ANOVA was used to determine significance; bars with different letters are significantly different.

changes calculated from M0505 and STOSE cell microarray data revealed that STOSE cells have differentially expressed genes that are consistent with human HGSC tumor samples and previous studies on ovarian cancer cell lines. Tumors with an immunohistochemical profile of HGSC formed in all immunocompromised SCID and syngeneic FVB/N mice following IP STOSE cell injections, confirming the potential for STOSE cells to be used as a syngeneic model of HGSC. Finally, STOSE cells that express SCA1 appear to be more aggressive, with increased colony forming efficiency *in vitro* and faster tumor initiation *in vivo*.

Recent reviews have discussed the pros and cons of current models of HGSC (5, 9, 10). Current models have focused on the use of transgenics, xenografts of human cancer cells, and OSE cells transformed by genetic engineering in attempts to model HGSC. These models have had some success in modeling HGSC as well as low-grade serous, endometrioid, and granulosa cell-derived tumors, although results of these studies are highly variable and commonly have strain-dependent phenotypes (5). Most transgenics have focused on the use of the anti-Mullerian hormone type II receptor (*Amhr2* or MISIIR) promoter to drive tumor suppressor knockout or oncogene activation, but its expression in granulosa cells as well as both ovarian epithelium and fimbria can confound the results and make the origins of such cancers unclear (5). Human xenografts into immune-compromised mice have provided much knowledge on the metastasis and chemoresistance of human tumors. The lack of an immune system can limit some uses of these models, which do not accurately represent the human tumor microenvironment in which the immune system has a critical role in tumor progression and response to treatment (9, 10). Genetically engineered OSE cells have provided much insight into genes that are sufficient to transform OSE cells (41, 42), but their involvement in HGSC initiation or progression

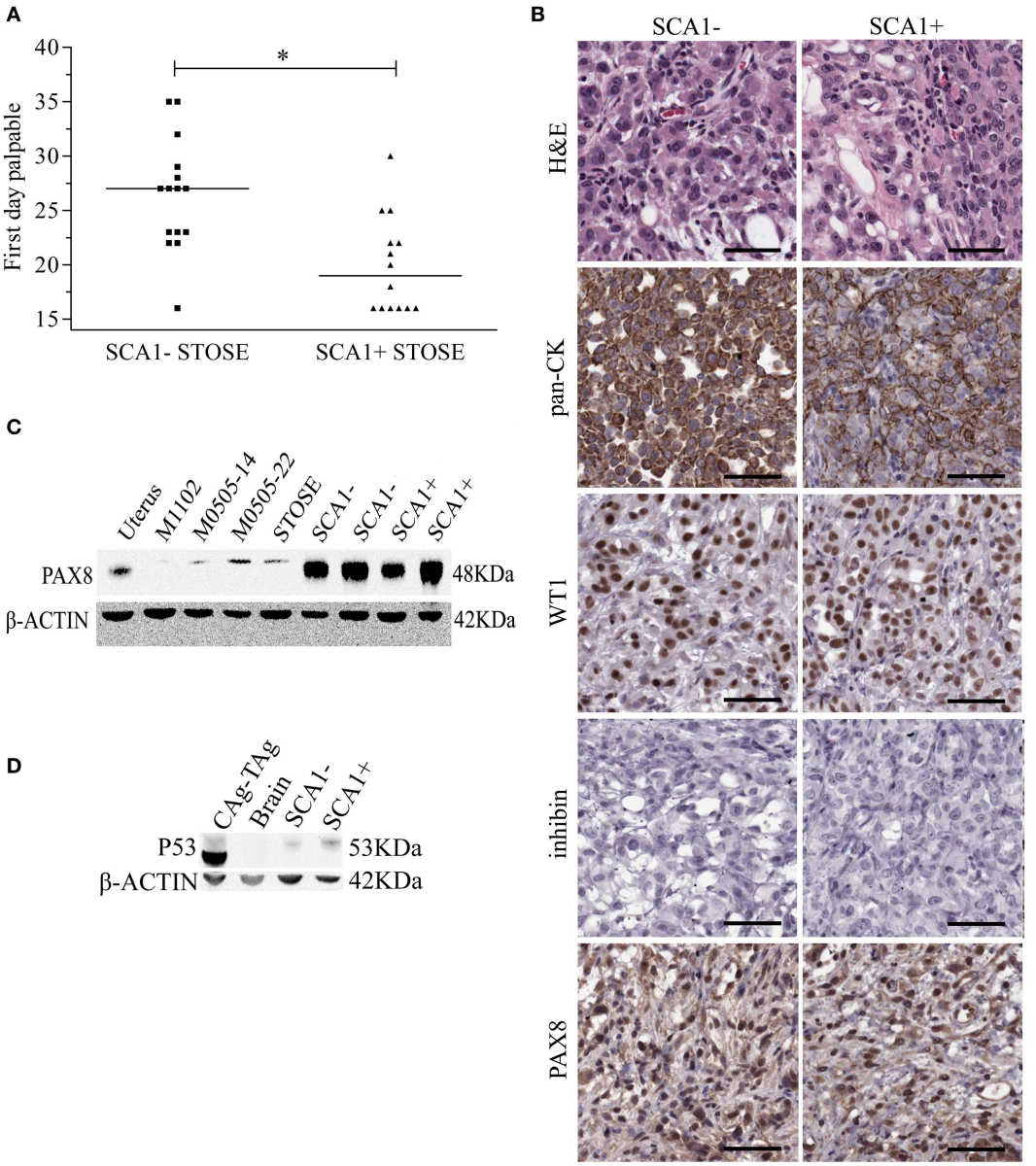

FIGURE 6 | SCA1+ STOSE cells initiate HGSC tumorigenesis faster than SCA1− STOSE cells. Flow cytometric sorting was used to separate SCA1+ and SCA1− STOSE cells. SCA1+ ($n = 14$) and SCA1− ($n = 15$) STOSE cells were injected intrabursally into FVB/N mouse ovaries (4×10^4 cells/ovary). **(A)** The first day ovarian tumors were palpable after day of injection (day 0) is presented and represents the initiation of tumorigenesis. Black lines represent median values. $^*p < 0.01$, Student's *t*-test. **(B)** Hematoxylin and eosin (H&E) staining and immunohistochemical staining of pan-cytokeratin (CK), WT1, inhibin, and PAX8, all commonly used markers to subtype ovarian carcinoma. Scale bars = 50 μm. **(C)** PAX8 (48 kDa) expression in cell lines and STOSE-derived SCA1+ and SCA1− tumors. Lane 1 is uterus from a wild-type FVB/N mouse as a positive control. Lane 2 is the normal M1102 OSE cell line as a negative control. Lanes 3–4 are passage 14 and 22 M0505 cells and lane 5 is STOSE cells. Lanes 6–7 and 8–9 represent tumors derived from SCA1− and SCA1+ STOSE cells, respectively. β-actin (42 kDa) was used as a loading control. **(D)** P53 (53 kDa) expression in STOSE-derived SCA1+ and SCA1− tumors. Lane 1 is a T-antigen expressing CAg–TAg tumor as a positive control. Lane 2 is brain from a wild-type FVB/N as a negative control. Lane 3–4 represents STOSE-derived SCA1− and SCA1+ tumors, respectively.

is unknown and manipulating such genes may not represent the natural progression of disease.

The STOSE cells reported here join a number of other spontaneously transformed rat (ROSE) (43, 44) and mouse OSE cell lines that have been previously reported. Syngeneic mouse models include ID8, IF5, IG10, L-MOSE, and MOSEC cells (45–48). These models are all tumorigenic in immunocompetent mice and allow the study of immunologic parameters as well as serve as a resource to test immunotherapies in ovarian cancer (48). Spontaneous models are beneficial since they arise from specific cell types, so their origins are clear (49). All of the models derived from spontaneously transformed OSE cell lines have yielded poorly

differentiated epithelial carcinomas, but have not been examined further to confirm their histologic identity as it compares with human tumors. Those lines tested have shown gene expression profiles similar to human (3, 50).

The STOSE model is the first spontaneous HGSC model, as confirmed by the expression of immunohistochemical markers (pan-CK+, WT1+, inhibin−, PAX8+), consistent with human ovarian carcinomas (2, 40). The expression of WT1 and PAX8, commonly used to diagnose HGSC, help to confirm that OSE cells have the ability to spontaneously transform into HGSC. PAX8 positivity in human HGSC is one of the characteristics used to support a fimbrial origin of HGSC (2). It is well-established as a marker of fimbrial epithelium and, due to its expression in HGSC, much research has now focused on the fimbrial epithelium (2, 4). Recently, a report has shown that PAX8+ tumors can be produced from transformed hilum cells that originate in the ovary, providing additional evidence that the OSE cells can be an origin of HGSC (4). Although OSE cells have little to no PAX8 expression, our results show that both the untransformed M0505 cells as well as the STOSE cells had a low level of expression of PAX8+ (**Figure 6C**), suggesting that early acquisition of PAX8 expression in the M0505 cells may have facilitated the transformation of these cells. Further study of PAX8 and its function in M0505 and STOSE cells will help delineate its role in the transformation process.

The STOSE model is also the first syngeneic ovarian cancer model in the FVB/N strain of mice. All previous spontaneously transformed mouse OSE cells have been derived from C57Bl/6 mice (32, 45, 46, 48). Most spontaneous models have been produced by IP injection into syngeneic hosts, abrogating the ability to study metastasis from a specific site. The ovarian bursa is a controlled and distinct microenvironment and we have previously shown that, while tumor histology is not different when cells are injected into this location, it is an effective means to identify more invasive cells, as only aggressive cells can invade the ovary and/or breach the bursal membrane (51). Injecting cells under the bursal membrane also provides the ability to study the immune parameters associated with metastasis that could enable the production of immune therapies to prevent metastasis. The spontaneous ID8 model has produced peritoneal metastases following IB injection into their syngeneic C57Bl/6 strain of origin (52). The STOSE model also forms extensive peritoneal metastases following IB injection, making STOSE the first metastatic HGSC model in the FVB/N strain. Having spontaneous models in multiple strains is an important resource to enable investigators to show that the efficacy of a therapeutic strategy is independent of strain background, greatly improving the translation of therapeutic strategies.

Spontaneously transformed OSE cells are aneuploid and have gene expression changes consistent with human ovarian cancer. Aneuploidy is common in many cancers including ovarian cancers (19, 39, 45, 46, 53). Aneuploidy is a prognostic determinant in HGSC since severe aneuploidy is associated with poor outcome (53). STOSE cells have a high degree of aneuploidy, characterized by triploid and polyploid cells. Furthermore, the loss of genomic stability in both M0505 and STOSE cells as seen by aneuploidy may have been an early event leading to transformation that may explain the tumorigenic capacity of STOSE cells. Loss of chromosome 3, which contains many tumor suppressors, is seen in both M0505 and STOSE cells. Haploinsufficiency of chromosome 3 tumor suppressors such as *Lrrc3b* (fold change of −2.69 in STOSE cells) may underlie transformation (54). Similarly, chromosome 8 is lost in M0505 and STOSE cells and it has been shown to contain ovarian cancer susceptibility loci, allelic loss of which may have contributed to transformation (55, 56). Three down-regulated genes in STOSE cells, *Enpp2, Sfrp1,* and *Star* are all located on chromosome 8. Loss of chromosome 8 in M0505 cells may have been an early event in transformation (30, 57).

Ingenuity pathway analysis of microarray data revealed gene expression changes related to Wnt/β-catenin signaling in STOSE cells suggesting signaling in the Wnt pathway might be aberrant. Many of the down-regulated genes in STOSE cells are associated with Wnt/β-catenin signaling and have been associated with loss of heterozygosity or promoter methylation in ovarian cancer, including *Fzd4*, *Sfrp1*, and *Axin2* (58–61). Interestingly, *Cdkn2a* is down-regulated in 30% of HGSC cases and *Ccnd1* is amplified in 4% of the cases, according to TCGA ovarian carcinoma array (35). STOSE cells have a similar expression pattern of *Cdkn2a* and *Ccnd1*. *Cdkn2a* and *Ccnd1* are both associated with Wnt/β-catenin signaling. *Ccnd1* is a well-established target gene of β-catenin signaling and has a role in promoting cell cycle progression, while *Cdkn2a* encodes a cell cycle inhibitor that is suppressed by β-catenin (35, 62, 63). Due to the association of these two genes with human HGSC and aberrant Wnt signaling in STOSE cells, further study of the role of *Cdkn2a*, *Ccnd1*, and Wnt/β-catenin signaling is needed to understand the role Wnt/β-catenin signaling in the transformation of M0505 cells into STOSE cells or in the tumorigenic capacity of STOSE cells. A greater understanding of this pathway may translate to greater knowledge on the initiation and progression of HGSC.

Interestingly, *Aldh1a2* is the most down-regulated gene in STOSE cells (−170.58 fold). *Aldh1a2* is involved in retinoic acid (RA) biosynthesis and has been shown to have ubiquitous expression in the human ovarian surface epithelium (2, 29). The RA-receptor β (*Rar*β) is also down-regulated in STOSE cells (−10.80 fold) suggesting multiple aspects of RA signaling are lost. RA signaling has been shown to crosstalk with Wnt/β-catenin signaling and *Aldh1a2* has also been identified as a tumor suppressor in prostate cancer, loss of which is an early event in the disease (64, 65). Further study of *Alhd1a2*, RA signaling, and the crosstalk between RA and Wnt/B-catenin signaling may help determine the mechanisms leading to transformation and tumorigenesis in HGSC.

Investigation of a potential TIC population in the STOSE revealed that STOSE cells have retained a SCA1+ population that appears to have a more malignant phenotype than SCA1− STOSE cells. TICs have been thought to be key contributors to HGSC etiology based on the heterogeneity and recurrence that are characteristic of the disease (3, 15, 50). TICs have been identified in both human and murine ovarian cancers by sorting for CD44, CD133, CD117, CD24, ALDH1, and SCA1 expression alone or in combination (3, 15). SCA1 has also been used for the enrichment of a stem cell population in leukemia, prostate, and breast cancers (15). STOSE cells were found to contain a SCA1+ population that exhibits increased malignancy both *in vitro* as assessed by colony

formation and *in vivo* as assessed by initiation of tumorigenesis. Interestingly, SCA1+ and SCA1− STOSE-derived tumors were positive for p53 expression by western blot analysis. DNA sequencing showed no mutations in the p53 gene, suggesting pathways that lead to p53 stabilization might also be aberrant in STOSE cells. Our findings that the SCA1+ population exhibits TIC characteristics is in line with a recent study on SCA1+ cells in the T2 mouse model of ovarian cancer, which showed that these cells have TIC characteristics that allow them to escape chemotherapy and produce heterogeneous tumors following treatment (15). The retention of a SCA1+ population with TIC characteristics allows us to compare tumorigenic SCA1+ STOSE cells with non-tumorigenic SCA1+ M0505 cells.

In summary, this study has led to the development of a spontaneously transformed syngeneic model of HGSC in the FVB/N mouse, the first spontaneous murine model with defined features of HGSC. The STOSE model has characteristics of human disease such as aneuploidy, gene expression, and the presence of a TIC population. This model also produces extensive metastases in the peritoneal cavity following IB injection allowing for the study of tumor dissemination. Further investigation is required to understand the contribution of Wnt/β-catenin signaling in STOSE cells. The STOSE model offers vast potential for testing of novel therapeutics, including immune therapies. This model will also allow for the discovery of new screening targets that are involved in the transition of normal cells to HGSC.

AUTHOR CONTRIBUTIONS

Experiments were designed by Curtis W. McCloskey and Barbara C. Vanderhyden. Olga Collins derived the cell line and performed initial validation of the transformation. All surgeries, IHC, microarray, and cell culture were done by Curtis W. McCloskey; western blotting was done by Reuben L. Goldberg; qPCR was done by Curtis W. McCloskey and Lauren E. Carter; Lisa F. Gamwell and Curtis W. McCloskey performed the flow cytometry; Kenneth Garson and Curtis W. McCloskey performed the cell cycle analysis. Elizabeth A. Macdonald and Curtis W. McCloskey performed the IP injections and necropsies. Manijeh Daneshmand performed the pathology assessment and Euridice Carmona did the IPA analysis. Curtis W. McCloskey and Ensaf M. Al-Hujaily performed DNA sequencing. The manuscript was written by Curtis W. McCloskey and Barbara C. Vanderhyden and edited by Manijeh Daneshmand and Euridice Carmona.

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Culture models to define key mediators of cancer matrix remodeling

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High grade serous epithelial ovarian cancer (HG-SOC) is one of the most devastating gynecological cancers affecting women worldwide, with a poor survival rate despite clinical treatment advances. HG-SOC commonly metastasizes within the peritoneal cavity, primarily to the mesothelial cells of the omentum, which regulate an extracellular matrix rich in collagens type I, III, and IV along with laminin, vitronectin, and fibronectin. Cancer cells depend on their ability to penetrate and invade secondary tissue sites to spread, however a detailed understanding of the molecular mechanisms underlying these processes remain largely unknown. Given the high metastatic potential of HG-SOC and the associated poor clinical outcome, it is extremely important to identify the pathways and the components of which that are responsible for the progression of this disease. *In vitro* methods of recapitulating human disease processes are the critical first step in such investigations. In this context, establishment of an *in vitro* "tumor-like" micro-environment, such as 3D culture, to study early disease and metastasis of human HG-SOC is an important and highly insightful method. In recent years, many such methods have been established to investigate the adhesion and invasion of human ovarian cancer cell lines. The aim of this review is to summarize recent developments in ovarian cancer culture systems and their use to investigate clinically relevant findings concerning the key players in driving human HG-SOC.

Keywords: high grade serous epithelial ovarian cancer, metastasis, culture models, 3D, synthetic scaffolds

High grade serous epithelial ovarian cancer (HG-SOC) is a devastating disease and the most lethal of the gynecological malignancies. Typically treatment consists of surgical debulking, followed by platinum/taxol chemotherapy regimens (1, 2). Treatment fails in up to 70% of patients, and patients with platinum resistant disease have a median survival of 6–12 months (1, 3). Some success has been observed in clinical trials for the palliative management of ascites accumulation using targeted antibody treatment (4), and while this symptom based therapy is clinically important, disease modifying/halting treatments are lacking. Other treatments have shown varied success, including those that target tumor angiogenesis such as bevacizumab alone or in combination with platinum agents and gemcitabine. Many other approaches have been taken including tyrosine kinase inhibitors, angiopoietin inhibitors, histone deacetylase inhibition, and EGF receptor targeting (5). The role of immune cells and interactions with tumor stroma are under intense investigation and may improve the future prospects for immunotherapy based regimes (5). However, response to treatment varies between patients and therefore, the development of personalized care through discovery of predictive molecular or protein markers becomes imperative for effective disease treatment.

Modeling HG-SOC as closely as possible to human disease to facilitate clinically relevant treatment testing is the "holy-grail" in research. A plethora of immortalized ovarian cancer cells and *in vitro* and *in vivo* model systems that utilize these cell lines have been described. Early disease events are arguably the most therapeutically relevant targets of preventative treatments and here, we discuss recently used model systems to identify pathways involved in the development of invasive malignancy.

ESTABLISHED EPITHELIAL OVARIAN CANCER CELL LINES AS MODEL SYSTEMS: A CONTROVERSIAL CHOICE

High grade serous epithelial ovarian cancer has long been thought to arise from the epithelial layer surrounding the ovary (6, 7). However, studies point to a different site of origin, the secretory cells of the fallopian tube fimbria. This highlights the lack of understanding of the histogenesis and molecular signature of this heterogeneous disease (8–14). Anglesio et al. suggested that the biomarker and molecular signatures of ovarian cancer cell lines may be a more accurate and relevant way of grouping "histotypes" over previously determined histological subtypes (15). However, discrepancies between the molecular profile of ovarian cancer cell lines and the tumor types they model have been identified. In fact, these profiles show more similarity between the cell lines themselves, despite differing tissues of origin (8, 16). Further, these reports have raised doubt on the use of a number highly cited ovarian cancer cell lines as models of clinically relevant HG-SOC, in particular A2780 and SKOV3 (8, 15). Cancer cell lines derived from patients who have undergone treatment will represent a population of cells that is intrinsically different from that of the original tumor due to the development of resistance. However, it has been suggested that cell lines derived from untreated tumors are enriched for resistant cells with up-regulation of multi

drug resistance associated genes via activation of stress responses during the primary culture process (16).

Immortalized normal ovarian epithelial cells and normal fallopian epithelial cells are increasingly being used to model early stages of cancer development (10, 11, 17–21). While the use of primary cancer cell cultures avoids issues associated with multiple passages (16), this is a labor intensive method, and differences between individual primary cultures leading to lack of reproducibility, may be a significant confounder. Immortalized cell lines offer the advantage of increased stable survival over longer periods in culture and can be manipulated to include many genetic alterations to mimic the disease of interest. Studies using immortalized cells derived from non-transformed normal human fallopian epithelial secretory cells, along with the induction of relevant genetic alterations, have been shown to successfully model human high grade serous cancer biology (10, 11, 19). The use of virally induced immortalization of cells is common; however this may also induce unappreciated effects on tumor development and virally induced tumor initiation is irrelevant to the pathogenesis of ovarian cancer. Non-viral methods using shRNA technology have also successfully targeted relevant genetic factors resulting in transformed cells (11).

Along with the method of cell line derivation, site of origin, and continuous passaging, culture conditions (monolayer, various 3D culture models, organ-like culture models) are also significant effectors of the characteristics of established ovarian cancer cell lines (8, 15, 16, 22). These issues are inherently difficult to address and there is likely no ideal way to completely control for all these changes. To date, particular HG-SOC cell lines have not been reported as being more relevant to 3D culture compared to 2D culture systems. SKOV3 and A2780 are the most commonly cited but may not be the best representations of HG-SOC with their use in 3D likely reflecting their popularity in 2D systems. Therefore at this stage there are no specific criteria for cell line selection for 3D systems and progression from 2D to 3D experiments with the same cell line can be a useful strategy. However, consistent use at a low passage number, of an appropriate cell line to model HG-SOC (via histological and molecular markers) is extremely important.

IN VITRO CULTURE MODEL SYSTEMS OF HGSEOC

2D VERSUS 3D CULTURE METHODS

Although it is well known that culturing cancer cell lines can drastically alter their genetic characteristics over multiple passages immortalized cancer cell lines remain the gold standard in cancer research and pre-clinical drug testing (22). This is largely because these cell lines display a consistent and relatively homogeneous phenotype over long periods of time, notwithstanding reports of minor side populations with cancer stem-like characteristics in some cell lines (23, 24). Evidence is accumulating that culturing these cells in 3D matrices is far more representative of disease than traditional 2D systems, as they provide structurally similar conditions for cell growth encompassing the ability to manipulate oxygen and growth factor/cytokine gradients as well as the material properties of the matrix (22, 25–30).

Common methods for assessing ovarian cancer cell proliferation/migration/invasion have included 2D culture growth studies, "scratch" wound healing assays, and penetration through transwell inserts. Scratch wound assays are relatively easy to set up, and very cheap to run and there are now many options for tracking and quantitating cell growth and migration, including the MetaMorph™ and Incucyte™ real-time Imaging systems (31). Migration assays through transwell inserts are more expensive and do not allow for real-time monitoring. Microfluidic assays have the advantage that cells can be grown in controlled chemotactic gradients (31). However, these systems have not to date been utilized widely for ovarian cancer cell culture studies. Cell spreading assays, in which a plastic culture surface is coated with various extracellular matrix (ECM) components (fibronectin or collagen type I) and cells are allowed to spread under serum free conditions for a short period of time, have been used to assess migration of ovarian cancer cells (32). While these methods may provide some useful information regarding the characteristics of certain cancer cell lines and their responses to stimuli (drug treatment, signaling molecules), they lack a 3D micro-environment to accurately mimic pathophysiological conditions. 3D environments containing relevant structural proteins (collagens, laminin, elastin) (**Figure 1A**), as well as defined tissue organization appropriate to site of tumor growth *in vivo,* are important considerations for recapitulating tumor cell behavior (**Figure 1B**).

Spread of ovarian cancer cells is complex with cells responding to stimuli from neighboring cells and ECM components and their ability to invade connective tissue is crucial for successful metastasis. In the absence of a requirement for ECM interactions and matrix degradation, 2D systems primarily evaluate the motility of cells, rather than a true invasive barrier removal (29). Care must also be taken when interpreting results based on incomplete 3D representations of a *bona fide* tumor/metastatic site ECM. For example, only a partial understanding of the involvement of proteases/MMPs in the spread and invasion of ovarian cancer cells can be drawn from experiments using matrices that lack structural properties of a relevant ECM. For example, matrigel is substantially less cross-linked and differs in overall composition compared to many tissues (29, 31, 33).

Omental models have been used, in which a primary culture of fibroblasts is grown in 2D with a confluent layer of mesothelial cells grown on top before fluorescently labeled ovarian cancer calls are seeded on a final layer to form a "mock" peritoneal environment. Invasion is typically measured by fluorescent microscopy after the cell layers are cultured in transwell inserts placed over growth promoting media. (26, 34–36). These models provide a more accurate representation of the tissue structure encountered by tumor cells, by supplying a barrier to test "metastatic" invasion of cells in presence of other cells such as fibroblasts that are important to disease processes. However, primary tumor development and the "metastatic cascade" are highly complex processes, and the 2D platforms that are currently used do not typify pathways involved, likely contributing to the unsuccessful translation of findings into *in vivo* systems and eventual failure of many treatments under clinical trial (37).

NATURAL VERSUS SYNTHETIC 3D PLATFORMS

The importance of recapitulating tumor ECM in model systems was highlighted by Infanger and others in their review (25). These authors stated that interactions between tumor cells and their

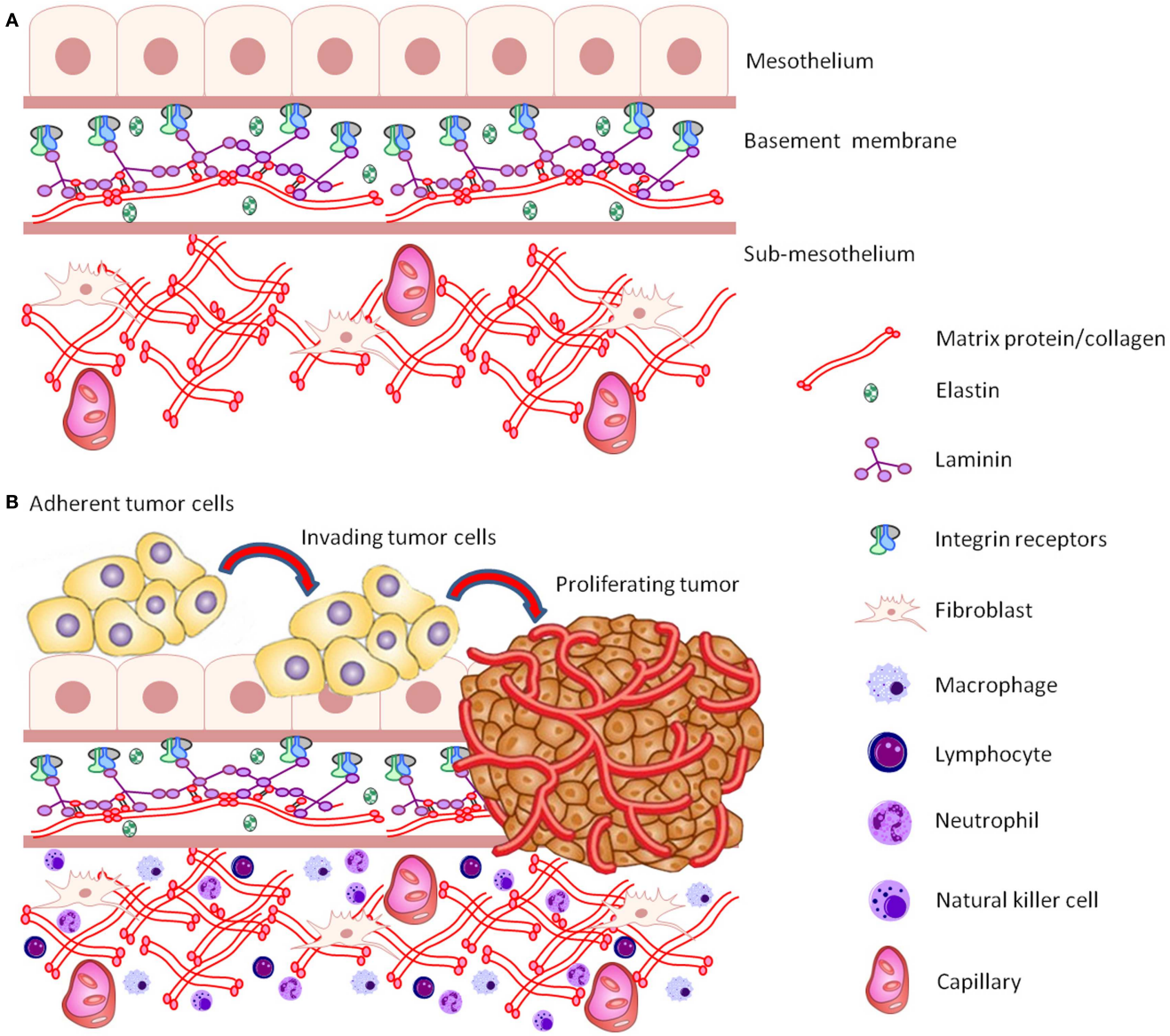

FIGURE 1 | (A) Schematic representation of the structure and components of the common peritoneal site of ovarian cancer metastasis. **(B)** Schematic representation of a cluster of adherent ovarian cancer cells invading, proliferating, and destroying basement membrane ECM tissue architecture.

surrounding micro-environment are as pivotal to tumorigenicity as oncogenic mutation (25). Normal homeostatic process and tissue structural properties control the dormancy required after malignant transformation of epithelial cells and when these pathways fail, along with the presence of certain genetic mutations, cells grow uncontrollably and tumors develop (25). Currently, there is a definite lack of studies that evaluate the combined effect of cell–cell, cell–ECM interactions as well as biochemical, biomechanical, and the specific processes that occur during the metastatic processes of ovarian cancer (25, 38).

Hydrogels, such as Matrigel, are commonly used for *in vitro* studies of ovarian cancer cell growth and invasion (29, 32, 39). Other substrates such as collagen gels (40), polyhydroxyethylmethacrylate coated plastics (22), algimatrix, and geltrex are also used to model ECM (16). Natural alternatives include human amniotic membranes (HAM) and chick chorioallantoic membranes (CAM). 3D culture systems incorporating amniotic membranes have been used to assess the spreading and invasive capacities of ovarian cancer cells. These offer the advantage of a physiologically relevant tissue barrier for assessment of cell behavior (41–43). Limitations of these materials are the batch to batch variation, presence of confounding growth factors and other biological components whose effects on culturing experiments are not well known (25, 44). Other non-biological considerations in these model systems, which to date have been largely ignored, are the tissue structural properties as well as

gradients of oxygen tension and effects from external physical stimuli (compression, shear stress) (25, 41).

Semi-synthetic matrices such as polyethylene glycol (PEG), hyaluronan, alginate-based, and peptide-based (Puramatrix™) hydrogels are amenable to experimental determination of matrix stiffness and integration of different binding sites and protease cleavage sites (31, 45). Matrix stiffness has been shown to influence endothelial cell behavior independently of matrix molecular composition, highlighting the relevance of matrix material properties in tumor modeling (46). PEG based hydrogels have been used to investigate the role of proteases in the migration of fibroblasts (47) and more recently to investigate cell–ECM interactions and drug resistance of epithelial ovarian cancer cells (48).

Semi-synthetic or synthetic matrices offer the greatest levels of experimental reproducibility due to the control that investigators have in the makeup of the ECM. The study by Loessner et al. is, to date, the most relevant study using a synthetic 3D scaffold to comprehensively investigate ovarian cancer cell growth and response to drugs in an anisotropic biomimetic hydrogel (48). This method enables combination of designed binding sites, protease substrates, other proteins including growth factors and an easily adjustable matrix stiffness. Cells seeded uniformly in the liquid scaffold precursor are exposed to similar levels of biomechanical and biochemical stimuli in all directions (48).

While these models are highly relevant, the addition of other cell types found in the cancer micro-environment (stromal cells, immune cells) would make these models more complete. The immune response has been shown to be clinically relevant in ovarian cancer. Traditionally, immune–cancer cell interactions have been studied in 2D cultures by the addition of immune components or immune stimulatory factors. The establishment of a physiologically relevant tumor micro-environment would enable all cells present (cancer, stromal, immune) to phenotypically resemble those found in disease (49–52). This would create a unique and powerful *in vitro* situation for testing the effects of different immune components and inflammatory responses relevant to disease. For example, TNF-β is known to effect ECM stability, and could therefore influence the capacity of tumor cells to migrate and invade (53). A biologically relevant *in vitro* representation of a tumor is also central for accurately testing drug efficacy, as the interaction of different cell types contributes to the drug response (54). Various 3D models (spheroid cultures, scaffold based 3D cultures, organotypic cultures) would be amenable to the addition of immune factors/cytokines, and although not yet in development, 3D co-culture of many cell types found in ovarian cancer including immune cells should be possible (55, 56).

Heterotypic culture to simulate the micro-environment of ovarian cancer has been shown to be a promising and representative method for investigating stromal–epithelial interactions during disease (57). It has been suggested that modeling ovarian cancer by using 3D cultures of fallopian tube secretory epithelial cells would be more relevant to early stage HG-SOC (58). Combining synthetic matrices, in heterotypic culture with the relevant cells that drive the initiation processes of disease to investigate potential therapeutic targets, would be ideal. A collaborative effort between the NIH, FDA, and the Defense Advanced Research Projects Agency has been instigated to develop and refine methods for functional organ microphysiological systems aimed at drug screening (59). These may also have potential for use in cancer biology. For example, a human liver-like model has been developed to study breast cancer metastases (60). It is possible that such models may, in the future, be adapted to investigate metastases to the liver in ovarian cancer. **Table 1** summarizes some of the factors to consider when choosing a method to model cancer cell growth.

3D modeling of early stage ovarian cancer, which the aforementioned systems aim to achieve, may be the most relevant for identifying potential targets for disease modifying therapies. The second stage of disease involves the spread of ovarian cancer cells from the primary tumor into the peritoneal space. Experiments to capture the behavior of ovarian cancer cells during metastasis focus on anchorage-independent models of cell migration (68–71). Multicellular aggregate, or spheroid formation is critical for shedding of cancer cells from the primary tumor, and it has recently been shown that the culture of ovarian cancer cells as spheroids in a biomimetic ECM, recapitulates the metastatic niche (72). Further, the biomechanical environment of the peritoneal space plays an important role on cancer cell behavior and spread, and so incorporation of physiological fluid mechanics are appropriate in these systems (41, 69). While the development of oxygen tension gradients limits the size of the multicellular spheroids in culture; it mimics the structure of solid tumors and the potential development of necrotic cores (73, 74). This representation of the physiological micro-environment is relevant and appropriate for the screening of drugs, as penetration into the tumor/spheroid is very different to 2D systems and consequently, the response will also be very different (75). A recent study by Jaeger et al. describes the development of a 3D culture system incorporating an oxygen permeable polymer and micro pillars, to mimic gas delivery via vessels (76). This system offers the potential of larger growth of organotypic models and more realistically represents vascularized tumors *in vivo*.

Tissue chips are a relatively new area of research aimed at incorporating as many components as possible to recapitulate the living tissue and study biological responses to many factors in concert (77, 78). Tissue chips allow the modeling of organ systems in a highly functional and controlled manner. They can incorporate many components relevant to tumor biology such as various 3D matrix components and hydrogels. These systems have the potential as tools for measuring metastatic potential, response to various growth stimulators or inhibitors, immune interactions, and drug responses. However, optimization of parameters such as endpoint data collection is still required in order to use these systems for complex tumor modeling (77, 78).

CONCLUSION AND FUTURE PERSPECTIVES

Many advances have been made in recent years in the development of representative 3D models to mimic ovarian cancer relevant to human HG-SOC. However, these systems are still limited and none to date combine all factors, biomechanical, and biological, to create a complete experimental culture system. This is compounded by recent controversy regarding the molecular characterization of HG-SOC cell lines, with several that are commonly used for research, being shown to be non-representative of this

Table 1 | Summary of factors contributing to the choice of model system for ovarian cancer cell culture.

	Natural/ synthetic	Control of ECM composition	Relevance to *in vivo* tumor	Comments/reference
COMPONENT/SYSTEM				
Human amniotic membrane (HAM)	Natural	Low	Medium	Physiologically relevant/provides ECM barrier/batch to batch variation high (42)
Chick chorioallantoic membrane (CAM)	Natural	Low	Medium	Physiologically relevant/provides ECM barrier/batch to batch variation high (43)
Collagen gel (acid extracted type 1 collagen from rat tail)	Synthetic	Medium	Low	Variable ECM stiffness/invasion assessment (binding sites/matrix interaction) (61, 62)
Matrigel (derived from mouse EHS cell secretions; laminin, collagen IV, enactin, various growth factors)	Synthetic	Medium	Low	Widely used (migration and invasion)/batch variation high/irrelevant matrix composition/properties (29, 31, 33)
Alginate/peptide-based (inert polysaccharide, β-d-mannuronic acid, α-l-guluronic acid, calcium ions)	Synthetic	High	Medium	Variable ECM stiffness/defined components/binding sites/matrix interaction (63, 64)
PEG (various cross-linked polyethylene glycol hydrogels) coasted plastics	Synthetic	High	Medium	Variable ECM stiffness/defined components/binding sites/matrix interaction/enzymatically degradable (31, 65)
Heterotypic/organotypic culture	Synthetic	High	High	Relevant micro-environment/cell interaction/combine with synthetic ECM (64, 66)
Spheroid culture	Synthetic	High	Medium	Biologically relevant/cell–cell interactions/combine with synthetic ECM (31, 58, 67)

grade of ovarian cancer. It has become clear that when modeling the micro-environment, it is particularly important to create an ECM that closely mimics that relevant to ovarian cancer, and so considerations of the origin of the cell line are important. For example, an ECM relevant to a primary tumor derived cell line may be different from that of a cell line derived from ascites. Likewise, generation of an appropriate ECM for early disease modeling may have different requirements for epithelial cells derived from the fallopian tube to those derived from the ovarian surface. Only through a comprehensive understanding of physiological tumor behavior will it be possible to identify key players in tumor progression, whether these are ECM proteins (MMPs, TIMPs), immune regulators or cytokines or upstream genetic changes in the cancer cells themselves.

While the sophisticated 3D culture models developed in the last few years have circumvented many problems associated with traditional methods, the use of these systems is still in its infancy in part due to the complex nature, cost, and specialized equipment that is often required. Thus these methods are not yet amenable for high-throughput experimentation and pre-clinical testing. However, technological progress in the coming years will hopefully reduce these limitations and see the widespread use of high-throughput screening using 3D culture systems that accurately recapitulate the tumor micro-environment.

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Recent technological advances in using mouse models to study ovarian cancer

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Serous epithelial ovarian cancer (SEOC) is the most lethal gynecological cancer in the United States with disease recurrence being the major cause of morbidity and mortality. Despite recent advances in our understanding of the molecular mechanisms responsible for the development of SEOC, the survival rate for women with this disease has remained relatively unchanged in the last two decades. Preclinical mouse models of ovarian cancer, including xenograft, syngeneic, and genetically engineered mice, have been developed to provide a mechanism for studying the development and progression of SEOC. Such models strive to increase our understanding of the etiology and dissemination of ovarian cancer in order to overcome barriers to early detection and resistance to standard chemotherapy. Although there is not a single model that is most suitable for studying ovarian cancer, improvements have led to current models that more closely mimic human disease in their genotype and phenotype. Other advances in the field, such as live animal imaging techniques, allow effective monitoring of the microenvironment and therapeutic efficacy. New and improved preclinical mouse models, combined with technological advances to study such models, will undoubtedly render success of future human clinical trials for patients with SEOC.

Keywords: mouse models, serous epithelial ovarian cancer, imaging techniques, immune therapy, cancer stem cells, reporter, metabolite profiling

INTRODUCTION

Mouse models provide a wealth of information for understanding tumor biology. Through the validation of *in vitro* findings, cancer progression, regression, and relapse in the physiological setting is better understood. The continued high mortality associated with serous epithelial ovarian cancer (SEOC) underscores a need for tailored disease models and improved technology to study such models. Several recent advancements promise to facilitate the success of preclinical models in refining our understanding and treatment of SEOC. This mini-review will focus on the latest mouse models of ovarian cancer and improved technologies for using these models to study SEOC initiation, progression, relapse, and therapeutic response.

Ovarian cancer is the most common cause of gynecological mortality in the United States, accounting for more than 14,000 deaths annually (1). Most patients initially respond favorably to platinum-based therapy, however, there is a high incidence of recurrent, chemoresistant disease. Our knowledge of the clinical and molecular attributes of epithelial ovarian cancer has improved greatly over the last few decades, but survival rates for women with this disease remain low. This is partially explained by the inability of clinical trials to replicate the therapeutic response observed in animal models. So far, about one-third of highly cited animal studies saw success in human trials, however, improvements in study design and data interpretation should increase that figure (2, 3). Animal models in the personalized medicine era highlight the availability of affordable genomic sequencing and molecular profiling. As the pharmaceutical industry relies heavily on mouse models, such new refinements will be critical for producing reliable preclinical data on personalized ovarian cancer therapeutic approaches.

In order to generate accurate models, the biology of the disease must be understood. High grade SEOC is thought to arise in a rapid fashion *de novo* from the surface epithelium of the ovary or from the mucosa of the fallopian tube (4, 5). The remaining ovarian carcinomas, categorized as low grade, follow a stepwise adenoma-carcinoma sequence (4, 6). Whether high or low grade, SEOC usually does not reach clinical detection until late stage where it has spread well beyond the ovaries. This feature has hampered efforts to identify the site of origin and understand the pathophysiology of SEOC. Most existing mouse models of SEOC present a disseminated abdominal phenotype, which closely resembles late metastatic disease, and therefore may only provide a good model for therapeutic response in the "average" patient. Some recent mouse models provide a phenotype of early progressive disease coming from defined genetic abnormalities identified from patient subtypes.

MOUSE MODELS OF EOC

An extensive analysis of every mouse model is beyond the scope of this mini-review, however, a summary of recent advances in mouse models of ovarian cancer to place the technological advances in context is presented here. Several recent reviews are available detailing epithelial ovarian cancer experimental models (7–9). Mouse models of ovarian cancer generally fall into one of three categories (xenograft, syngeneic, genetically engineered), the most suitable being dependent on the information being sought.

XENOGRAFT MODELS

A human tumor xenograft is the most widely used mouse model in which human tumor cells are transplanted under the skin (subcutaneous), into the abdominal cavity (intraperitoneal), or into the organ of origin (orthotopic) of an immune-compromised host. While intraperitoneal and orthotopic injections can mimic metastatic dissemination, subcutaneously injected cells are largely limited to tumor formation at the site of injection. The unique presence of a bursa, a sac encapsulating the ovaries and fallopian tubes, allows for intra-bursal injections in mice (10, 11). This technique permits the study of early, localized disease, tumor cell invasion, and dissemination in a more biologically relevant order of events (12).

Xenograft models are particularly useful for evaluating tumorigenesis in a timely fashion (13, 14). Within a few weeks, tumor formation can be measured *in vivo* with histology representative of the tumor of origin (12, 15, 16). Importantly, the pattern of spread to the ascites, liver, and spleen, typical in human disease, is replicated in many of these models and depending on the cell line used, tumors representing the different histological subtypes of epithelial ovarian cancer can be produced (8, 12). Xenografts are versatile and often used in parallel with *in vitro* studies to generate a majority of preclinical data.

Although quite valuable, xenografts carry important limitations. One major disadvantage is the lack of immune response inherent in these models. Nude mice are athymic and therefore have a limited T cell response, and severe combined immunodeficiency (SCID) strains lack both T and B cell responses. Because tumors can promote anti-tumor responses such as lymphocyte and macrophage infiltration, these models may not accurately represent disease progression and therapeutic response observed in otherwise immune-competent individuals (17–19). Furthermore, these models are not suitable for studying immunotherapy or mechanisms involving host–cell interactions. Cell line-derived xenografts have had little success in predicting therapeutic response in patients, thereby emphasizing a need for improvements to current models.

An alternative to traditional cell line-derived xenograft models involves the direct transfer of tumor fragments from individual patients. Minced fragments are delivered via orthotopic or intraperitoneal injection into immune-deficient mice to create "xenopatients" or tumor grafts (8, 20). Successful engraftment is higher in SCID mice compared to nude mice, likely due to the suppression of both cellular and humoral immunity (20–23). Several reports have demonstrated that tumor grafts stably maintain the histopathology, immunophenotype, and heterogeneity of the original tumor through multiple passages (21–26). Moreover, these models have the capacity to recapitulate the same therapeutic properties observed in patients (20, 25, 26). The better predictive response value makes these models superior to traditional cell line-based xenografts generated using a suspension of mostly homogenous cells. Engraftment of the native stromal extracellular matrix that would normally accompany a tumor graft may provide the most suitable microenvironment for replicating the biology of the original tumor. This feature renders tumor grafts more suitable for studying early metastasis, as it relies on dissemination of cells from a tumor fragment rather than dispersion of cells from a suspension (8). Thus, patient-derived tumor grafts provide a means to model inter-patient heterogeneity known to exist across high grade SEOC, and to study tumor evolution through exposure to therapy. Tumor grafts, although promising, are not without their own challenges. Generating a mouse model using a tumor graft is labor intensive and expensive and, as in traditional xenografts, the mice are immunocompromised; consequently immune responses cannot be studied. Although well suited for clinicians and personalized medicine, access to patient tumor samples can be challenging for many basic and translational investigators. Some research teams have generated banks of tumor grafts to make these models more accessible (20, 23, 24).

SYNGENEIC MODELS

Some challenges of xenograft models can be overcome using syngeneic mouse models, wherein tumors are established in immune-competent mice using cells from the same strain. In one of the most widely used syngeneic models, generated by Roby et al., ovarian surface epithelial cells isolated from immune-competent mice were repeatedly passaged *in vitro* until transformation occurred, and subsequently injected back into the same strain (27). Other syngeneic models have been created using genetically modified cells (28, 29) and highly metastatic cell lines stably expressing luciferase for monitoring disease (30). The histopathologic characteristics observed in the tumors of these models including the presence of papillary structures, nuclear atypia, and malignant ascites, closely resemble those seen in humans (29, 31).

The major advantage of this model is that the mice have an intact immune system; therefore the anti-tumor immune response can be examined and the risk of infection is minimized (19, 32). Syngeneic models provide the opportunity to study the tumor microenvironment, epithelial–stromal cell interactions, tumor-secreting factors, immune cell infiltration, and vasculature (28, 29, 31, 33). This model, however, is completely derived from the animal system and therefore may not mirror every element of human cancer. Although human and mouse tumors share similar features, the complexity of human disease coupled with the heterogeneity of cancer make it difficult to translate findings (34).

GENETICALLY ENGINEERED MODELS

Genetically engineered mouse models (GEMMs) are immune-competent mice with genetic defects introduced using RNA interference, inducible gene expression, viruses, or DNA recombination techniques. GEMMs provide a means for investigating the role of genetic alterations in cancer development. These models allow researchers to control and direct gene expression, which can be limited to the tissue of interest using a tissue-specific promoter to introduce the desired genetic alteration, or expressed throughout the organism using germ-line mutations (35). Furthermore, regulation of gene expression in the presence or absence of tetracycline and its receptor allows for inducible gene expression systems and provides the flexibility to turn on or off gene(s). For example, transgenic mice carrying both the tetracycline-regulated transcriptional transactivator and its respective binding site linked to a gene of interest permits amplified expression of that gene. If mice are provided with the tetracycline antibiotic in their drinking water, this expression is reversibly suppressed. Thus, GEMMs provide

opportunities to identify which genes are necessary for disease progression, regression, and/or resistance to treatment.

Extensive analyses of human ovarian cancer specimens have identified several genetic alterations associated with malignancy including *TP53*, *C-MYC*, *K-RAS*, *AKT*, and *BRCA1* and *BRCA2* (36–38). Subsequently, several genetically modified ovarian cancer models, summarized in **Table 1**, have been developed to explore the contribution of these different aberrations to ovarian cancer development (39–44).

Although GEMMs are labor-, time-, and resource-intensive, they provide information that cannot be attained in xenograft or syngeneic models. Early tumorigenesis and genetic events leading to tumor initiation, maintenance, and relapse can be analyzed. The flexibility provided by genetic manipulation permits the study of different mutation combinations. These models are ideal for target validation, treatment response, and chemoprevention (45). The major challenge with this model is the scarcity of tissue-specific promoters in ovarian surface epithelium or distal fallopian tube. It is also challenging to accurately replicate the contribution of genetic elements given that genes over-expressed in mice are often at non-physiological levels or deleted throughout the organism (46). GEMMs may fail to recapitulate the genetic complexity of human SEOC, and the varied genetic background of different mouse strains can influence findings and conclusions (8).

TECHNOLOGICAL ADVANCES IN USING ANIMAL MODELS

REPORTERS

Most ovarian cancer cell lines can be stably transfected with a fluorescent and/or bioluminescent reporter for monitoring tumor cell growth and dissemination, pathway activity, and receptor interactions.

This technology has been adapted to xenograft and syngeneic mouse models of ovarian cancer. For example, NF-κB activity was tracked in a syngeneic model of SEOC to confirm that activation correlated with progression and influenced immune cells of the microenvironment (47). Similarly, reporter-tagged tumor cells can be used to monitor tumor response in real-time using digital imaging following systemic targeted therapy (48–50). Using reporters in live animals to track tumor cell dissemination allows for studying cancer progression and therapeutic response, especially in syngeneic models where the immune response is integral.

Luciferase complementation-based assays measure receptor activation and protein interactions using monomeric enzyme components that have enzymatic activity only when complementation is induced by the interaction of binding partners or small molecules (51). Activation is proportional to the production of light that occurs upon complementation. The flexibility of this technology allows detailed quantitative measurements of complexes, assessment of nuclear translocation, and identification of pathway modulators (52). For example, this assay was successfully implemented for live imaging of the chemokine, CXCL12, interacting with its receptor, CXCR4, in animal models of ovarian cancer (53, 54).

IMAGING

Quantitative measurements of late-stage disease in ovarian cancer models are challenging due to the presence of varying levels of ascites and the poor correlation between total body weight gain and tumor burden. Diagnostic imaging is a reproducible means to

Table 1 | GEMMs for ovarian cancer.

Original reference	Genes altered	Ovarian specific expression	Cancer histology	Comments
(39)	*p53, c-Myc, Kras, Akt*	Oncogenes were delivered *in vitro* into ovarian epithelial cells from a transgenic *p53*-deficient mouse; modified cells were then introduced into ovarian bursa of the same mouse	Ovarian carcinoma	Illustrates necessity for p53 deficiency in combination with at least two other oncogenes for tumor induction
(41)	*p53, Rb1*	Adeno-Cre was introduced into ovarian bursa of transgenic mice carrying floxed alleles	EOC	p53 and Rb1 cooperate in EOC development
(40)	*p53, Brca1, c-Myc*	c-Myc and Cre were retrovirally delivered into ovarian explants from floxed *Brca1* and *p53* transgenic mice; modified cells were then introduced i.p. into recipient syngeneic mice	SEOC	Identifies the requirement for *Myc* in p53 and Brca1-induced transformation
(42)	*Pten, Apc*	Adeno-Cre was introduced into ovarian bursa of transgenic mice carrying floxed alleles	OEA	Illustrates the role of Wnt and PI3K signaling in development of ovarian endometrioid adenocarcinoma (OEA)
(43)	*Pten, Kras*	Anti-Mullerian hormone receptor directed Cre-expressing mice crossed with mice carrying floxed alleles	Low-grade serous adenocarcinoma	Demonstrates role of Kras transformation and loss of Pten for elevated p53 levels and associated low-grade phenotype
(44)	*p53, Rb, Brca1 or Brca2*	Adeno-Cre was introduced into ovarian bursa of transgenic mice carrying floxed *p53* and *Brca* alleles and *Rb* deficiency directed to epithelium by Keratin18 promoter for T-antigen expression	SEOC	Genetic modifications recapitulate human SEOC stages

quantify tumor mass, monitor tumor progression, and interrogate the tumor microenvironment. Imaging techniques used in the clinic [e.g., magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), ultrasound] have been adapted for use in animals (55, 56). These modalities are especially informative as they can be performed in intact living animals. Interval imaging reduces the number of animals needed for experiments as measurements are taken without sacrificing the animal. The major challenge to imaging ovarian cancer in animal models, as in humans, is the difficulty in detecting early disease; by the time mice begin to show signs of morbidity the cancer has often spread beyond the ovaries and throughout the peritoneum.

Positron emission tomography imaging is a standard diagnostic radiological technique commonly used to monitor drug action in cancer patients. This modality allows the measurement of metabolic activity in cancer cells and is especially useful in quantitative monitoring of tumor response to anti-cancer therapies (56). PET imaging can assess targeted therapies in both transgenic (57) and xenograft (58) models of ovarian cancer.

Ultrasound imaging is another common tool used in small animal models and is often combined with other imaging techniques for a more comprehensive analysis (57). Ultrasound is cost-effective and convenient for measuring individual tumors in live animals (59). Doppler ultrasonography can measure changes in blood flow and angiogenesis associated with disease progression or response to anti-angiogenic therapy (59, 60).

Magnetic resonance imaging with gadolinium-based contrast agent permits high-resolution serial imaging with minimum scanning duration, allowing quantification of tumor volume over time. MRI data are comparable to caliper-based measurements taken at necropsy. This longitudinal imaging protocol is well suited for monitoring therapeutic response (61). MRI can also be combined with fluorescence molecular tomography (FMT) to monitor tumor-specific biology, such as protease and integrin activity (62). When coupled with a reporter gene such as ferritin heavy chain (FHC), MRI can evaluate recruitment of other cell types, such as fibroblasts, to the tumor site (63). Alternatively, MRI combined with magnetic resonance spectroscopy (MRS) can characterize tumor physiology and metabolic profiles over time (64).

METABOLIC PROFILING

Measurement of metabolites and their intermediates can illustrate the response of an organism to a genetic manipulation or therapy. Metabolites are small, low molecular weight analytes and include amino acids, oligopeptides, sugars, fatty acids, and various intermediates of biochemical pathways, in contrast to large proteins and nucleotides that are assessed using proteomics and genomics, respectively (65). Nuclear magnetic resonance (NMR) spectroscopy, liquid and gas chromatography, and mass spectrometry (MS) are generally used to analyze serum, urine, or tissue extracts. Such measurements provide insight into drug mechanisms and toxicities. Metabolic profiles represent a snapshot of the biochemical reactions occurring at a point functionally downstream of genome, transcriptome, and proteome (65).

Commonly used in human studies, (66, 67) this technology was adopted in a GEMM of SEOC. The metabolic profile overlapped with human SEOC and showed a temporal correlation with disease progression (44, 68), highlighting the feasibility of metabolic profiling for identifying biomarkers and monitoring treatment response in animal models (44).

TUMOR-INITIATING CELLS

The cancer stem cell (CSC) or tumor-initiating cell (TIC) hypothesis suggests that a small population of chemoresistant cells reside in the tumor, capable of reconstituting the tumor. These cells share properties of normal stem cells, such as self-renewal and multipotency. Given the high recurrence of ovarian cancer, the TIC hypothesis is an attractive model for explaining ovarian cancer relapse.

Mouse models have been especially useful in evaluating TICs. When injected into mice, these cells must recapitulate the heterogeneity of the original tumor. Animal models are essential for defining TICs and for evaluating drugs and pathways important for eradicating these cells. Patient-derived xenografts might allow further characterization of the frequency of TICs in human tumors, and their relevant biomarkers.

A number of markers have been used to identify and isolate ovarian cancer TICs including CD133, CD44, CD117, and ALDH activity; however it is unlikely that a single marker defines ovarian TICs (69, 70). Several studies have demonstrated heterogeneous tumor formation in xenograft mice after subcutaneous injection of sorted ovarian cancer cells from primary tumors, cell lines, or ascites (71–74). TICs have also been propagated *in vitro* using low attachment culture plates and specialized serum-free media to enhance the formation of multicellular spheroids with stem-like features (69, 73).

Although much research has focused on characterizing tumorigenesis of human TICs in xenograft models, recent studies evaluated endogenous TICs in mice (75–77). Syngeneic or GEMMs offer the possibility of studying the role of the immune system in TIC biology. Furthermore, with direct or indirect labeling of the TICs, each of these models can facilitate tracking of the cells to monitor tumor initiation and dissemination.

IMMUNE THERAPIES

The role of the immune system in ovarian cancer is studied extensively using animal models (19). Representing a robust predictor of outcome, tumor-infiltrating lymphocytes are associated with better survival for ovarian cancer patients (78, 79). Immune therapies involving vaccines, dendritic cell therapy, engineered T cells, and immune modulators thus hold promise for ovarian cancer treatment (80–86).

Current goals aim to enhance the anti-tumor immune response through increased immune activation and decreased immune suppression. Programed death-1 (PD-1) and CTL antigen-4 (CTLA-4) signals silence the immune response in tumors. A syngeneic mouse model of ovarian cancer showed that simultaneously blocking these pathways enhanced T cell infiltration into the tumor and increased long-term survival (81). A related model found that the therapeutic effect of gemcitabine is limited because of the immunosuppressive network of CTLA-4 (83). Gemcitabine plus anti-CTLA-4 antibody exhibited synergy in a strong anti-tumor immune response. Likewise, anti PD-1 therapy shows synergism

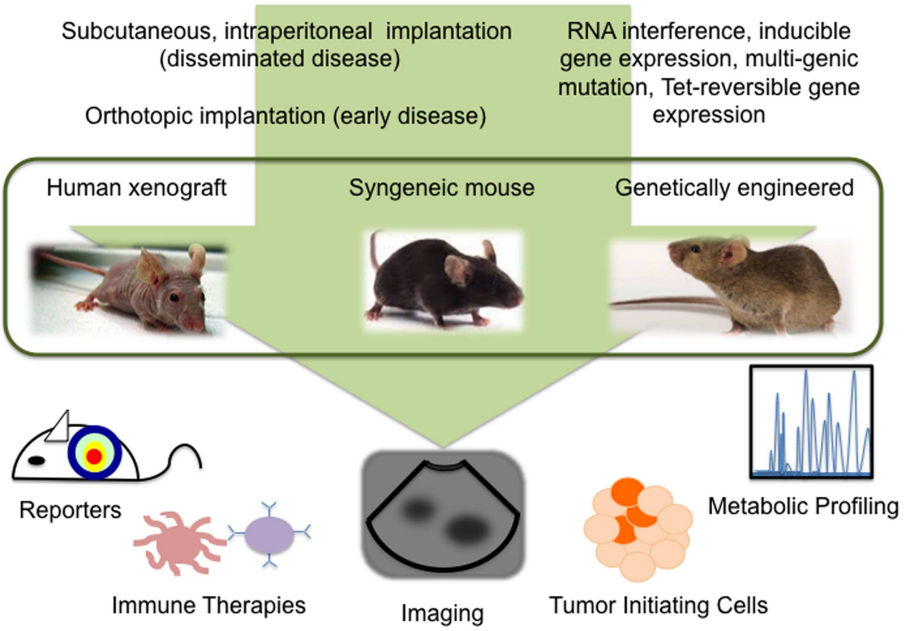

FIGURE 1 | Technological advances in mouse models allow detailed study of ovarian cancer biology.

with a variety of immunotherapies or vaccines (86). These findings have translated well and are currently under evaluation in the clinic.

Genetically modified T cells engineered to over-express receptors for tumor-associated antigens have shown great success in mouse models of ovarian cancer (87, 88). This emerging technology is a logical avenue for ovarian cancer, an apparently immunogenic disease where T cell infiltration is associated with improved survival (19, 88).

CONCLUSION

Despite our progress in understanding ovarian cancer biology, there remains a high mortality associated with this disease. Exciting advances in reporter assays, live imaging, metabolomics, TICs, and immune therapies, provide new information about the tumor microenvironment and further our understanding of SEOC development, progression, and recurrence (**Figure 1**). Further refinement of mouse models of ovarian cancer, an awareness of the limitations each model presents, and taking advantage of the technologies available to study these models will undoubtedly expedite the success of new treatments.

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Patient-derived xenograft models to improve targeted therapy in epithelial ovarian cancer treatment

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Despite increasing evidence that precision therapy targeted to the molecular drivers of a cancer has the potential to improve clinical outcomes, high-grade epithelial ovarian cancer (OC) patients are currently treated without consideration of molecular phenotype, and predictive biomarkers that could better inform treatment remain unknown. Delivery of precision therapy requires improved integration of laboratory-based models and cutting-edge clinical research, with pre-clinical models predicting patient subsets that will benefit from a particular targeted therapeutic. Patient-derived xenografts (PDXs) are renewable tumor models engrafted in mice, generated from fresh human tumors without prior *in vitro* exposure. PDX models allow an invaluable assessment of tumor evolution and adaptive response to therapy. PDX models have been applied to pre-clinical drug testing and biomarker identification in a number of cancers including ovarian, pancreatic, breast, and prostate cancers. These models have been shown to be biologically stable and accurately reflect the patient tumor with regards to histopathology, gene expression, genetic mutations, and therapeutic response. However, pre-clinical analyses of molecularly annotated PDX models derived from high-grade serous ovarian cancer (HG-SOC) remain limited. *In vivo* response to conventional and/or targeted therapeutics has only been described for very small numbers of individual HG-SOC PDX in conjunction with sparse molecular annotation and patient outcome data. Recently, two consecutive panels of epithelial OC PDX correlate *in vivo* platinum response with molecular aberrations and source patient clinical outcomes. These studies underpin the value of PDX models to better direct chemotherapy and predict response to targeted therapy. Tumor heterogeneity, before and following treatment, as well as the importance of multiple molecular aberrations per individual tumor underscore some of the important issues addressed in PDX models.

Keywords: ovarian cancer, patient-derived xenografts, pre-clinical models, targeted therapy, clinical trials

INTRODUCTION

Cell lines and archival tumor tissue have provided a platform for discovery and validation of novel therapeutic targets in epithelial ovarian cancer (OC). However, despite increasing evidence that precision therapy targeted to the molecular driver(s) of a tumor has the potential to impact overall survival (1), patients with high-grade epithelial OC are currently treated with a "one-size fits all" approach, without consideration of molecular phenotype or biomarkers of response that could better inform treatment. Pre-clinical models to predict those patients who will benefit from targeted therapy are imperative to implement effective precision therapy strategies. Classic cell line-derived xenograft models have provided invaluable mechanistic insight toward the key signaling pathways and oncogenic drivers of OC tumorigenesis, malignant progression, and chemotherapeutic resistance. The translational potential of models, generated from either human OC, many years ago, with scant histo-pathologic data about the source OC tumor from which they were derived or from human ovarian surface epithelial (OSE) cell lines, remains questionable (2). In both cases, the cell lines used to generate xenografts have been expanded *in vitro*, and as such, have likely acquired significant alterations in morphology, motility, and proliferation that do not necessarily reflect the physiologic state of the tumor (3). More importantly, recent evidence suggests that the ovarian surface may not be the origin of "ovarian cancer" (4, 5).

The majority of epithelial OC are serous in sub-type (50% of OC) and display Fallopian tube-like or "endosalpingeal" characteristics. Endometrioid OC (20% of OC) and mucinous OC (10% of OC) represent additional epithelial sub-types displaying features of epithelia from other Mullerian tract (developmental female genital tract) organs, resembling endometrial and endocervical epithelia, respectively (6). Two main phenotypic groupings of human EOC have been described. Type I EOC includes low-grade, mucinous, and clear cell cancers, with progression identifiable from adenoma-borderline-cancer. Type II EOC comprises lethal, high-grade serous (HG-SOC), endometrioid, and undifferentiated EOC. Although previously thought not to have recognizable precursor lesions (7), the current consensus indicates an association

with early lesions being found in the distal Fallopian tube in carriers of *BRCA1/2* mutations (5, 8). The Fallopian tubes are derived from the Mullerian ducts (also of mesodermal origin) and consist of muscular, ciliated, and secretory epithelia (9). Fallopian tube cancer and primary Peritoneal cancer (the latter is derived from the coelomic epithelium, as is the OSE), behave in a clinically similar fashion to serous EOC and while often studied together, distinct molecular differences are evident (10, 11).

The OSE has long been postulated to be the source of putative cancer initiating cells for epithelial "ovarian" cancer (12), with an alternative origin postulated to derive from the distal ends of the Fallopian tube and malignant or pre-malignant cells migrating to and settling on contiguous OSE (5, 8). A proportion of malignant lesions originate in the Fallopian tube and may potentially metastasize to the OSE (13). The OSE persists as a single layer of squamous to cuboidal epithelium that covers the ovary (14), is derived from coelomic epithelium (of mesenchymal origin), has a basement membrane and, unusual for a surface epithelium, can undergo epithelial-mesenchymal transition (EMT) (15). The OSE has been described as a facultative stem cell niche, with cells retained within this niche maintaining pluripotency and expressing markers typical of a stem cell-like quiescent state (12). However, it is possible that the OSE provides a suitable "niche" for the development of "ovarian" cancer and that the majority of HG-SOC in fact derive from secretory cells from the fallopian tube (16). Many reports of OC xenografts and patient-derived xenografts (PDXs) have mixed together OC sub-types, which may have very different implications for cell of origin or treatment approaches. By not taking into account sub-type, the likelihood of deriving useful information is greatly diminished.

Furthermore, traditional *in vivo* pre-clinical models do not accurately recapitulate the complexity and heterogeneity of patient tumors (17). As each tumor's molecular phenotype impacts prognosis and response to treatment, detailed genomic annotation of each xenograft is necessary for comprehensive evaluation of targeted therapies. Xenografts derived from a cell line originating from OC have to undergo extensive selection. More often these lines reflect the *in vitro* culture system and are devoid of the complex pathology and molecular attributes of the original patient tumor. A compelling example of this discordant phenomenon was reported in a study involving 41 cell lines, each of which rarely contained *BRCA1* and/or *BRCA2* mutations (18). The authors concluded that the use of these cell lines for xenograft studies would not accurately reflect the patient population. Moreover, xenografts derived from cell lines cultured from potentially irrelevant tissues (e.g., non-surface epithelium), may be even more flawed as models of human HG-SOC.

Xenografts may be derived directly from patient tissue without prior *in vitro* culture (PDXs), in which tumor tissue excised at the time of surgery is immediately transplanted into immune-deficient mice. Importantly, digestion of tumor material using protocols known to involve harsh cell dissociation buffers may inadvertently strip the cell surface of molecules integral toward *in vivo* cell–cell interactions. Alternatively, methods such as mincing of tumor fragments or the use of whole fragments may more closely model the heterogeneity of clinical disease. PDXs derived from non-digested OC can provide extremely flexible models for pre-clinical analysis of novel therapeutics (19). Primary OC and resultant serial PDX can be histologically assessed for known diagnostic and prognostic markers and characterized by molecular techniques including genome sequencing. These PDX models can therefore be extensively annotated and serve as powerful models for pre-clinical studies of targeted therapeutic strategies, thus bridging the gap between lab bench discoveries and clinical translation. As such, there has been an increase in characterization and application of PDX models for drug screening across a range of cancers [reviewed in Ref. (17)].

Thus, major concerns regarding OC PDX literature to date are as follows: numerous papers lack detail regarding histologic subtype, molecular phenotype, a detailed description of the methods used to generate and maintain the PDX, limited genomic characterization has been performed (e.g., CGH or CNV analysis), and the stability of various phenotypes over successive generations is noteworthy. As a result, a substantial barrier to the study of OC is the paucity of translationally (e.g., transient *in vitro* primary cell lines) and clinically (e.g., archived tissues from retrospective analyses) relevant models, thereby highlighting the salient need for an alternative, clinically relevant means to rapidly translate results from bench-to-bedside. The development of personalized PDX models, with each patient having a PDX generated across her disease progression (primary tumor, metastasis, recurrence) and stage of treatment (prior to treatment, at relapse), with availability of source biospecimens (germline DNA, serum, frozen, and FFPE tissue, etc.) and prospective clinical annotations could overcome many of the current hurdles (e.g., the dependence on isolation/digestion and subsequent amplification *in vitro* prior to establishment and testing in animals). These PDX models recapitulate primary patient tumors (e.g., formation of bowel metastases, obstruction, ascites, etc.), reproducibly engraft, retain the molecular and gross phenotypic characteristics of the donor OC patient, can be accurately monitored for tumor detection and progression (e.g., gross tumor palpation, calipers, ultrasound-guided imaging, etc.) and represent a practical and highly translatable medium to study the effects of both standard chemotherapy and precision targeted therapeutics.

METHODOLOGY

As previously noted, standard OC xenografts are derived from established, highly annotated, and widely available cell lines. While OSE models are commonly utilized and have become a mainstay workhorse to investigators, their uncommon origin (e.g., murine-derived) brings into question functional significance. For example, the ID8 cell line was originally developed as a syngeneic mouse model to study the early molecular and immune events related to ovarian carcinogenesis (20). As a result, greater attention toward patient-derived OC models has been expended.

Patient-derived xenograft (digested) have played a key role toward the study of the cancer stem cell (CSC) niche and identified tumor-initiating cells (TICs) as key players in primary patient ovarian xenografts (21). The frequency of TICs represents an intrinsic property of the primary patient tumor. However, the integrity of the TIC landscape (e.g., proportion of CD133 positive versus negative cells) is altered in PDX models. It is plausible

that the extensive *ex vivo* digestion prior to PDX engraftment is the confounding source of TIC PDX discrepancies.

Patient-derived xenograft (fragments) are generated by sectioning of fresh tumor tissue and engrafting ($1–2 \times 1–3\ mm^3$) pieces either subcutaneously or orthotopically into immuno-deficient mice (e.g., NOD-SCID IL2R$\gamma^{-/-}$). Engraftment rates for this generation (T1) range from 25 to 80% depending on tumor type (22), and growth usually takes 2–6 months. Once the T1 PDX tumor has reached $\sim$700–1500 mm^3 (23), it is harvested and directly re-transplanted for expansion in later serial generations (T2, T3) which are used for *in vivo* drug response, biomarker studies and generating cell lines for additional drug response and molecular studies. Alternatively, the fresh patient tumor can be minced and cryo-preserved in DMSO for later thawing and transplantation, thus ensuring the renewability of the resource. For molecular comparisons, the original patient tumor and the PDX models can undergo extensive histo-pathological and genomic analysis. In addition, for OC PDX models, the T1 PDX can be analyzed for platinum response and homologous recombination (HR) activity, which are key clinical indicators of drug response.

PDX MODELS IN OVARIAN CANCER

Patient-derived xenograft models have been applied to pre-clinical drug testing and biomarker identification in a number of cancers including pancreatic cancer (24), NSCLC (25, 26), melanoma (27), breast cancer (28, 29), and prostate cancer (30). As reviewed by Tentler et al. comprehensive genomic analysis including sequencing, expression, and copy number, have demonstrated that PDX models maintain overall global gene expression and activity as the source tumor (17). In OC, PDX models have been developed that accurately reflect the patient tumor and have successfully been used to examine drug response and effects of targeted treatment (22).

Some of the earliest applications of OC PDXs in studies of drug response were reported by the Repasky group. They developed 20 different PDX models in severe combined immuno-deficient (SCID) mice. Histo-pathologic and *in situ* hybridization analyses were carried out to confirm similarity to the source tumor. While all implanted PDXs eventually formed tumors, 65% (13/20) of them reached 1–2 cm within 2–6 months and were further expanded. Three of the later generation PDX models developed metastases and two developed ascites, representing clinical progression of the disease (31). The group then applied their subcutaneous PDX models in two separate studies to examine the effects of IL-12 and Flt-3 ligand on ovarian tumor growth. Following engraftment, PDX mice were treated with either placebo or IL-12 (32), or placebo or Flt-3 ligand (33), and tumor volume was measured over time. Treatment with IL-12 or Flt-3 ligand resulted in decreased tumor growth compared to control-treated mice, with increased NK cells and necrosis in the tumors of IL-12 or Flt-3 ligand treated mice. These findings suggest an immunologic reaction in response to IL-12 and Flt-3 ligand, supporting their potential therapeutic roles in the treatment of OC (32, 33).

Ghamande et al. followed up these studies by examining the effect of CD40 ligand therapy, previously shown to decrease growth in OC cells, on CD40 receptor-positive PDX serous OC models (34). PDX mice with subcutaneous or intra-abdominal tumors were treated with vehicle or increasing concentrations of recombinant CD40 ligand and tumor growth was assessed over time. Tumor growth in both locations was decreased following as little as one cycle of treatment, regardless of concentration. In addition, once tumors were excised following treatment, histological analysis revealed disruption of tissue architecture and increased fibrosis and apoptosis, providing further insight into the mechanism of therapy. Furthermore, the authors utilized these PDX models to examine the effect of combination therapy using standard chemotherapeutic agents and CD40 ligand therapy, further demonstrating an augmented effect when both drugs were used in treatment of CD40-positive tumors (34). These studies highlight the utility of PDX models in evaluating drug efficacy and mechanism of action.

While a majority of HG-SOC patients initially respond to first-line treatment (generally, a platinum drug in combination with a taxane), a large proportion eventually relapse and develop platinum-resistant disease. OC PDX models can be useful for screening drug sensitivity, which in turn provides guidance for clinical management of the patient who presents with recurrent disease. Kolfschoten et al. established 15 subcutaneous OC PDX models and examined sensitivity to standard chemotherapy (35). They reported that response rates in the PDX models correlated with those in OC patients (e.g., 40% of PDXs responded to cisplatin while 48% of patients respond). As detoxification by glutathiones has been demonstrated to render cells resistant to platinum treatment, the authors also investigated the glutathione-based mechanisms involved in the development of resistance. They measured levels of glutathione and glutathione-related enzymes in the PDX models and related them to drug response. They identified a correlation between glutathione reductase activity and efficacy of chemotherapeutic agents cisplatin and cyclophosphamide, suggesting that glutathione-related enzymes may be useful as predictors of drug sensitivity (35). These findings speak to the value of PDX models for expanding *in vitro* findings of drug response and relating them to patient tumors.

Because HG-SOC patients frequently develop resistance to platinum-based chemotherapy, it is imperative to identify novel therapies with efficacy toward tumors with *de novo* or acquired resistance. In an effort to investigate the efficacy of lurbinectedin, a new DNA binding drug, Vidal et al. generated serous PDX models by engrafting primary tumor tissue directly onto the mouse ovary surface (36). They included tumors with cisplatin sensitivity, as well as a tumor selected for acquired cisplatin resistance by repeated *in vivo* exposure. They reported a high correlation of histo-pathologic features between the patient and the platinum-sensitive and -resistant PDX tumors. The platinum-sensitive PDX displayed a dose-dependent response to cisplatin treatment, characterized by significant tumor volume reduction. Interestingly, 30–50% of treated mice relapsed at 6 months following treatment, and histo-pathologic features of the relapsed tumors were similar to the un-treated xenografts (36). As expected, cisplatin treatment did not significantly inhibit tumor growth in the cisplatin-resistant PDX model compared to control-treated mice. However, lurbinectedin treatment alone significantly decreased tumor growth in both cisplatin-sensitive and -resistant PDX models, and lurbinectedin in combination with cisplatin was more effective

than either drug alone (36). Additional studies demonstrated an increase in apoptosis and mitotic catastrophe in lurbinectedin-treated PDX mice, providing further insight into its mechanism of action. Thus, drug-resistant PDX models can be used to identify therapies that may be effective in patients with tumors resistant to standard agents.

In addition to subcutaneous and intra-peritoneal (IP) engraftments, OC PDX models have been established in mice by sub-renal capsule xenografts, allowing for follicle maturation. Lee et al. have demonstrated a high take-rate (95%) in sub-renal capsule PDX models, including low- or moderate-grade OC tissues that are typically difficult to engraft in subcutaneous or IP models (37). The authors compared histo-pathologic features in the original patient tumor, pre-graft tissue, and post-graft tissue and found no architectural or cytological differences, nor any major differences in immunomarker expression including CK20, CK7, or WT-1 (87–91% overall concordance). This group then investigated five individual sub-renal PDX models for drug response and genetic stability over subsequent passages (38). The authors analyzed the primary tumor and corresponding PDX by array CGH and reported similar gene copy numbers, with the primary tumors consistently clustering with their matching PDX. Furthermore, there was no significant difference in copy number changes between the primary tumor and corresponding PDX (38). These findings further support the accurate reflection of the patient tumor in PDX models. Furthermore, the high engraftment rate of sub-renal capsule PDX models may provide the opportunity to investigate the differences in tumor progression between low- and high-grade ovarian tumors.

As most HG-SOC tumors present at advanced stage, following peritoneal dissemination, IP PDX models are useful for investigation of tumor progression and metastasis. Bankert et al. generated IP PDX models from five different OC patients to examine metastasis and the microenvironment of human ovarian tumors (39). In these mice, tumor growth and spread reflect the patterns that occur clinically whereby tumors grew on surfaces within the peritoneal cavity including the omentum, spleen, ovaries, pancreas, and liver. In addition, these PDX mice formed distended abdomens with ascites fluid containing viable tumor cells, and CA-125 was present in their ascites and blood. Thus PDX OC models not only accurately reflect the histo-pathologic features of the tumor, but also present with clinically relevant disease, making them excellent models to investigate tumor progression.

Patient-derived xenograft models generated from patient ascites may also be useful for investigation of tumor progression and metastasis, and are readily transplanted. Ascites-derived PDX models have been used to characterize genome-wide chromosomal aberrations in *BRCA1*-mutated tumors (40). In addition, Stewart et al. generated ascites-derived PDX models to identify and characterize ovarian TICs (21), and found that these cells are molecularly heterogeneous across different tumors. Direct comparisons of PDX models generated from primary tumors and associated ascites would be helpful to determine whether ascites-derived PDX models accurately reflect the heterogeneity of the solid tumor. Not all patients develop ascites during their clinical course, suggesting that PDX derived only from ascites may not reflect the full disease spectrum.

PDX MODELS TO TEST TARGETED THERAPY

A targeted therapy currently under clinical investigation in OC treatment is inhibition of poly(ADP-ribose) polymerase (PARP), which targets cells with HR defects. PARP inhibition leads to accumulation of single-strand breaks, which generates double-strand breaks in DNA at replication forks. While double-strand breaks are effectively repaired in normal cells by HR repair, cells with deficiencies in *BRCA1/2* use error-prone mechanisms resulting in chromosomal instability and cell death (41). Germline mutations in *BRCA1/2* are present in 17% of HG-SOC cases (42) and in 25% of HG-SOC patients under the age of 50 (43). In addition, loss of BRCA function by genetic or epigenetic processes has been reported in 50% of HG-SOC cases (44). This high frequency of *BRCA* deficiency makes HG-SOC patients ideal candidates for PARP inhibition. PARP inhibitors have been shown *in vitro* and in Phase I/II clinical trials to be an effective treatment in some *BRCA*-deficient tumors, although it is still unclear why all patients with *BRCA1/2* mutations do not respond to PARP inhibitors (45). Furthermore, a proportion of those who do respond eventually progress and thus studies are still necessary to determine the mechanism of resistance to PARP inhibitors. HG-SOC PDX models, generated from tumors before and after treatment, are extremely useful to better understand the mechanisms of therapeutic response and resistance.

The Wang group further analyzed two of their sub-renal PDX models that carried alterations in *BRCA1* and *BRCA2* (38). In one case, DNA sequencing revealed a germline mutation in exon 2 of *BRCA1*, as well as loss of heterozygosity. In the other case, promoter hypermethylation of *BRCA1* was identified in the primary tumor, as well as a sequence variant in intron 2 of *BRCA2*. All of these alterations were maintained in the xenograft tumor. In assessing drug response in these models, tumor volume was decreased in carboplatin/paclitaxel-treated versus control-treated mice; however treatment with the PARP inhibitor PJ34 did not affect tumor growth in the *BRCA* models, despite decreased PAR expression in these tumors (38). It is unclear whether the lack of response was due to poor potency of this PARP inhibitor or due to additional tumor biology causing PARP inhibitor resistance.

In a more recent study to investigate targeted therapy, Kortmann et al. established PDX models from a *BRCA* wild-type and a *BRCA2* germline-mutated HG-SOC tumor to examine response to the PARP inhibitor, olaparib (46). First generation (T1) xenografts were analyzed following daily olaparib treatment of 50 mg/kg for 4 weeks. Immunohistochemical analysis of the *BRCA2* mutated model demonstrated decreased tumor cell proliferation and increased numbers of dead cells following olaparib treatment, while wild-type tumor characteristics were not affected. Moreover, olaparib treatment significantly decreased tumor volume in the *BRCA2* mutant PDX while having no effect on the *BRCA* wild-type PDX (46). These studies demonstrate the value of PDX models for characterizing response to targeted therapy.

In addition to PARP inhibitors, signaling molecules make attractive targets to inhibit tumor growth in HG-SOC and other cancers. The Hedgehog (Hh) pathway promotes proliferation, regeneration, and differentiation in adult somatic tissues, and aberrant activation of the Hh pathway is associated with malignant transformation in several cancers. Combination treatment

Table 1 | Summary of ovarian cancer PDX models.

Reference	Histotype (*n*)	Culture	Method	Treatment	Molecular annotation
Repasky group (31–33)	Serous (14), endometrioid (1), mucinous (2), clear cell (1), unspecified (2)	No prior *in vitro* culture	Minced, implanted on GFP or SC, SCID mice	IL-12, Flt-3 ligand (in serous models)	Not reported
Ghamande et al. (34)	Serous (6)	No prior *in vitro* culture	Minced, implanted SC, SCID mice	CD40 ligand, cisplatin, paclitaxel	Not reported
Kolfschoten et al. (35)	Serous (5), mucinous (4), clear cell (2), undifferentiated (3), carcinosarcoma (1)	No prior *in vitro* culture	Fragmented, implanted SC, athymic nude mice	Cisplatin, cyclophosphamide, doxorubicin, hexamethylmelamine, methotrexate, 5-fluorouracil	Glutathione content and glutathione-dependent enzyme activity
Vidal et al. (36)	Serous (1)	No prior *in vitro* culture	Implanted on ovary surface, athymic nude mice	Lurbinectedin (PM01183), cisplatin	Not reported
Wang group (37, 38, 44)	Serous (6), mucinous (2), granulosa cell tumor (2), leiomyosarcoma (1), clear cell (1), unspecified (1)	No prior *in vitro* culture	Fragments implanted sub-renal, NOD/SCID mice	Carboplatin, paclitaxel, PARP-1 inhibitor (PJ34) (in *BRCA1* null model)	CGH, *BRCA1/2* mutations (three serous, one clear cell, one leiomyosarcoma)
Bankert et al. (39)	Serous (4), undifferentiated (1)	No prior *in vitro* culture	Aggregates injected IP, NSG mice	IL-12	Not reported
Kortmann et al. (46)	Serous (2)	No prior *in vitro* culture	Fragments implanted sub-renal, NOD/SCID mice	Olaparib, carboplatin	*BRCA1/2* mutations, copy number
McCann et al. (49)	Serous (4)	No prior *in vitro* culture	Single cell suspensions injected SC, NOD/SCID mice	Cyclopamine (1), Hedgehog inhibitor (IPI-926), paclitaxel, carboplatin (3)	Not reported
Hylander et al. (50)	Serous (2)	No prior *in vitro* culture	Fragments implanted SC, SCID mice	Not reported	Stromal annotation by IHC
Stewart et al. (21)	Serous (31 primary tumor or ascites)	No prior *in vitro* culture	Digested, single cell suspensions injected as 1:1 HBSS:Matrigel in mammary fat pad, NOD/SCID mice	Not reported	Tumor-initiating cell markers CD133, CD44, CD117, EpCAM, ALDH1
Indraccolo et al. (40)	Serous (2 ascites)	No prior *in vitro* culture (T1), 2–3 passages (T2)	Ascitic fluid collected at recurrence, IP injection in SCID mice	Not reported	*BRCA1* mutations and expression, LOH, chromosomal aberrations by MLPA

GFP, gonadal fat pad; SC, subcutaneous; IP, intra-peritoneal; NSG, NOD-SCID IL2Rγ$^{-/-}$; MLPA, multiplex ligation-dependent probe amplification.

with standard chemotherapy plus Hh pathway inhibitors has been demonstrated to be effective against proliferation in basal cell cancer, medulloblastoma, and small cell lung cancer, amongst others (47). In OC, it has been reported that 20–50% of cases include Hh pathway activation (48). Furthermore, ectopic expression of Hh factors results in increased proliferation and motility of OC cells, while Hh inhibition impairs the growth of OC cell lines *in vivo*. Thus the Hh pathway may be a potential therapeutic target in OC treatment.

McCann et al. further examined the potential of Hh inhibitors as OC treatment using subcutaneous PDX models of serous OC (49). In this study, they treated a serous PDX model reported to have an activated Hh signaling pathway with the Hh pathway inhibitor cyclopamine. The cyclopamine-treated PDX mice had significantly decreased tumor volume compared to control-treated mice. In addition, the authors tested the efficacy of IPI-926, a derivative of cyclopamine that has increased oral bioavailability and potency and is currently in Phase I/II clinical trials, alone and in combination with standard first-line chemotherapy. In three different serous PDX models with activated Hh signaling pathways, Treatment with IPI-926 alone, or in combination, resulted in decreased tumor growth similar to results from chemotherapy alone, compared to controls (49). Interestingly, when the PDX mice were maintained on IPI-926 alone following combination

treatment, tumor regression was retained for up to 50 days following initial therapy. These studies indicate the utility of PDX models in examining combination therapy as well as maintenance therapy, in clinically relevant models.

PDX COHORTS

One of the most useful features of PDX models is their renewability, providing a repository of xenografts, tissues, and cell lines for researchers to access, along with relevant clinical and molecular data (**Table 1**). These cohorts provide fully annotated, genome-specific PDX models as training and test sets, providing the opportunity to efficiently bring molecular targeted drugs into clinical trials for the treatment of OC. In order for these cohorts to be fully beneficial, comprehensive annotation is essential, particularly two major characterizations: (a) the methods utilized to generate and maintain the PDX models (e.g., source tissue to confirm HG-SOC origin, fragmentation or mincing versus digestion to limit selection and possibly allow retention of infiltrating stroma, no prior *in vitro* culture to ensure reflection of primary tumor, method of implantation, etc.); (b) histotype, molecular, and genomic characterization (mutation, gene expression, CGH, CNV analysis), as well as response to standard therapy, of the PDX models (**Table 1**). Upon complete characterization and annotation, this resource will greatly accelerate the development of newly targeted therapies and identification of predictive biomarkers in OC, further bridging the gap between laboratory-based discoveries of novel therapeutic targets and clinical care.

LIMITATIONS

Probably the most noted limitation of PDX models involves the use of immunocompromised mice, which may attenuate the impact of the tumor microenvironment on tumor growth and drug response. In addition, stromal components such as vasculature or secreted stromal factors are increasingly being targeted by novel therapies. Thus, it is imperative that PDX models recapitulate the heterogeneity of the patient tumor in order to accurately test these novel therapies. In a recent study, Hylander et al. investigated vascularization and stromal formation in 37 subcutaneous PDX models in SCID mice created from a range of tumor types, including ovary, pancreas, kidney, and colon cancers (50). Successfully engrafted tumors were histologically examined for stromal factors and blood vessels. Their findings demonstrated that at the first passage (15–25 weeks), tumors no longer contained human stromal factors or vasculature; indeed, the stromal fibroblasts and vessel markers within the tumor were of murine origin (50). Kinetic studies suggested that loss of human vascularization markers occurred within 3–9 weeks, depending on tumor type. The authors conclude that in PDX models in which tumors are engrafted directly into immunocompromised mice, tumor growth is supported by host stroma and vasculature, suggesting that studies of therapies targeting human stromal components may not be adequate in these models.

To overcome these challenges, various approaches have been applied and additional models have been generated with the aim of recapitulating the tumor microenvironment. Engraftment of whole, non-disrupted chunks of human tumor helps to preserve tumor microenvironment components including leukocytes, fibroblasts, extracellular matrix, and vasculature (51). In addition, the use of NOD-SCID IL2R$\gamma^{-/-}$ mice provides improved PDX models for tumor–stromal interactions as they maintain tumor-associated leukocytes and stromal fibroblasts for up to 9 weeks after implantation (52). In the previously described IP OC PDX study by Bankert et al. performed in NOD-SCID IL2R$\gamma^{-/-}$ mice, the authors identified functional human lymphocytes and fibroblasts in tumors from multiple organs within the peritoneal cavity up to 177 days following engraftment (39). These findings suggest that future PDX models would provide greater value if generated in NOD-SCID IL2R$\gamma^{-/-}$ mice, particularly for studies involving drug response or microenvironment-targeting treatments.

CONCLUSION

The establishment of PDX models that recapitulate the complexity and genetic heterogeneity of HG-SOC will guide personalized cancer therapy and be invaluable toward establishing research priorities and strategies for developing new and more effective approaches to treatment in patients with recurrent OC. A repository of extensively characterized HG-SOC PDX models can be used for drug screening and discovery as well as biomarker development and testing. Furthermore, PDX models generated at initial diagnosis as well as at the time of recurrence will not only permit personalized treatment options, but in the long-term serve to enrich the recruitment and accrual of patients into early phase clinical trials.

AUTHORS CONTRIBUTION

Clare L. Scott, Marc A. Becker, Paul Haluska, and Goli Samimi all contributed to the writing and editing of the manuscript.

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Development of a mouse model of menopausal ovarian cancer

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Despite significant understanding of the genetic mutations involved in ovarian epithelial cancer and advances in genomic approaches for expression and mutation profiling of tumor tissues, several key questions in ovarian cancer biology remain enigmatic: the mechanism for the well-established impact of reproductive factors on ovarian cancer risk remains obscure; cell of origin of ovarian cancer continue to be debated; and the precursor lesion, sequence, or events in progression remain to be defined. Suitable mouse models should complement the analysis of human tumor tissues and may provide clues to these questions currently perplexing ovarian cancer biology. A potentially useful model is the germ cell-deficient Wv (white spotting variant) mutant mouse line, which may be used to study the impact of menopausal physiology on the increased risk of ovarian cancer. The Wv mice harbor a point mutation in c-Kit that reduces the receptor tyrosine kinase activity to about 1–5% (it is not a null mutation). Homozygous Wv mutant females have a reduced ovarian germ cell reservoir at birth and the follicles are rapidly depleted upon reaching reproductive maturity, but other biological phenotypes are minimal and the mice have a normal life span. The loss of ovarian function precipitates changes in hormonal and metabolic activity that model features of menopause in humans. As a consequence of follicle depletion, the Wv ovaries develop ovarian tubular adenomas, a benign epithelial tumor corresponding to surface epithelial invaginations and papillomatosis that mark human ovarian aging. Ongoing work will test the possibility of converting the benign epithelial tubular adenomas into neoplastic tumors by addition of an oncogenic mutation, such as of Tp53, to model the genotype and biology of serous ovarian cancer. Model based on the Wv mice may have the potential to gain biological and etiological insights into ovarian cancer development and prevention.

Keywords: ovarian cancer, epithelium, menopause, mouse models, ovarian follicles, pre-malignant lesions, Tp53

INTRODUCTION

Most ovarian cancers are epithelial-derived, and of the four major histological subtypes, serous ovarian cancer accounts for approximately 70% of the tumors (1–4). Serous ovarian carcinomas usually present as high-grade, with limited therapy options (5–7). Standard treatment regimens involve surgery to remove all visible disease, followed by a combination of taxane and platinum-based chemotherapy. Most patients who respond to first line chemotherapy will eventually relapse and die from drug-resistant disease. Despite intensive research and improvements in surgery and chemotherapy, the 5-year survival rate for ovarian cancer patients has languished around 30% for the past 30 years (5–7). This dismal survival rate attests to the urgency for a clear, more accurate understanding of basic ovarian cancer biology and etiology.

In the last several decades, great effort has been devoted to understanding ovarian cancer and the research has yielded significant knowledge and information about the biology and genetics of the disease (1–4). BRCA1 and BRCA2 mutations are associated with hereditary breast and ovarian cancers (1–4), which account for only a small fraction (estimated to be around 5–10%) of ovarian cancer cases. Recently, the Cancer Genome Atlas Project has provided a molecular profile of serous cancers (8): the tumor suppressor Tp53 is frequently mutated, but no other somatic mutation is consistently or frequently found. Nevertheless, Tp53 deletion alone is insufficient to induce epithelial tumors in mouse models (9–14). Thus, the molecular mechanism of ovarian serous cancer is not completely understood. In all the many types of ovarian tumor mouse models published so far, none reflects both the genetic (p53 mutation) and serous histology of human cancer.

Another key question in ovarian cancer biology related to reproductive etiology remains unanswered (1–4). Reproductive factors, such as increased parity and use of oral contraceptives, reduce the risk of ovarian cancers. Age and menopausal statues are even more important factors in ovarian cancer risk (1–4). Most ovarian cancers are diagnosed in menopausal women; fewer than 15% are diagnosed in women younger than 50 years of age, and the histological subtype of those cancers may not be epithelial but derived from germ cells or granulosa cells (15). The risk of ovarian

cancer increases greater than fivefold during the peri-menopausal years (16–23).

In laboratory studies, few of the developed ovarian tumor models incorporate the epidemiological evidence that reproductive factors and age influence the risk of ovarian cancer. Consequently, the mechanism for the well-established impact of reproductive factors on ovarian cancer risk remains obscure and not well explored. Thus, a reasonably good model to understand the etiology of ovarian cancer should incorporate the genetics and the reproductive physiology of the disease, such as menopausal stage. Here, we discuss the development of a unique mouse model to study menopausal ovarian cancer.

OVARIAN CANCER EPIDEMIOLOGY AND ETIOLOGY

Epidemiological evidence suggests that the risk of ovarian cancer associates with reproductive history and hormonal factors (16–23). Increased parity decreases the risk by 50% over nulliparity, as does oral contraceptive use for 5 years (17, 19, 20). The most significant risk factors for developing ovarian cancer are age and menopausal status (16–23). The majority of ovarian cancers are diagnosed in post-menopausal women in their late 50s and early 60s. The average age of diagnosis for sporadic ovarian cancer is about 63 years, although women with genetic or familial risk factors tend to be diagnosed at a younger age (average age of diagnosis is 54 years). Thus, it appears that age and menopausal status closely associate with ovarian cancer risk.

Several theories have been proposed to explain the epidemiological data associated with ovarian cancer risk. One idea holds that incessant ovulation, or the repeated wounding and subsequent proliferation that occur to repair the surface epithelium at the site of ovulation, results in mutations accumulating in the ovarian surface epithelial cells (24–26). Ultimately a tumor mass develops. This idea would explain the reduction of risk associated with pregnancy, extended breastfeeding, some oral contraceptive formulations, and early menopause, all of which reduce the number of ovulatory events.

Supported by the same epidemiological evidence, the gonadotropin stimulation hypothesis postulates that the surges of pituitary gonadotropins [including follicle stimulating hormone (FSH) and luteinizing hormone (LH)] that initiate each ovulation also stimulate the ovarian surface epithelium and induce cell transformation (20, 21). The speculated role of gonadotropins is also consistent with the fact that ovarian cancer occurs most frequently in post-menopausal women, when ovulation ceases yet plasma gonadotropins are elevated (21–23). However, since FSH and LH have unremarkable effects on growth of ovarian surface epithelial cells in culture (27–29), a direct effect of the hormones on ovarian epithelial transformation is unlikely to be sufficient. Thus, neither theory completely or satisfactorily explains the epidemiological observation of an association between ovarian cancer incidence and the menopausal transition.

A more recent idea posits that the depletion of ovarian follicles disrupts ovarian epithelial homeostasis and may be the true cause of an increased cancer risk in menopause (30). The idea that loss of ovarian function may underlie the link between reproductive factors and ovarian cancer was also proposed previously (31). The follicle depletion hypothesis explains the association between menopause and ovarian cancer risk, and can potentially unify "incessant ovulation" and "gonadotropin stimulation" as mechanisms. Specifically, incessant ovulation leads to the depletion of the ovarian reserve, which in turn leads to the increased level of gonadotropins that characterize menopause. Thus, the two theories explain the cause and consequence, respectively, of ovarian follicle depletion. The studies of a germ cell-deficient Wv mouse line provided basis for the follicle depletion theory (30, 31).

BIOLOGY OF MENOPAUSE

By the end of the reproductive age, germ cells and follicles are depleted from the ovaries and the ovulatory cycle ceases, resulting in menopause. Menopause is defined as the permanent cessation of menstruation resulting from depletion of germ cells and loss of ovarian follicular activity (32–34), and has become a woman's health issue as a by-product of modern health advances and the extension of lifespan that occurred in the last century (32–34). The peri-menopausal period commences when the first features of menopause begin until at least 1 year after the final menstrual period, generally lasting an average of 5 years. In humans, the transition to menopause is a set of gradual changes, in which ovarian function, reproductive capacity, and hormonal status are altered long before menses stops completely. Menopause generally occurs between 45 and 55 years of age, and the symptoms vary among women.

Hormonal changes characterize the menopausal transition. In the normal reproductive ovary, following ovulation and release of the ovum, the follicle converts into a corpus luteum, where sex steroids, predominately estrogen and progesterone, are produced and released. The steroid hormones act to inhibit the release of FSH and LH. With the depletion of follicles and cessation of ovulation, estrogen and progesterone levels fall and normal feedback inhibition of FSH and LH release stops. As a result, FSH and LH reach highest serum levels in peri- and post-menopausal periods and remain elevated (32–34). These changes precipitate a number of menopausal-associated symptoms and disorders.

MECHANISMS FOR MENOPAUSE AS A RISK FACTOR

Among the physiological changes associated with menopause, the ovarian tissues undergo morphological transformation, known as "ovarian aging" (25), and this is implicated in the high incidence of ovarian cancer that occurs during the peri-menopausal and immediate post-menopausal periods (30, 31). One feature associated with ovarian aging is the accumulation of ovarian morphological changes such as deep invaginations, surface papillomatosis, and inclusion cysts (35–37), which are thought by some to be the histological precursors of ovarian cancer (38–43). Presumably, acquisition of an oncogenic mutation (such as Tp53 mutation) in these proliferative ovarian epithelial cells would promote the development of ovarian cancer.

From the analysis of pre-cancerous ovarian tissues obtained from prophylactic oophorectomies, pre-neoplastic lesions and microscopic carcinomas were identified in the ovaries or fimbria of fallopian tubes from women with a family history of ovarian cancer or identified BRCA mutations (38, 44, 45). Several studies reported the increased ovarian morphological changes in high-risk ovaries (35, 37, 38, 46, 47), though some found negative results

(48–50). In one analysis, we found that no significant increase in the presence of non-neoplastic ovarian morphological changes is associated with BRCA1/BRCA2 mutations (35). Rather, the frequency of these histological features, especially inclusion cysts, associates with age or menopausal status. We propose that ovarian morphological changes increase in the peri-menopausal period, and these histological features may promote the transformation of genetically compromised epithelial cells in the development of ovarian cancer. The results suggest age-dependent pre-neoplastic morphological changes may be a risk factor, and support the idea that ovarian aging-related epithelial morphological changes provide precursor cells that may transform upon acquisition of oncogenic mutation(s) (42).

The fallopian tube origin of ovarian cancer suggests that tubal epithelial cells from the normal fimbria, which envelops the ovary and contacts the ovarian surface, dislodge and seed, or implant on, the surface of the ovary (51–58). Inclusion cysts form by membrane engulfment. Likewise, transformed cells of the fimbria may shed and implant on the ovarian surface. The tumor that establishes appears to arise from the ovary but originates, in fact, from the fallopian tube. It may be that age and follicle depletion alter the receptivity of the ovarian surface to seeding by the fallopian fimbria epithelial cells, i.e., its ability to accept the fimbria cells, and also make it a more permissive substratum for engulfment or proliferation of the seeded cells. Thus, the idea of follicle depletion as a risk factor for ovarian cancer may also be adapted to the fallopian tube cell of origin model, in addition to that originally proposed considering only cancer derived from the ovarian surface and/or surface-derived inclusion cysts (30, 31). Additionally, follicle depletion may also encourage the proliferation of stromal epithelial cells of Müllerian origin, which have also been considered to be possible cells of origin of ovarian serous carcinomas (59, 60). The epithelial cells of both fallopian tube fimbria and extra-ovarian Müllerian glands may be responsive to the menopausal increase of gonadotropins.

MOUSE MODELS IN OVARIAN CANCER RESEARCH

In the past decade, a number of technical breakthroughs have led to the establishment of several mouse models as described briefly here. First, a genetically defined model of ovarian cancer was established by Orsulic and colleagues (13), in which mouse ovarian surface epithelial cells were isolated and transfected with defined genetic changes such as k-Ras, v-Akt, v-myc, etc. The cells were then re-implanted into the ovarian bursa of mice and malignant ovarian tumors developed. Using the MIS II R promoter, a mainly ovarian-restricted transcript, Connolly, Hamilton and colleagues developed the T-antigen transgenic line that develops malignant bilateral ovarian tumors (61). Presumably, T-antigen expression results in the inactivation of both p53 and Rb. Indeed, using adenoviral delivery of cre to ovaries of mice with floxed p53 and Rb, Flesken-Nikitin et al. demonstrated the development of malignant ovarian tumors when both p53 and Rb are deleted (11). Mice with conditional expression of K-ras and deletion of pten in ovarian surface epithelial cells were made and found to develop endometriosis and endometrioid carcinomas (62). Since both mutations are associated with endometriosis and endometrioid ovarian cancer in humans, this model appears to recapitulate the genotype and histomorphology of the human disease. Another mouse model of endometrioid carcinomas was established by combining beta-catenin activation and pten loss (63). Based on the understanding that the majority of serous ovarian cancer may be derived from fallopian tube fimbria, the reproductive tract tumor models were produced by targeting SV40T using the promoter of the mouse oviduct-specific glycoprotein (OGP) (64). In another study, fallopian tube-derived tumors were produced by Amhr2-Cre mediated deletion of pten and Dicer (65). Likely there are additional ovarian cancer animal models that are not mentioned here (66–69).

However, the modeling of genotype and phenotype of human serous cancer has not been successful. Although p53 mutation is the only common genetic mutation in ovarian cancer (8), p53 null mice do not develop ovarian cancer. When p53 null ovaries were transplanted into wild type mice to allow prolonged aging, the tumors that developed were of granulosa rather than epithelial origin (9). In several recent studies, concomitant inactivation of Tp53 and BRCA1 produced leiomyosarcomas, which likely originated from the ovarian bursa (10, 12–14). Further investigation of these animal models should lead to a better, more thorough understanding of ovarian cancer development. Nevertheless, none of these models has components related to the etiology of ovarian cancer. Also, few investigations on early lesions or cells of origins were reported in these ovarian tumor models.

To investigate reproductive factors, mouse models that mimic or incorporate menopausal biology may be useful. Most female mammals, except for humans, live only a relatively short time after ceasing reproduction, and normal rodents or other animals do not adequately model the menopausal state (32). In the laboratory setting, surgical removal of ovaries is used to mimic menopause on the physiology. Another method is to kill germ cells and ovarian follicles using toxins such as such as busulfan and 4-vinylcyclohexene diepoxide (70–72). These "menopausal mouse models" may be useful for some purposes, for example, to investigate breast tumor xenografts under menopausal conditions and to study chemical-induced breast carcinogenesis (73, 74). Mutant mice that contain gene mutation affecting ovarian function were also suitable to investigate ovarian cancer. A notable mouse model of restricted BRCA1 deletion in granulose cells was produced to investigate the association between menstrual cycle and ovarian cancer risk (75, 76). Mice with FSH receptor knockout were reported to exhibit some phenotype of ovarian failure and have been proposed as a potential model of menopause (77). In this article, we highlight the use of a natural mutant mouse line, the white spotting variant (Wv) mouse, to model menopause and associated ovarian cancer risk. In the Wv females, the ovarian follicles are gradually depleted early in life because of a reduced c-kit activity and resulted oocyte reserve, and the mice mimic the phenotypes in both the cause (ovarian follicle depletion) and many consequences (such as changes in heart, bone, lipids, ovarian epithelia) of menopause (78, 79).

THE Wv GERM CELL-DEFICIENT MOUSE MODELS

The Wv mice harbor a point mutation in the kinase domain of the c-kit gene, resulting in developmental defects in germ cells, pigment-forming cells, red blood cells, and mast cells in

homozygous mutant mice (68–83). The Wv/Wv mice have a similar lifespan as wild type, are sterile, white-coated with black eyes, and predisposed to ovarian neoplasms (84). The Wv/Wv mice contain less than 5% of the normal number of oocytes at birth and the remaining germ cells are depleted by about 8 weeks of age (**Figure 1**). Consequently, ovulation ceases to occur and an increase in pituitary gonadotropins follows (85). Compared to wild type littermates, in which ovaries contain a large number of follicles at various developmental stages, Wv ovaries are depleted of follicles by 2–3 months of age (**Figure 1**). Ovarian surface epithelial dysplasia and tubular adenomas develop in Wv/Wv mice (79, 85). The Wv mice appear to model several aspects of postmenopausal biology, including a long post-reproductive lifespan, increased serum gonadotropins, decreased sex steroids, and physiological changes, such as decreased bone density, elevated serum cholesterol, and altered cardiac function (78).

The ovarian lesions in the Wv mice distribute throughout the ovarian stroma, and are known as stromal tubular adenomas (85). The contiguous connection to ovarian surface epithelium is evident (**Figure 2**), and is especially pronounced in early ovarian lesions from younger (7–10 weeks) mice when lesions begin to develop (79). The majority if not all the tubular adenomas in Wv/Wv ovaries appear to be derived from ovarian surface epithelial cells. However, rete ovarii structure is also very prominent in Wv ovaries. At 4 months, epithelial lesions permeate the entire ovary, and rete ovarii appear to form distinct lesions (**Figure 2**, arrow). At 8 months of age, the Wv ovarian tumor is extensive, and surface versus rete ovarii epithelia are no longer distinguishable. The majorities of the lesions either exhibit inclusion cyst-like structures or resemble surface deep invaginations/papillomatosis (**Figure 2**) (79).

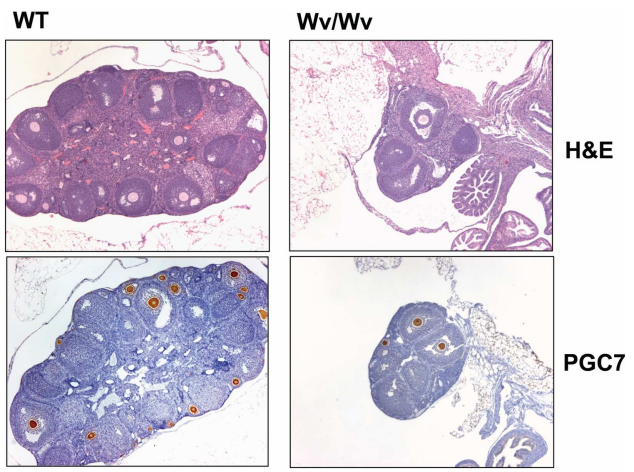

FIGURE 1 | Germ cell deficiency in Wv ovaries. Ovaries from 6-week-old wild type and Wv/Wv littermates were harvested and subjected to histological analysis. PGC7 staining was used as a marker for germ cells and follicles (86, 87). Wild type ovaries contain abundant PGC7-positive germ cells and follicles of various developmental phases, and in particular, germ cells and primary follicles are found immediately beneath the surface. Wv/Wv mutant ovaries are smaller and contain a greatly reduced number of germ cells.

POTENTIAL DEVELOPMENT OF THE Wv MICE TO MODEL MENOPAUSAL OVARIAN CANCER

The germ cell-deficient Wv mutant mouse line mice may be explored to gain additional understanding and verification of the impact of menopausal physiology on the increased risk of ovarian cancer.

Tp53 deletion, alone or in combination with other genetic changes, does not seem to produce ovarian epithelial tumors in mouse models. Since mutations in Tp53 that result in accumulation of mutated Tp53 protein occur frequently in ovarian cancer and are more relevant than deletion (8, 88), it may be possible to add the Tp53 mutation in the Wv ovarian tubular adenomas to test if Tp53 mutation can convert the benign epithelial tumors to malignant adenocarcinomas (**Figure 3**). If successful, such a

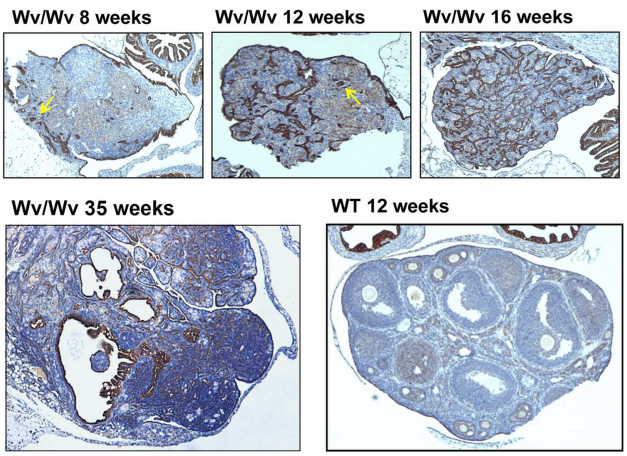

FIGURE 2 | Epithelial ovarian tumors in Wv/Wv mice. Ovarian tissues were harvested and subjected to histological analysis. Cytokeratin-8 staining was used as a marker for epithelial cells. Representative ovaries are shown for 8-, 12-, 16-, and 35-week-old Wv/Wv mice, indicating the progressive increase in epithelial lesions. In comparison, a wild type ovary from a 16-week-old littermate has a single layer of cytokeratin-8-positive ovarian surface epithelium. The arrows in the two left panels indicate the putative lesions that developed from rete ovarii.

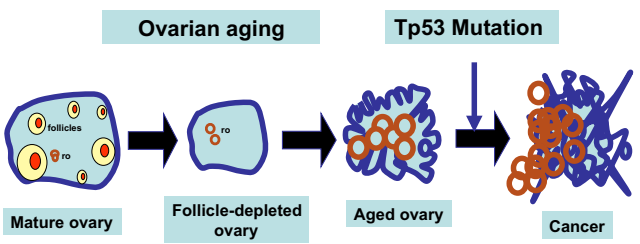

FIGURE 3 | Working model for follicle depletion and ovarian aging in ovarian tumorigenesis. A schematic illustration of the consequences of follicle depletion in the development of ovarian tumors is presented. Upon depletion of ovarian follicles, ovarian surface and rete ovarii (ro) epithelia undergo remodeling and morphological changes. Wv ovarian epithelial tumors may be derived from both surface and rete ovarii (ro). Genetic mutations, such as Tp53 mutation, will promote the benign epithelial lesions to develop into malignant tumors.

model may mimic the development epithelial ovarian cancer in both genetic and reproductive aspects. Using the Wv mice, we are currently performing experiments to determine if adding a Tp53 point mutation in the epithelial cells of the Wv ovarian tumor generates a malignant tumor that resembles ovarian cancer.

By deleting a transcription stop signal in the floxed Tp53 mutant (88) in ovarian surface epithelial cells through injection of adenovirus expression Cre, we predict that the model mimics both reproductive factors (postmenopause) and genetic mutation (Tp53). Preliminary studies indicate that these Wv/Wv:p53(R172H) (fl/fl):Adv-Cre ovarian epithelial tumors appear malignant. We are currently characterizing in more detail these mouse ovarian epithelial models and expect to report these findings in the near future.

POSSIBLE STRATEGIES FOR DELAYING MENOPAUSE AND OVARIAN CANCER RISK REDUCTION

The neoplastic ovarian tumor models following addition of Tp53 mutation in the benign Wv tumors may be used to explore several questions regarding the etiology and possible preventive strategies for ovarian cancer. Several potential preventive approaches, such as inhibition of cyclooxygenases and use of progestin to mimic oral contraceptive usage, have been proposed and can be tested in Wv mouse models.

Genetic suppression of cyclooxygenase 2 produced a significant alleviation of ovarian lesions in the Wv/Wv:Cox-2 (±) ovaries analyzed, although the degree to which the tumor phenotype was suppressed varied greatly (79). Hemizygous reduction of the Cox-2 gene resulted in a complete or partial rescue from the epithelial adenoma phenotype. Thus, a reduction in Cox-2 gene dosage rescued the ovarian epithelial morphological alteration, but deletion of both copies was less sufficient in reversing the adenoma phenotype. Reducing the Cox-2 gene dosage on ovarian tumor phenotype can be achieved by using pharmacological agents. Thus, cyclooxygenase inhibitors are able to prevent ovarian epithelial morphological transformation and tumor phenotypes. Inhibition of both Cox-1 and Cox-2 with indomethacin is more effective than inhibition of Cox-2 alone with celebrex. When indomethacin was given for a period of 1 month to Wv/Wv mice at 3 months of age when ovarian tumors were already established, the tumors were not reduced compared to controls, suggesting inhibition of cyclooxygenases prevents the development of ovarian tumors but has no suppressive effect on established tumors (79). Furthermore, inhibition of Cox-1 was superior to inhibition of Cox-2, and inhibition of Cox-1 reduced the development of ovarian adenomas in Wv mice by delaying ovarian follicle maturation and thus depletion (89) occurs. The conclusions of these studies are consistent with the notion that the ovarian follicle depletion, rather than ovulation and gonadotropin stimulation, is a major determinant of an increased ovarian cancer risk in menopause. The experimental results provide explanation for the epidemiological observations that use of non-steroidal anti-inflammatory drugs (NSAIDs) reduce ovarian cancer risk (90–96). Inhibition of Cox-1 and Cox-2 may have different mechanisms. Cox-1 inhibition may delay follicle depletion and ovarian cancer risk. Cox-2 inhibitors may reduce the cancer promoting activity of the inflammation-like ovulatory processes that are stimulated by gonadotropins (95, 96). The mechanism predicts that use of NSAIDs may be more effective in reducing the risk of ovarian cancer in pre-menopausal compared to post-menopausal women, since Cox-1 inhibition can delay ovarian follicle depletion (89). In post-menopausal women, inhibition of Cox-2 may slow epithelial remodeling and thus still reduce ovarian cancer risk.

Because endogenous hormones play a major role in the risk of breast, endometrial, and ovarian cancer, the impact on risk for oral contraceptives and hormonal therapy given at about the time of menopause has been a major concern (97, 98). Numerous studies provide insights into cancer risk associated with use of these preparations. Generally, use of oral contraceptives reduces ovarian cancer risk (19, 99). Many studies attribute the preventive effect on its suppression of gonadotropin level and ovulation. Also, this risk reduction may differ between pre- and post-menopausal women. Recent studies suggest that prolonged oral contraceptive pill use provided a greater protective effect against pre-menopausal ovarian cancer than against post-menopausal cancer (100). Furthermore, suppression of pituitary gonadotropin release with hormone replacement therapy may not reduce ovarian cancer risk in post-menopausal women (97, 100). These findings substantiate that intact ovarian function may be an important determinant of ovarian cancer risk, and the timing of progesterone administration may differentially alter its preventive capacity depending upon follicle reserve and menopausal status. The Wv mouse model will be useful in experiments to test the suppressive activity of progesterone/progestin on gonadotropin levels and the role of increased gonadotropins on ovarian tumorigenesis.

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Advances in tumor screening, imaging, and avatar technologies for high-grade serous ovarian cancer

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The majority of high-grade serous ovarian carcinoma cases are detected in advanced stages when treatment options are limited. Surgery is less effective at eradicating the disease when it is widespread, resulting in high rates of disease relapse and chemoresistance. Current screening techniques are ineffective for early tumor detection and consequently, *BRCA* mutations carriers, with an increased risk for developing high-grade serous ovarian cancer, elect to undergo risk-reducing surgery. While prophylactic surgery is associated with a significant reduction in the risk of cancer development, it also results in surgical menopause and significant adverse side effects. The development of efficient early-stage screening protocols and imaging technologies is critical to improving the outcome and quality of life for current patients and women at increased risk. In addition, more accurate animal models are necessary in order to provide relevant *in vivo* testing systems and advance our understanding of the disease origin and progression. Moreover, both genetically engineered and tumor xenograft animal models enable the preclinical testing of novel imaging techniques and molecularly targeted therapies as they become available. Recent advances in xenograft technologies have made possible the creation of avatar mice, personalized tumorgrafts, which can be used as therapy testing surrogates for individual patients prior to or during treatment. High-grade serous ovarian cancer may be an ideal candidate for use with avatar models based on key characteristics of the tumorgraft platform. This review explores multiple strategies, including novel imaging and screening technologies in both patients and animal models, aimed at detecting cancer in the early-stages and improving the disease prognosis.

Keywords: fallopian tubal secretory epithelial cell, high-grade serous carcinoma, genetically engineered mouse models, avatar models, serous tubal intraepithelial carcinoma, near-infrared fluorescence, the Cancer Genome Atlas, BRCA

TREATMENT APPROACHES FOR PATIENTS AND HIGH-RISK WOMEN

Ovarian cancer is the most lethal gynecological malignancy, with the majority of high-grade serous carcinoma (HGSC) cases being diagnosed in late stages of disease [for a comprehensive review see Bast (1)]. The current American Cancer Society statistics estimates that the 5-year survival is 44% when all disease stages are included but declines to 25% if only advanced stage cases are considered (1–3). Initial treatment for HGSC patients involves debulking surgery, which typically includes a combination of hysterectomy, bilateral salpingo-oophorectomy, and removal of the omentum, followed by therapy using platinum compounds and taxanes (4, 5). While platinum-based chemotherapy improves patient survival, the treatment is also very toxic; in addition, disease relapse following treatment and the acquisition of platinum chemoresistance are frequent events, suggesting a need for alternative treatment modalities (6). One strategy involves the addition of bevacizumab, a monoclonal antibody targeting VEGF-A, which has been tested together with standard chemotherapy in the setting of platinum-resistant recurrent disease. Indeed, the combination of an anti-angiogenic compound and single-agent chemotherapy improved objective response rates and progression free survival in comparison with chemotherapy alone, but the overall survival trend was not significant (7). An alternative strategy, which is aimed at reducing both the treatment toxicity and recurrence rates, proposes the administration of lower doses of carboplatin plus paclitaxel given once a week for 18 weeks instead of standard doses administered every 3 weeks for six cycles. Interestingly, this modified weekly regimen of reduced chemotherapeutic doses has recently been found to not only be an effective option for first-line treatment but, most importantly, to be associated with an enhanced quality of life as assessed physically, socially, emotionally, and functionally (8).

New treatment approaches could be beneficial not only for patients but also for women at increased risk for developing the disease. A recent study, which included a broadly spanning, exome-wide analysis of both ovarian cancer somatic and germline mutations, has estimated that more than 20% of women

Abbreviations: FR-α, folate receptor α; FTSEC, fallopian tubal secretory epithelial cell; GEMM, genetically engineered mouse models; HGSC, high-grade serous carcinoma; NIRF, near-infrared fluorescence; STIC, serous tubal intraepithelial carcinoma; TCGA, the Cancer Genome Atlas.

likely have an inherited predisposition to ovarian cancer (9). The most studied mutations involve the *BRCA* family of tumor suppressor genes, which confer an increased risk for developing breast and ovarian cancers (10). The lifetime risk for developing breast cancer in *BRCA* mutation carriers varies between 56 and 84% while the ovarian cancer risk ranges from 36–46% to 10–27% for women with *BRCA1* and *BRCA2* mutations, respectively (11). In addition, lifetime risks for both breast and ovarian cancers due to *BRCA* mutations appear to have increased over time, possibly due to lower physical activity and higher BMIs (12). When assessing individual patient risks for ovarian cancer it is important to note that irrespective of genetic risk, women with irregular menstrual cycles experience a 2.4 times greater incidence of death due to ovarian cancer (13).

QUALITY OF LIFE AFTER PROPHYLACTIC SURGERY IN HIGH-RISK WOMEN

The mean age at the time of diagnosis for the average population is 63 years; by comparison, the mean age for BRCA mutation carriers is considerably lower at 50.8 years. For both groups, the cancer risk increases with age, especially after menopause. Consequently, the current recommendation for *BRCA* carriers is to undergo risk-reducing surgery once childbearing is completed, since they tend to be diagnosed at an earlier age than sporadic ovarian cancer (14). Standard prophylactic surgical options include bilateral mastectomy and salpingo-oophorectomy, which confer the most substantial reduction in cancer risk and increase in life expectancy (10, 15, 16). Studies looking at the efficacy of bilateral prophylactic salpingo-oophorectomy in *BRCA* mutation carriers concluded that it not only resulted in a 96% reduction in the risk of developing coelomic epithelial cancers but also decreased the breast cancer risk by 53% in comparison with *BRCA* mutation carriers who chose not to undergo the procedure (14). Despite the drastic risk-reduction associated with prophylactic surgery, it is far from an ideal treatment approach. In addition to being stripped of their inherently female characteristics, women who choose to undergo a combination of mastectomy, bilateral salpingo-oophorectomy, and hysterectomy are faced with a significant decrease in their quality of life, premature menopause, hot flashes, decreased cognition and sexual function, and increased risk of osteoporosis and cardiac mortality (14, 17–19). The use of hormone therapy and other medications may mitigate some of these adverse effects. Nevertheless, hormone replacement therapy is controversial, especially in high-risk women, as it has been linked to an increased risk of breast cancer (20). While an elective oophorectomy procedure may correlate negatively with life expectancy in women at average risk, the procedure remains beneficial for women at increased risk (21). Despite the possible negative physiological and psychological impacts of prophylactic oophorectomy, women at increased risk for developing HGSC report less anxiety about developing the disease, which they believe compensates for undesirable side effects (16). The primary cause of depression reported amongst post-surgical women is due to sexual dysfunction (21). In addition, *BRCA* mutation carriers may also experience the fear of transmitting a hereditary disease to their children. Women with these reproductive concerns could choose to investigate alternative methods, including *in vitro* fertilization and screening of embryos via preimplantation genetic diagnosis in order to eliminate the chance of transmitting faulty *BRCA* genes to their children (17).

NON-SURGICAL RISK-REDUCTION APPROACHES IN *BRCA* MUTATION CARRIERS

There are limited risk-reducing approaches for women with *BRCA* mutations who choose not to undergo prophylactic surgery (22). Current strategies include early breast cancer screening consisting of annual mammogram and breast MRI. In addition, gynecologic cancer screening consists of baseline transvaginal ultrasound and CA-125 serum level measurements followed by frequent monitoring using ROCA evaluation protocols. Breast cancer screening results in early tumor detection and a survival advantage; in contrast, ovarian cancer screening has yet to be associated with a significant reduction in mortality (23). For example, 63% of ovarian cancers detected were stage IIC or higher in a comprehensive study of over 6000 high-risk women (23). This can be partially attributed to the fact that current screening options are limited and not best suited to detect early-stage cancers (24). An alternative strategy involves the use of chemopreventive methods, including selective estrogen receptor inhibitors, tamoxifen and raloxifene, in addition to oral contraceptives (15). Oral contraceptives have indeed been shown to decrease the risk of developing ovarian cancer in the general population (25). However, the use of oral contraceptives in *BRCA* mutation carriers, while beneficial for ovarian cancer prevention, may be associated with an increased risk for developing breast cancers (26, 27).

DEVELOPMENT OF IMAGING TECHNOLOGIES AIMED AT EARLY DETECTION AND IMPROVING PATIENT OUTCOME

As mentioned above, the vast majority of both familial and spontaneous HGSC cases are diagnosed in advanced stages, at which point the prognosis is poor (28). Because there are few clear early symptoms, developing effective methods for early detection is paramount to improving long-term patient survival (29). Current methods of detection include non-invasive screenings using serum CA-125, ultrasound, sonography, CT, and MRI scans (30, 31). A laparoscopic procedure may also be used to provide an image of the lower abdominal organs, as well as attain a biopsy, which is necessary in order to confirm cancer diagnosis. These tools can give information regarding the size, composition, and location of the tumor and whether it has spread, which will be important for disease staging and treatment plans (28, 32). In terms of early detection, it is important to note that current imaging technologies are not able to distinguish precursor lesions inside the fallopian tube or ovary, two of the tumor initiation sites for HGSC. Novel *in vivo* imaging devices, such as confocal microlaparoscopes, which are instrumental in providing live images of abnormal regions and guiding biopsies, may be better equipped to assist with early tumor diagnosis (33). Images obtained by these probes in real-time during surgery have shown a clear distinction between normal and abnormal regions within the ovarian surface epithelium (33). Furthermore, studies using a flexible microlaparoscope have demonstrated the ability to provide high-resolution images of early stage cancer inside the fallopian tube in the intra-operative setting, thereby facilitating both an earlier

diagnosis and accurate disease staging (34). Since these procedures have shown merit in surgical settings, they may become a viable complementary option to traditional biopsies as future modalities of disease confirmation (33). Nevertheless, the specificity and sensitivity of this method have yet to be determined in clinical trials and there are also drawbacks associated with this procedure. For example, the image quality produced by the microlaparoscope is diminished when compared to traditional laparoscopes due to a smaller scope size, focal distance, and a reduced light output. Procedural complications may also arise from a lack of tight correlation between the movement of the instrument handle and instrument movement in the surgical field due to its increased flexibility (35). Other considerations include a reduced imaging depth, which may limit the ability to view cells below the tissue surface layer, as well as the development of non-toxic and non-mutagenic contrast-enhancing agents (33). Nevertheless, the device can successfully image organs *in vivo* without major complications (33) and needs to be carefully evaluated in future clinical trials.

Another widely used primary diagnostic tool for women seeking evaluation of a pelvic mass is transvaginal sonography (TVS). Traditional TVS technologies are being replaced by contrast enhanced transvaginal sonography (CE-TVS) and transvaginal color Doppler sonography (TV-CDS), which can better depict the tumor morphology by analyzing its microvasculature (31). In a study aimed at evaluating the diagnostic ability of contrast enhanced 3-dimensional power Doppler sonography (CE-3D) relative to conventional 3D Doppler sonography, the CE-3D technology showed 95.6% accuracy in distinguishing between benign and cancerous tumors compared to 86.7% accuracy for the conventional method (36). Contrast-enhancing agents coupled with transvaginal and Doppler sonography may merit further investigation as an early screening tool for women at risk. It is worth mentioning that the development of selective means for tumor imaging is critical not only for early detection but also for improving patient outcome (29). Thus, the use of selective intraoperative tumor imaging devices has the potential to both improve disease staging and enhance precision during cytoreductive surgery as it allows for better visualization of tumors (37). For example, a combination of functional *in vivo* and anatomical *ex vivo* X-ray micro-computed tomography (μCT) can provide a highly detailed three-dimensional analysis of the tumor micromorphology, vascularization, and accurately quantify relative blood volumes (rBV) in tumors, which can further inform treatment plans (38). Most importantly, the study has found a direct correlation between microvascular parameters (i.e., vessel size, the complexity of vessel branching) and tumor angiogenesis and aggressiveness (38).

Further advances have been made in ultrasound technology as well. Photoacoustic imaging is an emerging technology based on the photoacoustic effect, which is generated when tissues are pulsed with non-ionizing lasers. This results in a transient thermoelastic expansion and emission of an acoustic wave, which is detected by ultrasonic transducers and converted into images (39). A study evaluating biodegradable photoacoustic imaging agents in animal models of ovarian cancer found that cellulose nanoparticles produced high contrast signals. Interestingly, cellulose nanoparticles demonstrated a significant increase in signal when compared to gold nanoparticles, which are commonly used in photoacoustic imaging. Unfortunately, this imaging agent only proved to be biodegradable *ex vivo*. For the purpose of reducing toxicity and to facilitate clinical translation, further research will need to focus on nanoparticles that biodegrade within the mammalian circulatory system (40).

A strong emphasis is currently placed on evaluating imaging agents that are safe to use in patients and allow the visualization of early or recurrent tumors. Current research in ovarian cancer investigates the use of the folate receptor α (FR-α) and HER-2 as targeted agents for tumor-specific fluorescence imaging. Thus, FR-α is overexpressed in the majority of epithelial ovarian cancers, especially in HGSC tumors with a high risk of recurrence. Increased FR-α expression is detected not only in primary tumors but also in metastatic foci and recurrent tumors (41). Most importantly, chemotherapy does not appear to significantly alter FR-α expression in patient tumors (37, 41). In addition, FR-α-targeted fluorescent agents are able to selectively enhance imaging of tumor cells (37). PPF, an FR-targeted probe that is well suited for both PET and optical imaging was investigated *in vivo* in a trial using primary cell xenografts, *in vitro* with primary human ovarian cancer cells, and *ex vivo* with omentum removed from xenografts (42). PPF injected either intraperitoneally or intravenously was able to identify FR-positive primary HGSC tumors and their metastases to the omentum. As FR is overexpressed in HGSC, FR-targeted probes, such as PPF, may bear great utility in the clinical setting. This method could be ideal for guiding surgery due to the non-invasive, high-resolution, real-time images it produces (42). The unique features of this novel imaging tool may prove useful for ovarian cancer detection and monitoring. Furthermore, fluorescence imaging using FR-α-targeted agents could play a critical role during debulking surgery, as current methods of imaging are not tumor-specific.

Fluorescence imaging has been shown to detect a greater number of tumors when compared to conventional methods. Interestingly, a vinblastine folate-targeted drug, vintafolide, when used in combination with a diagnostic imaging tool, etarfolatide, may merit attention as a means to advance personalized treatments. Thus, etarfolatide imaging has been used successfully to select patients with FR-positive platinum-resistant ovarian tumors who may benefit from folate-targeted therapy (43). This novel combination of folate-targeted agents for imaging and treatment resulted in a marked increase in progression free survival for platinum-resistant patients. Based on the highly selective nature of this treatment, the drug efficacy was reported to be greater and its toxicity decreased compared to standard therapy (43). An alternative strategy for selective tumor imaging involves the use of the HER-2 biomarker, which is overexpressed in advanced HGSC cases (44). Imaging using HER-2-targeted magnetic iron oxide nanoparticles allows both magnetic resonance and optical imaging of peritoneal tumors when used in orthotopic ovarian xenograft models (45). This technology, which enables tumor imaging with high specificity and resolution, can be instrumental in drug delivery and image-guided surgery (44, 45). A third strategy involves the development of near-infrared fluorescence (NIRF) imaging technologies, which have been successfully tested *in vivo* in both pancreatic and ovarian cancer models as an alternative to ultrasound, CT, and MRI scans (46). Gene expression profiling was instrumental in

identifying proteases relevant to tumors, which further enabled the development of protease-specific NIRF probes. Such probes can provide not only a higher resolution for molecular-guided detection of early tumors but also the ability to distinguish between inflammation and cancer (46). Similarly, an alpha(v)beta(3-) integrin targeted NIRF probe was used successfully in ovarian xenograft models to optimize debulking surgery (47). Furthermore, the increased target to background ratio, high sensitivity (95%), specificity (88%), and diagnostic accuracy (96.5%) of this imaging system suggest that the NIRF-targeted platform is well suited for clinical translation and may be able to provide highly accurate images of small tumor lesions that are otherwise difficult to detect (47).

Plasma tumor biomarkers for the detection of early tumors and precursor lesions have been difficult to identify. CA-125 is presently used to help diagnose ovarian cancer, mainly for late stage cases, and to predict the chance of tumor recurrence (48). Nevertheless, CA-125 is not always a reliable biomarker for early stage tumors due to its lack of sensitivity (48). Large screening studies are currently underway to determine the sensitivity and specificity of a combined monitoring protocol using serum CA-125 levels and TVS for early diagnosis. The screening is based on an improved algorithm designed by Dr. Steven Skates to identify cancer risk based on rising trends in individual CA-125 levels (1). An alternative strategy involves the combined evaluation of multiple biomarkers in addition to CA-125. Research suggests that the addition of HE4, leptin, prolactin, osteopontin, insulin-like growth factor-II, CEA, and soluble vCAM cancer biomarkers to CA-125 serum surveillance protocols may result in a better diagnostic reliability when compared to CA-125 alone (1, 25). It is worth noting that recent studies suggest that a significant proportion of HGSC tumors originate from precursor lesions [serous tubal intraepithelial carcinoma (STIC)] located within the fallopian tube rather than the ovary, and this is the case especially in *BRCA* women (1, 49). Consequently, the development of serum screening tests and methods of diagnostic imaging need to include markers characteristic for the fallopian tube/fimbria in addition to the ovary. Currently, small early tumors within the fallopian tube cannot be detected via ultrasound or by measuring serum CA-125 levels (50). Comprehensive screening of such lesions through endometrial cytological testing may be a promising method for the early detection of HGSC in high-risk women and BRCA mutation carriers. Otsuka et al. reported that tumor cells shed from tubal STICs could be detected through careful examination of endometrial cytological samples (50). This would enhance the early detection rates for HGSC tumors as the occurrence of false positives through cytological testing appears to be low (50). Interestingly, this pilot study was able to detect malignant cells in five patients for whom imaging results were normal; three of them presented with no symptoms and were later diagnosed with early-stage HGSCs (50). In addition to further confirming the tubal site of origin for HGSC cases, the study also reported a 4-fold increase in the number of high-grade serous tumors being detected using this method when compared to other ovarian cancer subtypes (50). In contrast, the direct testing of cervicovaginal cytological samples yielded positive results in only one of five patients, suggesting that this is not an efficient means of detecting early-stage HGSC tumors.

GENERATION OF IMPROVED ANIMAL MODELS THAT CLOSELY RESEMBLE HGSC

In addition to aiding in the development of new imaging techniques, murine models of ovarian cancer are integral to drug development and disease pathogenesis studies (46, 51). While several genetically engineered mouse models (GEMM) have been previously developed for endometrioid ovarian cancer (52–54), clinically relevant models for HGSC have been difficult to generate. This could be due to model designs based solely on a traditional view of disease pathogenesis, such as the ovarian origin hypothesis. New clinical protocols, which involve the sequential sectioning and examination of the fimbrial (SEE-FIM) end of the fallopian tube pioneered by Dr. Christopher Crum, have identified precursor lesions for HGSC arising in secretory cells of the fallopian tube, namely p53 signatures and STICs (55–58). In addition, several groups have been instrumental in leading efforts for refining murine HGSC models. Recently, the first genetic model of *de novo* HGSC originating in fallopian tubal secretory epithelial cells (FTSEC) has been generated, which recapitulates key genetic alterations (*BRCA*, *TP53*, and *PTEN*) and precursor lesions (STICs) that are hallmarks of the human disease (59). In addition to offering mechanistic insight into the origin and pathogenesis of HGSC, this model provides a platform to explore tumor sensitivity to novel therapeutic agents and diagnostic imaging methods that include the distal fallopian tube in addition to the ovary (59). Recently, a second genetically engineered model of HGSC was described using the Ovgp-1 promoter to target SV40 large T-antigen-induced tumorigenesis in the fallopian tube (60). This model also displays neoplastic lesions of the fallopian tube that resemble human STICs and p53 signatures. The murine ovarian carcinomas have molecular characteristics that strongly resemble the human disease as well. Furthermore, gene expression analysis studies of Ovgp-1-driven tumors have identified a novel biomarker, topoisomerase II-alpha, which is overexpressed with mutant TP53 in both murine and human STICs and HGSCs but not in normal adjacent tissues (60). Most importantly, this model provides independent support for the hypothesis that HGSC may be primarily tubal in origin and mirrors the clinical progression of human HGSCs (61). The development of FTSEC-driven animal models will be instrumental in providing a platform to test newly emerging data from The Cancer Genome Atlas (TCGA) in an *in vivo* relevant system. The goal of the research community is to develop ovarian cancer models that recapitulate not only novel genetic/genomic alterations but also the histopathology and clinical behavior of HGSCs. Resolving the pathogenesis of HGSC and its precursor lesions will likely enable more efficient methods for early detection, tumor imaging, and cancer prevention. Furthermore, by using a combination of murine model studies and epidemiological data from patients, it will be important to determine if premenopausal women with *BRCA* mutations can be offered risk-reduction surgery in a multi-step procedure without undergoing surgical menopause and loss of fertility in their younger years.

As a complement to genetically engineered models, personalized patient-derived murine xenografts ("avatar mice") have been developed, which are able to more accurately predict tumor responses to therapy. Xenograft tumor models have been used

for decades to examine the behavior of various types of therapies within a living system (62). They can closely resemble the molecular and histological characteristics of the human cancer they are derived from (63, 64) and demonstrate clinical relevance by predicting the activity and effects of trial therapies (65). However, their predictive ability varies dramatically based on the cancer being studied, and multiple therapies, which tested well in xenografts, did not ultimately result in successful clinical trials (66–68). While traditional xenografts are created by generating and then engrafting established cell lines (51), a subcategory of xenografts produced by direct transfer of patient tumor tissue into immunocompromised mice (also known as explant xenografts or tumorgrafts) has mimicked the drug effects seen in humans much more closely than cell line xenografts (69). Furthermore, one study of personalized tumorgrafts involving a broad range of human-derived tumors and anticancer therapies demonstrated a positive clinical predictive value for drug resistance over 90% of the time (70). Unlike human cell cultures, which tend to result in increasingly homogenous populations with successive passages, direct tissue transplants more accurately represent the heterogeneous makeup and genetic diversity of the original tumor, including its relative cell proportions and overall genomic profile (71, 72). These tumor sections can be implanted orthotopically within the homologous source tissue in addition to being dispersed into the body cavity via intraperitoneal injection, as is the case for cell line xenografts (73, 74). Using these techniques, avatar models have been generated that closely recapitulate the tumor of a specific donor patient. This has led to the identification of personalized therapeutic regimens by creating a tailored stand-in for patient tumors prior to or alongside treatment (75). The use of tumorgraft testing surrogates, which are generated by using a specific patient's own tissue, has increased over the last decade. This trend was initiated by a recent study that described the use of xenograft technologies to create personalized tumorgrafts for a total of 14 patients with a variety of cancer types (76). The study results identified optimal, non-obvious treatment choices with a high rate of clinical success (76). This process has been performed with similar success in models of lung (77), pancreatic (78), prostate (79), breast (80, 81), and fallopian tube (82) cancers. In addition to being highly representative of the morphology and progression of human cancers, it was found that the success of tumor engraftment is by itself a prognostic indicator of disease outcome for women with newly diagnosed breast cancer (80).

While GEMM and xenografts both strive to generate accurate models of human disease and their usage at times overlaps, they have individual features best suited to distinct roles in cancer research. Tumors that develop from a xenograft retain the natural genetic alterations derived from the original source (63, 83). Conversely, GEM models must recreate these changes based on the result of investigation or hypothesis, and therefore they often cannot replicate the complexity and genomic diversity found in patient tumors (74). In cancers with high variance in molecular alterations between patients, tumorgrafts should be used to test therapies in models that more accurately represent individual tumors (77), since generalized results from GEMM studies will not be applicable. Personalized tumorgrafts have also been used to identify changes in drug resistance at specific stages of disease by grafting repeatedly from the same patient at different time points (63). Furthermore, avatar models could also allow the preemptive identification of new treatment strategies necessary when a patient develops resistance to clinically available therapies (84). Being able to determine tumor sensitivity and drug resistance for each individual patient upfront would allow oncologists to attempt experimental treatments with a higher probability of success while retaining conventional therapies as an option (85). In contrast, GEMMs have attributes superior to avatar models when it comes to studying the origin of the disease, precursor lesions, tumor progression, and the contribution of the immune system to cancer pathogenesis by allowing the inducible targeting of key genes in a tissue-specific manner in immunocompetent mice (86). There are several challenges in creating avatar mice relative to GEM and cell line xenograft models, including the need for surgical extraction of adequate tumor samples from the patient, ideally including accessory tissue (72), and a high rate of implantation failure (85). While tumor heterogeneity is represented, the microenvironment is typically not. This drawback, combined with the use of immunocompromised mice, restricts how similarly avatar models behave when compared to human disease. Such limitations could be overcome by incorporating recent xenograft advancements. The tumor microenvironment can be retained in a xenograft by grafting stroma alongside the tumor (75) and the use of "humanized" models preserve immune system functionality after engraftment (87). Besides technical concerns, there is a high financial barrier for creating new avatar lines (75). However, once tissues have been extracted and implanted, human tumors can be serially passaged in mice, archived, and later repropagated from tumor banks (82). The generation of tumor banks enables the repeated testing and study of patient tumors from a small number of original extractions.

Further development of representative mouse models of HGSC is an urgent need for the field (88) and tumorgrafts have attributes suited to this subtype. HGSCs are characterized by rapid metastasis (89) and tumorgraft models were reported to metastasize to regions similar to those seen in patients (90). HGSCs are also characterized by genomic instability (51) and tumorgrafts were shown to accurately retain the genomic profile of the original patient sample in animal models throughout multiple grafts (72). Avatar mice have been generated for fallopian tube carcinoma (82), which supports their viability for ovarian cancer, particularly in conjunction with the tubal origin hypothesis (91). This year, the first large-scale tumorgraft mouse study of ovarian cancer was published, consisting of 168 engrafted models from patient samples, which were representative of the entire spectrum of the disease (83). As in previous tumorgraft studies, the models closely resembled the patients they were derived from. The majority of models that developed ascites originated from patients with ascites, which is notable as the development of ascites is characteristic of HGSC (89). Consistent with prior comparisons between cell line xenografts and donor patient platinum sensitivity in ovarian cancer (92), all of the ovarian models tested for platinum sensitivity had the same type of response as the donor patient (83). It has been suggested that the tumor microenvironment may play a role in the high rate of relapse and increased drug resistance seen in HGSC (93). These ovarian tumorgrafts strongly

resemble the source microenvironment by inducing the formation of tumor stroma (83). As shown in a larger tumorgraft study (72), the ovarian models closely resembled the source patient's genomic alterations after engraftment and the grafts implanted in clinically relevant sites. Finally, just as demonstrated previously (80), the initial success of a graft tended to correlate negatively with patient survival (83). This extensive study demonstrates the feasibility of ovarian tumorgrafts as patient surrogates, particularly given the reasonably accurate representation of the disease diversity (94). Consequently, the generation of avatar mouse models for HGSC is expected to assist oncologists with establishing individual resistance profiles quickly in a surrogate model following biopsy and informing patients of therapy choices in real-time (85).

Avatar tumorgrafts have been found to be highly predictive models clinically and the generation of such models can aid with drug design on an individual patient basis (67, 76). The use of avatar models alongside patients in concurrent clinical trials has already been proposed (75), though it is noted that variance in how accurately these strains reflect human disease, including the contribution of the immune system, can be a confounding factor. In recent years, there has been renewed interest in harnessing the immune system to target cancers (95, 96), often achieved by blocking the inhibitors of immune reactions elicited by tumor cells. This approach has led to considerable success, such as the use of ipilimumab immunotherapy in metastatic melanoma (96). Ipilimumab is a monoclonal antibody against CTLA-4 and promotes effective antitumor targeting by cytotoxic T lymphocytes. Therapies targeting CTLA-4 have also been investigated in ovarian cancer; a combined treatment, which involves blockade of CTLA-4 and PD-1 and boosts the immune system, was found to induce tumor rejection in 75% of tumor models examined (97). In addition, the endothelium of many tumors may function as a primary defense against immune system activation by creating a physical protective barrier for the tumor and resisting immune cell invasion (98). It remains to be seen whether the development of effective immunotherapies will increase the efficacy of conventional therapies and achieve durable remissions in patients (96). Notably, it was recently demonstrated that traditional therapeutic regimens could be modified to effectively recruit the immune system in the setting of platinum-resistant relapsed disease (6). While platinum and paclitaxel are often delivered at maximum tolerable doses, the dose-dense chemotherapy study demonstrates that delivery of conventional drugs in low doses within frequent intervals can enhance natural antitumor immune responses and reduce immunosuppression, thus leading to increased treatment efficacy (6). Thus, a dose-dense chemotherapy regimen was successful in promoting the antitumor $CD8^+$ T-cell response in both mouse models and patients and reduced the tumor ability to suppress the immune system. This bodes well for further optimization of such treatments for individual needs, particularly as an extended weekly, dose-dense carboplatin and paclitaxel regimen has been shown to be effective in heavily pre-treated, recurrent, platinum-resistant ovarian cancer patients (99). Clearly, optimizing these treatments for patients requires a clinically translatable graft model. Xenograft models with an implanted functional human immune system have been previously developed to investigate viral and immune disease (100). Interestingly, such models were shown to be functional in cancer as well (87). A key benefit of GEMMs over xenografts has always been that they retain normal immune function (51). However, a xenograft with a humanized immune system, which is interacting with human tumor cells, may be more translatable to designing immunotherapies for patients based on individual needs. While GEMMs may ultimately prove more useful than avatar models for understanding the intricate details of HGSC origin and progression, personalized tumorgrafts will be key for the design of individual therapeutic regimens.

CONCLUSIONS

Advances in tumor screening and imaging may help determine the optimal time to employ risk-reducing surgical approaches in women at high risk for HGSC, including *BRCA* mutation carriers. Prophylactic surgery offers the most significant reduction in the risk of developing breast and ovarian cancers, as current surveillance methods are not effective enough to lend support for ovarian conservation in premenopausal women at high risk. Bilateral salpingo-oophorectomy is, however, associated with multiple physiological and psychosocial side effects that may contribute to a decrease in the quality of life and a loss of fertility in younger women. Consequently, the use of endometrial cytological testing in high-risk women or improved *in vivo* imaging devices (i.e., confocal microlaparoscopes, photoacoustic imaging, μCT, and contrast enhanced 3D Doppler sonography) could prove to be more effective for both early detection and treatment. In addition, alternative strategies for tumor-specific imaging, which involve the use of FR-α-targeted fluorescent agents, HER-2-targeted magnetic iron oxide nanoparticles, or protease-specific NIRF probes, merit further investigation as selective tools for early tumor detection, monitoring, and image-guided surgery. It is worth noting that animal models are a valuable tool in ovarian cancer research for the purpose of developing and testing novel imaging technologies, biomarkers, and experimental treatments. However, there are currently a limited number of animal models available for HGSC. Advances in tumor xenograft technologies have enabled the development of personalized avatar mouse models, which have emerged as an ideal drug-testing platform, especially in concurrent clinical trials. Additionally, tumorgrafts appear to have qualities well suited to model HGSC. We suggest that avatars have the potential to improve patient outcome and quality of life by reducing the cost and toxicity of ineffective imaging and treatment.

AUTHOR CONTRIBUTIONS

Anders W. Ohman, Noor Hasan, and Daniela M. Dinulescu wrote the manuscript.

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The role of the tumor stroma in ovarian cancer

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The tumor microenvironment, consisting of stromal myofibroblasts, endothelial cells, and leukocytes, is growingly perceived to be a major contributor to the pathogenesis and disease progression in practically all cancer types. Stromal myofibroblasts produce angiogenic factors, proteases, growth factors, immune response-modulating proteins, anti-apoptotic proteins, and signaling molecules, and express surface receptors and respond to stimuli initiated in the tumor cells to establish a bi-directional communication network in the microenvironment to promote tumor cell invasion and metastasis. Many of these molecules are candidates for targeted therapy and the cancer stroma has been recently regarded as target for biological intervention. This review provides an overview of the biology and clinical role of the stroma in ovarian cancer.

Keywords: ovarian carcinoma, stromal myofibroblasts, metastasis, tumor progression, prognosis

INTRODUCTION

Cancer is characterized by uncontrolled cell growth due to the combined effect of growth-promoting and cell death-suppressing signaling. Tumor growth and progression in carcinomas characteristically involves a pre-invasive phase, followed by invasion of the surrounding stroma, entry into blood and lymphatic vessels, and metastasis. It is growingly perceived that all these phases require cross-talk between tumor cells and their microenvironment, which consists of immune system effectors, endothelial cells, and stromal myofibroblasts. The latter cell population, often referred to as cancer-associated fibroblasts (CAF), has a particularly important role in tumor biology, due to its ability to dynamically modify the composition of the extracellular matrix (ECM), thereby facilitating invasion and subsequent metastatic colonization, and to produce and secrete tumor-promoting factors (1–3). This has impacted on the development of therapeutic strategies designed at targeting stromal myofibroblasts in cancer (4).

Ovarian cancer, the most lethal gynecologic malignancy (5), is a heterogeneous group of malignant tumors, of which ovarian carcinoma (OC) is the most common one. The common histological types of OC – serous, endometrioid, clear cell, and mucinous carcinoma, are distinct morphological entities that are growingly perceived to be of different etiology, with unique genetic and phenotypic characteristics and different clinical behavior, including response to chemotherapy (6). OC patients are diagnosed with advanced-stage disease in the majority of cases, and despite aggressive surgery combined with platinum-based chemotherapy often succumb to their disease, primarily due to chemoresistance in recurrent tumors (7).

As in other cancers, the OC stroma produces and expresses myriad molecules relevant for tumor biology, and the mere presence of a large stroma component in OC was reported to be associated with poor survival in advanced-stage disease (8). This review summarizes current data regarding the expression and clinical relevance of molecules related to the cancer microenvironment in OC stromal cells. Data related to the immune system or to the tumor vasculature are not discussed. Studies of areas which remain controversial, such as the role of mesenchymal stem cells in OC biology, are similarly not the focus of this paper.

PROTEASES

Proteases are critical mediators of invasion and metastasis and are the cancer-associated molecules which have been most frequently studied in the OC stroma. Studies have predominantly focused on the matrix metalloproteinase (MMP) family, but a significant number of papers have focused on urinary-type plasminogen activator and cathepsin D.

Matrix metalloproteinases are a family of at least 23 membrane-bound (MT-MMP) or secreted zinc-dependent endopeptidases involved in invasion, tumor growth, inflammation, and angiogenesis. MMP family members share several domains, including a signal peptide required for secretion, a propeptide which keeps the enzyme latent, catalytic domain, and hemopexin-like domain, the latter required for binding tissue inhibitors of metalloproteinases (TIMP) and MMP activation. MMP-2 (Gelatinase A, 72 kDa type IV collagenase) and MMP-9 (Gelatinase B, 92 kDa type IV collagenase) additionally contain a collagen-binding area adjacent to their catalytic domain. In addition to ECM molecules, MMP substrates include proteases (other MMPs, plasminogen), growth factors (transforming growth factor; TGF), tyrosine kinase receptors (epidermal growth factor receptor, fibroblast growth factor receptor; EGFR, FGFR1), adhesion molecules (CD44, E-cadherin, αV integrin), chemokines, and the metastasis inhibitor KISS-1. MMPs are negatively regulated by various proteins, including TIMP-1–4, α2 macroglobulins, thrombospondins, and RECK. However, MMP-2

activation requires the formation of a complex with TIMP-2 and MT1-MMP (MMP-14) (9–11).

Collagen I and an anti-β1 integrin antibody induced activation of proMMP-2 in OC-derived fibroblasts *in vitro* (12). OC cell lines implanted in the peritoneal cavity of mice lacking the MMP-9 gene had fewer and smaller tumors than cells injected into mice with wild-type MMP-9 (13). MMP-2, MMP-9, MT1-MMP, and MT2-MMP were detected in the mouse stroma in animals inoculated with OC cells, but only MMP-2 and MT1-MMP levels were increased compared to normal mouse ovaries. Stromal expression of these molecules was unrelated to metastasis, the latter being rather related to tumor MT1-MMP levels (14).

The presence of stromal MMP-1, MMP-2, MMP-9, MT1-MMP, and TIMP-2 mRNA and/or protein has been shown in multiple studies of clinical OC specimens (15–35). However, the clinical significance of MMP and TIMP expression in the OC stroma remains controversial. In analysis of 90 primary OC, MMP-2, MMP-9, and MT1-MMP protein expression in stromal cells by immunohistochemistry (IHC) was significantly related to advanced-stage disease and poor disease-specific survival (DSS). Stromal MMP-9 and MT1-MMP were independent prognosticators in multivariate analysis (28). Higher stromal MMP-9 protein expression was similarly related to poor DSS in univariate, though not multivariate, analysis in another study (31). Stromal MMP-2 protein expression was related to shorter overall and disease-free survival (OS, DFS) in endometrioid, but not in serous OC in a third report (27). In contrast, in a smaller study of 33 OC, absence of MMP-2 from the OC stroma was associated with more aggressive disease (20). TIMP-2 mRNA expression in stromal cells of both primary OC and OC metastases was associated with poor outcome in univariate analysis, whereas the presence of MT1-MMP mRNA in stromal cells in metastases correlated with significantly longer survival. The association between stromal TIMP-2 mRNA expression in primary carcinomas and poor survival retained its significance in a multivariate analysis. Stromal MMP-2 and MMP-9 mRNA expression in primary or metastatic disease was unrelated to survival (19). In contrast, stromal TIMP-2 protein expression was significantly related to better chemoresponse and longer progression-free survival (PFS) and OS in analysis of 43 tumors (33).

Stromal expression of MMP-2 (30–32, 34), MMP-7 (34), MMP-9 (34), MMP-11 (32), MT1-MMP (34), TIMP-1 (34), and TIMP-2 (34) proteins was unrelated to survival in several studies.

The glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN; CD147) is member of the immunoglobulin superfamily of adhesion molecules, which stimulates the synthesis of several MMPs and binds MMP-1 and integrins on the surface of tumor cells.

Extracellular matrix metalloproteinase inducer was detected in tumor cells in primary OC, solid metastases, and malignant effusions in OC, as well as in stromal cells and endothelial cells. In solid lesions, EMMPRIN mRNA by *in situ* hybridization (ISH) was significantly co-expressed with β1 integrin mRNA in stromal cells. In survival analysis, EMMPRIN protein expression in stromal and endothelial cells of primary carcinomas correlated with poor survival (36).

Extracellular matrix metalloproteinase inducer protein expression by immunofluorescence was found in both tumor and stromal cells in a study of 120 primary OC and 40 intraperitoneal metastases. The monocarboxylate transporters MCT1 and MCT4, reported to be associated with EMMPRIN expression and drug resistance, were additionally detected in these specimens (37).

Urokinase-type plasminogen activator (uPA) is a serine protease that is synthesized as a latent pro-enzyme and activated by several proteases, including plasmin, cathepsins B and L, and kallikreins (KLKs). uPA and its homolog tissue-type PA (tPA) cleave plasminogen to plasmin, thereby mediating degradation of fibrin and other ECM proteins and the activation of several MMPs, as well as growth factors such as basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and TGF-β. The uPA receptor uPAR additionally binds ECM proteins and integrins. The plasminogen activator inhibitors PAI1 and PAI2 and the plasmin inhibitor α2 antiplasmin negatively regulate this system (38, 39).

Analysis of uPA mRNA and protein expression in 57 ovarian tumors and 8 abdominal metastases showed expression of uPA mRNA in epithelial cells in benign and borderline tumors, whereas poorly differentiated primary OC and metastases of different histological grade had predominantly stromal expression. In contrast, uPA protein expression was seen in both compartments (40). In another paper by this group, increased expression of uPA, uPAR, and PAI1 mRNA was found in poorly differentiated primary OC with solid growth pattern and in metastases compared to cystic, better differentiated tumors (41). Protein expression of uPA and uPAR, as well as several MMP members, was frequently seen in the OC stroma in both primary carcinomas and metastases, though uPA and uPAR were absent in the stroma of well-differentiated tumors (42). In a murine OC model, $uPAR^{-/-}$ mice lacking uPAR in host mesothelial cells had reduced tumor and ability to form peritoneal metastases, as well as reduced ascites formation and longer survival compared to $uPAR^{+/+}$ mice. In clinical specimens, higher stromal uPAR protein expression was seen in OC compared to normal ovaries, with higher expression associated with higher histological grade (43).

The ETS family of transcription factors regulates the transcription of a large number of cancer-associated molecules, including uPA, uPAR, MMP-7, and MMP-9, as well as the apoptosis inhibitor Survivin, the tumor suppressor Maspin, the cell cycle protein p21/CIP1, and Slug, mediator of epithelial-to-mesenchymal transition (EMT), thereby affecting many cellular processes, including angiogenesis, invasion and metastasis, and cell survival (44).

Ets-1 mRNA is co-expressed with MMP-1 and MMP-9 mRNA in the OC stroma (22). In analysis of 66 primary and metastatic OC from long-term and short-term survivors, Ets-1 mRNA was detected in stromal cells in 33% of cases using ISH (**Figure 1**), more often in tumors of short-term survivors, and was co-expressed with vascular endothelial growth factor (VEGF) mRNA. Ets-1 mRNA expression in both tumor and stromal cells was associated with poor survival in univariate analysis, and expression in stromal cells was an independent prognostic factor in a multivariate analysis (45).

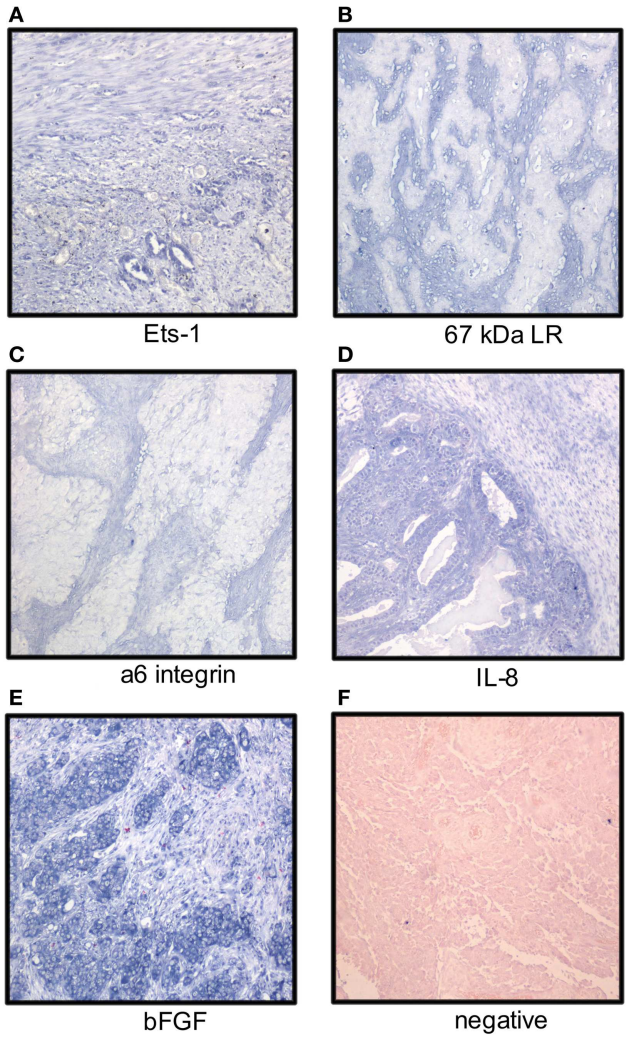

FIGURE 1 | Localization of mRNA of cancer-associated molecules to the ovarian carcinoma stroma. OC stromal cells express mRNA of the Ets-1 transcription factor **(A)**, laminin receptors **(B,C)**, and the angiogenic factors IL-8 and bFGF **(D,E)**; **(F)** negative control. Tumor cells express Ets-1, IL-8, and bFGF (NBT-BCIP as chromogen, nuclear fast red as counterstain).

In another study of the same cohort, the expression of PEA3, another Ets family member, was assessed using ISH. PEA3 mRNA was detected in stromal cells in 89% of tumors, but strong expression was limited to the stroma of grade 2–3 tumors. PEA3 mRNA expression in stromal cells was significantly related to MMP-2 mRNA expression in carcinoma cells, whereas PEA3 expression in carcinoma cells was significantly related to mRNA expression of the β1 integrin subunit, bFGF, and EMMPRIN in stromal cells. PEA3 mRNA was detected significantly more often in both carcinoma and stromal cells in tumors of short-term survivors and PEA3 expression in stromal cells correlated with shorter DFS and OS in univariate and multivariate survival analysis (46).

The clinical role of cathepsins, another family of proteases, was investigated in several studies. The level of cathepsin D, a lysosomal aspartyl protease, measured by immunoradiometric assay in OC tissue homogenates, was unrelated to clinical parameters or survival, with similar results for protein expression in tumor and stromal cells by IHC (47). In a study limited to stage III tumors ($n = 185$), tumor cell cathepsin D expression was related to longer OS in univariate analysis, with no such role for stromal expression. However, combined epithelial and stromal expression was an independent prognostic factor in multivariate analysis (48). No association was found with PFS. In contrast, cathepsin D expression in stromal cells was an independent prognostic factor of longer DFS, but not OS, in IHC analysis of 80 OC, with no prognostic role observed for tumor cell expression (49).

Cathepsin B, a cysteine protease, and the cysteine protease inhibitor cystatin C were detected in OC cells and their stroma, and were absent in cystadenomas (50).

Tissue KLKs are a family of 15 serine proteases encoded by a single gene cluster located at chromosome 19q13.4. Analysis of KLK4 expression in 43 primary and 63 metastatic OC showed stromal KLK4 expression in 48/103 specimens, which was significantly higher in primary tumors compared to metastases, with no prognostic role for this protein (51).

ECM PROTEINS AND THEIR RECEPTORS

The ECM composition in OC and its clinical relevance has been the subject of several studies.

Analysis of mRNA expression of the proα1(I) and proα2(I) chains of type I procollagen and of the proα1(III) chain of type I procollagen by ISH demonstrated their localization to the OC stroma, whereas expression was weaker or absent in the stroma of benign cysts. In poorly differentiated carcinomas ($n = 2$), signals were additionally detected in tumor cells (52). Differences in the density of collagen type I fibers were observed between cystadenomas, borderline tumors, and OC of different histological grade in another study (53). Oncofetal fibronectin was detected in the OC stroma, but not in endometriosis, suggesting this protein was selectively expressed by the tumor microenvironment (54). Fibulin-1, an estrogen-regulated calcium-binding and acidic ECM glycoprotein, was localized to the OC stroma, with strongest expression in proximity to tumor cells, and its mRNA was localized to the latter compartment. Staining increased from normal ovaries through benign and borderline tumors to OC, and was associated with progesterone receptor, but not estrogen receptor expression (55).

Analysis of the expression pattern of laminin γ2 chain in mucinous ovarian tumors with gastrointestinal differentiation by IHC showed basement membrane localization in adenomas, borderline tumors, intraepithelial carcinomas, and adenocarcinomas with expansile growth pattern, whereas expression was cytoplasmic or stromal in carcinomas growing with infiltrative pattern (56). Stromal expression of laminin-5 γ2 chain with concomitant presence of MT1-MMP on the tumor cell surface was reported in clear cell OC (57). Galectin-1, a laminin-binding protein regulating tumor cell proliferation and adhesion to matrix, was overexpressed in OC compared to normal ovaries and co-localized with laminin-1 and fibronectin. Its levels were increased in fibroblasts cultured with OC cells *in vitro* with effect on tumor cell proliferation and adhesion (58). Analysis of the expression of two laminin receptors, the 67-kDa laminin receptor precursor (LBP) and the α6 integrin subunit, in 41 primary OC and 75 solid metastases showed

mRNA expression by ISH in stromal cells in 68 and 20% of cases (**Figure 1**), respectively. No association with clinicopathologic parameters or outcome was found (59).

Analysis of additional integrin subunits in primary OC and solid metastases showed stromal expression of the β1 integrin subunit mRNA by ISH in 2 independent tumor series, whereas the αV subunit mRNA was found in the stroma in only one of the series. While tumor αV subunit mRNA expression was associated with poor survival in one of these studies, the presence of these subunits in stromal cells had no prognostic value (60, 61).

The mRNA expression of angiogenic cytokines and growth factors was analyzed in two studies. bFGF, interleukin-8 (IL-8), and VEGF mRNA was expressed in both tumor and stromal cells with no significant difference between primary carcinomas and metastases. bFGF was the most strongly and frequently expressed transcript in primary OC and in solid metastases in both series, with intermediate expression of IL-8 and low expression of VEGF (**Figure 1**). None of these factors was related to clinicopathologic parameters or disease outcome (62, 63). In another series, IL-8 mRNA expression was higher in tumor compared to stromal cells in OC specimens, whereas the protein was expressed in both compartments. IL-8 receptor B, but not A, was expressed in stromal cells (64). In a study of FGF-8 expression in OC, this cytokine was localized to tumor cells, whereas its receptors FGFR1, FGFR2, and FGFR4 were expressed by tumor cells, and to lesser extent, in stromal cells (65).

Hyaluronan (also termed hyaluronic acid or hyaluronate; HA), a large, linear, negatively charged polysaccharide with strong capacity to attract water, maintains tissue hydration and osmotic balance under normal condition. It additionally regulates cell adhesion, migration, apoptosis, and proliferation via interaction with specific cell surface receptors, which include the adhesion molecule CD44. HA has been shown to be involved in tumor progression of multiple cancers, through its effect on the above processes, as well as angiogenesis, invasion, and EMT (66).

HA is expressed in the stroma of both stage I and stage III OC, and its expression is increased in peritoneal metastases from patients with stage III disease compared to primary carcinomas (67). Analysis of 309 primary OC showed significant association between stromal HA expression and high histological grade, serous histology, advanced-stage and large residual disease volume, with no relationship to tumor cell CD44 expression. High stromal HA expression was further significantly related to poor relapse-free survival (RFS) and OS, and HA was more highly expressed in 45 patient-matched metastases additionally studied (68). Allelic imbalance at chromosome 3p21.3, a region harboring the hyaluronidase genes *HYAL1-3*, was found in microdissected tumor and stromal cells of borderline tumors and OC (69).

The unique stroma of clear cell OC was reported to contain both HA and collagen type IV, and these components were involved in its formation or modification (70, 71).

Proteoglycans, composed of a core protein to which glycosaminoglycan chains are attached, are a family of highly conserved macromolecules localized to the cellular membrane or the ECM. Proteoglycans are expressed by multiple cancers and mediate angiogenesis, tumor growth, invasion, and metastasis (72, 73).

Davies et al. analyzed the expression of syndecan-1–4, glypican-1, and perlecan in 147 ovarian specimens, including 115 OC, using IHC. Syndecan-1 was expressed in tumor and stromal cells of benign ovarian tumors, borderline tumors, and OC, with most intense staining in areas of invasion in OC, and was absent in normal ovaries. Syndecan-2 and -3 and glypican-1 were expressed in the stroma of all types of specimens, as was true for syndecan-4 in epithelial cells. Stromal perlecan expression was frequently seen in benign tissue and borderline tumors, but was lost in 67% of carcinomas. Stromal syndecan-1 expression was significantly associated with poor PFS and OS, though not independently (74).

In another study, stromal syndecan-1 and versican expression were associated with advanced-stage, serous histology, massive ascites, positive peritoneal cytology, and sub-optimal cytoreduction, as well as poor PFS and OS, though not independently (75). Ghosh et al. reported on overexpression of versican in OC compared to normal ovaries, as well as in advanced-stage compared to early-stage disease. Stromal versican expression was associated with higher microvessel counts, platinum resistance, and poor PFS and OS in univariate analysis (76). In another study, stromal versican expression was related to non-mucinous histology, advanced-stage, and reduced 5-year survival rate (77).

Decorin protein was reported to be expressed by the OC stroma, whereas tumor cells were negative, despite the presence of its mRNA in both cellular compartments (78). Periostin was overexpressed in the OC stroma compared to borderline and benign tumors and its presence in OC was associated with advanced-stage, disease recurrence, and poor OS, the latter also in multivariate analysis (79).

TGF-β is a ubiquitous cytokine with a dual role as both growth suppressor and promoter, effects which are largely mediated by the stroma and immune system. TGF-β acts predominantly as tumor promoter in several cancer types, including OC, and is consequently under consideration as a potential therapeutic target (80).

Comparative analysis of TGF-β1 and latent TGF-β1 binding protein 1 (LTBP-1) expression in serous and mucinous OC and adenomas showed strong stromal expression of these proteins limited to the former group (81). Transcriptome analysis of microdissected tumor and stromal cells from OC specimens and TGF-β-treated normal ovarian fibroblasts recently identified versican as an upregulated gene in CAF, and versican expression was upregulated by TGF-β, with resulting activation of the NF-κB signaling pathway and increased levels of CD44, MMP-9, and the hyaluronan-mediated motility receptor (82). Chloride intracellular channel 4 (CLIC4) was shown to mediate conversion of fibroblasts to myofibroblasts following stimulation with TGF-β1 *in vitro* and was frequently expressed in the OC stroma (83). Expression of TGF-β in the stroma of primary and recurrent OC was reported in another study (84).

Protein expression of the βA-subunit of activin A, member of the TGF-β superfamily, which regulates migration and invasion during EMT, metastasis, and MMP expression, was increased in stromal cells from OC specimens compared to adenomas (85).

Stromal protein and mRNA expression of secreted protein, acidic and rich in cysteine (SPARC; a.k.a osteonectin), a

matricellular protein involved in angiogenesis and tumor invasion, was higher in OC compared to normal ovaries and borderline tumors. Tumor cells expressed SPARC protein, but not mRNA (86, 87).

Endothelins, mitogenic peptides with autocrine and paracrine effect, stimulated the growth of fibroblast cell lines isolated from ascites specimens of OC patients, and were found in both the tumor cell and stromal compartments in clinical specimens (88).

The platelet-derived growth factor receptors PDGFRα and PDGFRβ were expressed in stromal cells in 32 and 44% of OC in analysis of 170 tumors, but their expression was unrelated to clinical parameters or survival (89).

The granulin–epithelin precursor (GEP/progranulin/PC-cell-derived growth factor) is a 68-kDa secreted protein with several higher molecular weight forms due to glycosylation, most commonly of 88 kDa. GEP was shown to be a growth factor in OC (90). Analysis of 189 solid OC specimens (64 primary OC, 125 metastases) showed GEP expression in stromal and endothelial cells 52 and 67% specimens, respectively. Stromal GEP expression was significantly lower in metastases sampled during or following chemotherapy compared to chemo-naïve tumors, and the presence of GEP-positive stromal cells in untreated primary tumors correlated with worse OS (91).

Insulin-like growth factor-1 was detected in the OC stroma, with strongest expression around vessels, with less frequent and weaker expression in tumor cells (92).

TRANSCRIPTIONAL REGULATORS

HOX transcription factors constitute a large family of proteins that regulate embryogenesis and organogenesis via spatial cues, as well as by regulating apoptosis, proliferation, differentiation, motility, and angiogenesis. HOX members are differentially expressed in adult tissues and regulate the expression of cadherins, integrins, NCAM (CD56), and p53. Deregulation of HOX members has been shown in different cancers (93, 94).

HOXA7 was overexpressed in the tumor cell nuclei and in the stroma of clear cell OC compared to other OC histotypes, and expression was lowest in serous OC (95). HOXA9 expression in OC cells induced normal peritoneal fibroblasts and adipose tissue- and bone marrow-derived mesenchymal cells to develop CAF features, a process shown to be mediated by TGF-β2 upregulation of CXCL12, IL-6, and VEGF-A (96). HOXA10 expression in OSE cells stimulated interaction with the ECM proteins fibronectin and vitronectin, with omental mesothelial cells and fibroblasts (97).

DNA topoisomerase IIα (TOP2α), an enzyme involved in DNA replication, RNA transcription, chromosomal condensation, and mitotic chromatid separation, is the target of chemotherapeutic drugs such as etoposide and doxorubicin. Comparative analysis of primary and recurrent OC specimens showed reduced TOP2α expression in tumor cells in the latter group, whereas stromal expression was increased (98).

Vestigial like 3, a putative tumor suppressor, was expressed in high-grade serous OC cells, and to a lesser extent in stromal cells, in a series of 182 tumors, and higher stromal expression was associated with a trend for longer survival (99).

Nuclear expression of Snail1, one of the key regulators of EMT, was observed in tumor and stromal cells in 23 and 24% specimens, respectively, in a series of 74 OC. Snail1 expression was minimal in borderline tumors and absent in adenomas and normal ovaries. Snail1 tumor cell and stromal expression was unrelated to clinicopathologic parameters or survival (100).

Expression of two of four studied members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, reported initially to regulate adipocyte proliferation and differentiation, was observed in the OC stroma, whereas all four proteins (C/EBP-α, -β, -δ, and -ζ) were expressed in tumor cells (101).

Nuclear expression of adrenal 4-binding protein/steroidogenic factor-1 (Ad4BP/SF-1) and dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1 (DAX-1), nuclear receptor superfamily members involved in the regulation of steroidogenesis, was shown in stromal cells in OC. Enzymes involved in ovarian steroidogenesis, including steroidogenic acute regulatory protein (StAR), P450 side chain cleavage enzyme (P450scc), and 3-beta-hydroxysteroid dehydrogenase (3b-HSD) were detected in the stromal cell cytoplasm (102). Stromal protein expression of PPAR-β, another nuclear receptor superfamily member, was reduced in OC compared to borderline tumors, benign tumors, and normal ovaries, whereas expression of its target protein 3-phosphoinositide-dependent protein kinase 1 (PDK1) was limited to epithelial cells and increased in OC (103).

OTHER MOLECULES

Various molecules related to other biological pathways have been localized to the OC stroma and are discussed in this section.

IMMUNE RESPONSE EFFECTORS

Several studies have investigated the expression of molecules related to the immune response in OC stromal cells. Proteins reported to be expressed by stromal cells include IL-11 receptor (104), the pro-inflammatory peptide LL-37 and its precursor human cationic antimicrobial protein-18 [hCAP-18; (105)], lymphotoxin-β receptor and the chemokine CXCL11 (106), and CD277 (107), as well as IL-6, COX-2, and CXCL1 (108). The clinical role of these biomarkers in this cellular compartment remains to be established.

IL-1β was recently reported to suppress nuclear p53 expression in CAF. High IL-1β and its receptor IL-1R1 and low p53 expression in CAF were associated with poor OS. p53 knockdown in ovarian fibroblasts resulted in increased expression and secretion of IL-1β, IL-6, IL-8, VEGF, and growth-regulated oncogene-α (GRO-α) and increased tumor growth *in vivo* in a NF-κB-dependent manner (109). Induction of senescence in fibroblasts by GRO-α was previously reported to mediate tumor promotion in a previous study by the same group (110).

Ribonuclease-2 (RNASET2), an extracellular RNase expressed in the OC stroma, was shown to mediate recruitment of macrophages to the tumor microenvironment and its silencing enhanced tumor growth of OVCAR-3 cells *in vivo*. Genes altered following *RNASET2* silencing were involved in pathways related to the immune response and cell adhesion (111).

CELL CYCLE AND APOPTOSIS-RELATED PROTEINS

Protein expression of the cell cycle inhibitor p16 in stromal cells was reported to be associated with improved prognosis, whereas

the presence of this protein in tumor cells was a poor prognostic marker (112). Stromal expression of another cell cycle inhibitor, p27, was significantly reduced in OC compared to normal ovaries, as was the expression of lung resistance protein (LRP), a protein associated with multidrug resistance (MDR), whereas multidrug resistance protein (MRP) expression was not significantly different (113). Expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the death receptors DR4, DR5, and DcR1 was found in OC stromal cells (114). TRAIL was detected in the OC stroma in an additional study (115).

VARIOUS MOLECULES

The RNA-binding protein HuR and COX-2 were expressed in the OC stroma in 24 and 7% of specimens in a study of mucinous OC, with no clinical role observed for expression in this cellular compartment (116). Analysis of proteins related to the prostaglandin synthesis pathway using IHC showed expression of COX-2, microsomal prostaglandin E synthase-I (mPGES-I), and the prostaglandin E_2 receptors EP_1 and EP_2 to the OC stroma, particularly in tumors of higher histological grade (117).

Expression of the α, β, and π sub-types of the detoxification enzyme glutathione *S*-transferase was observed in the stroma of OC specimens and different benign tumors (118).

Somatostatin and its receptors sst_1, sst_2, sst_3, and sst_5 were expressed with variable frequency in OC tumor cells and in their surrounding stroma, as well as in the stroma of different benign conditions. Somatostatin was significantly co-expressed with sst_1, sst_2, and sst_5 in the stromal compartment in analysis of the entire cohort (119).

The serotonin receptors 5-HT1A, 5-HTA2, 5-HT2B, and 5-HT4 were expressed, to variable extent, in the stroma of normal ovaries, benign ovarian tumors, borderline tumors, and OC specimens, with 5-HT2B being the most expressed receptor (120).

Retinoic acid receptor-α was found in stromal fibroblasts, tumor-infiltrating lymphocytes, and OC cells in analysis of 16 tumors of serous or mixed histology (121).

Neural endopeptidase (CD10) was expressed in the stroma of serous borderline tumors and in OC of different histotype, whereas no staining was observed in mucinous borderline tumors, in benign tumors, and in normal ovaries (122).

Luteinizing hormone receptor mRNA expression analysis by RT-PCR and ISH was reduced in both tumor cells and the OC stroma compared to benign tumors, with intermediate levels for borderline tumors. Expression in grade 2–3 tumors was less frequent then in their grade 1 counterparts, and the receptor was absent in five analyzed metastases (123).

The expression of six different isozymes of aldehyde dehydrogenase, an enzyme implicated in stem cell biology in OC, was investigated in normal ovaries, adenomas, borderline tumors, and OC specimens. Stromal and tumor cell expression of several isozymes was found to differ between normal tissue and ovarian tumors, as well as between OC of different histotype (124).

Expression of class III β-tubulin was reduced, though not significantly, in the OC stroma following neoadjuvant chemotherapy in analysis of 22 paired tumors obtained pre- and post-chemotherapy. Tumor and stromal class III β-tubulin expression was associated with poor OS (125).

Graphical illustration linking molecules known to have biological association, including HA, bFGF, MMP members, uPA, ETS transcription factors, HuR, and HOXA is shown in **Figure 2**.

CONCLUDING COMMENTS

Ovarian carcinoma is a highly lethal cancer characterized by considerable heterogeneity across different histological sub-types, as well as within the same morphological entity. In order to achieve noticeable improvement in the outcome of this disease, better understanding of the microenvironment of this tumor at both the primary site and metastatic locations is critically in need.

The above-discussed papers provide compelling evidence regarding the synthetic capacity of CAF in OC and emphasize the cross-talk between tumor cells and the stromal compartment; the latter interaction recently demonstrated *in vitro* (126). They additionally highlight the fact that the clinical relevance of a given molecule may be different or even opposite when expressed in carcinoma cells or in stromal cells. Nevertheless, many of these studies constitute single reports of the expression and clinical role of a given molecule, which need to be confirmed in series from other institutions, preferably studies in which each of the histological types of OC is studied separately.

Recent studies have applied high-throughput technology to the identification of central regulatory pathways in OC fibroblasts, often following microdissection, which allows for analyses focused on the target cell population. Qiu et al. studied genome-wide copy number and loss of heterozygosity (LOH) in CAF isolated from 25 OC and 10 breast carcinoma samples using SNP arrays. LOH and copy number alterations were rarely observed (127). Microarray analysis of microdissected stroma from 24 OC identified 52 candidate genes related to PFS, of which early growth response 1 (*EGR1*) and FBJ murine osteosarcoma viral oncogene homolog B (*FOSB*) were validated in an independent series of 50 tumors and found to be independent prognostic markers of poor PFS (128).

The role of miRNAs in reprograming of normal fibroblasts into CAF through downregulation of miR-31 and miR-214 and upregulation of miR-155 was recently shown, and the chemokine CCL5 was identified as target of miR-214, suggesting a role in modulation of the tumor microenvironment (129).

Exosomes are 30–100 nm lipoprotein vesicles containing proteins, mRNAs, and miRNAs that are secreted from cells and present in most circulating body fluids (130). Exosomes from SKOV-3 and OVCAR-3 cells induced adipose tissue-derived stem cells to acquire characteristics of myofibroblasts, with activation of the TGF-β pathway (131).

Lili et al. studied the stroma of 45 OC by microarray analysis and found two distinct signatures for the stromal compartment, characterized by different pairs of receptors and ligands (132).

Many of the molecules discussed in this review are expressed by both tumor and stromal cells and thereby present the possibility to target both cellular components in order to maximize the tumor-suppressive effect. While clinical studies aimed at inhibiting some of these cellular targets, e.g., proteases and COX-2, have been largely disappointing, other pathways, particularly receptor

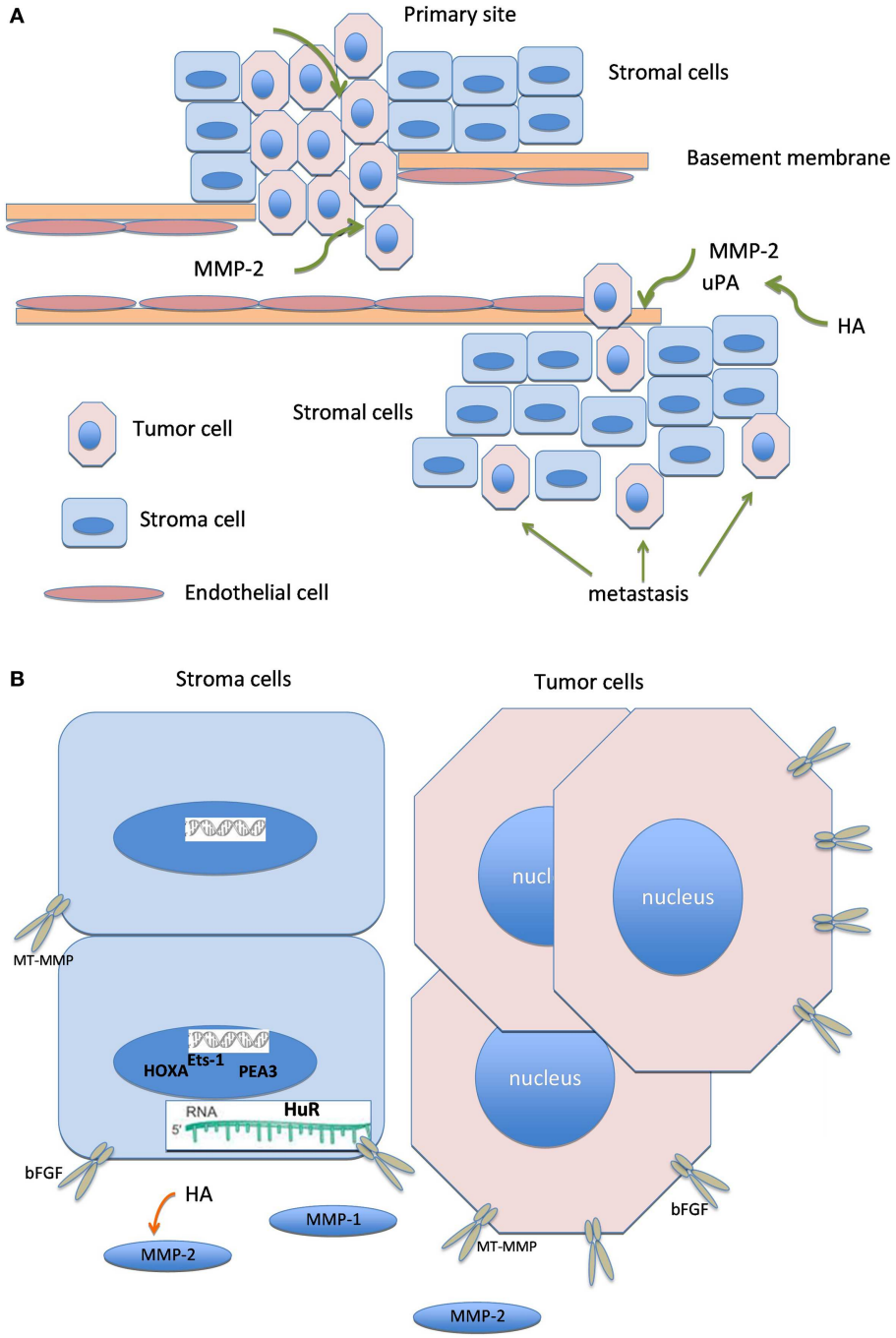

FIGURE 2 | Biologically linked cancer-associated molecules in ovarian carcinoma cells and the tumor stroma. Graphical illustration linking molecules known to have biological association in this cancer, including hyaluronic acid (HA), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP), urinary-type plasminogen activator, ETS transcription factors, HuR, and HOXA.

tyrosine kinase-driven pathways mediating angiogenesis and other tumor-related processes, are highly relevant (133, 134).

Therapeutic approaches are likely to focus to a larger extent on the tumor stroma in the future, as in the recent study by McCann and co-workers, in which inhibition of *Gli1*, part of the Hedgehog pathway, using the cyclopamine derivative IPI-926 in combination with chemotherapy was assessed (135). Whether such approaches could change the clinical course of OC is yet to be determined.

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Heterotypic cellular interactions in the ovarian tumor microenvironment: biological significance and therapeutic implications

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The majority of women who are diagnosed with epithelial ovarian cancer present with extensive peritoneal carcinomatosis and are rarely cured by conventional chemotherapy. Ovarian cancer cells typically disseminate by shedding into the peritoneal fluid and implant on the mesothelium-lined peritoneal surfaces that overlie connective and white adipose tissues. Emerging evidence indicates that ovarian tumor progression is orchestrated by dynamic interplay between tumor cells and a variety of stromal cells such as adipocytes, endothelial cells, fibroblasts, mesenchymal stem cells, macrophages, and other immune cells. This mini-review discusses the biological significance of the heterotypic cellular interactions in the ovarian tumor microenvironment and the therapeutic implications of targeting these interactions.

Keywords: ovarian cancer, tumor microenvironment, mesothelium, endothelial cells, adipocytes, fibroblasts, mesenchymal stem cells, macrophages

INTRODUCTION

The lethality of epithelial ovarian cancer primarily stems from late diagnosis. Women who are diagnosed with early-stage, ovarian-confined tumors have a 5-year survival rate of more than 90% (1). However, 60% of ovarian cancer patients present with advanced-stage, disseminated disease, and these women have a 5-year survival rate of less than 30% (1). Despite optimal tumor-debulking surgery and initial high response rates to platinum–taxane chemotherapy (70–80%), most patients with advanced-stage ovarian cancer relapse within 18 months (2). The biological behavior of ovarian cancer differs markedly from the hematogenous or lymphatic metastasis found for many other types of tumors. Ovarian cancer can initially progress by extending to adjacent pelvic tissues, but mainly disseminates by shedding into the peritoneal fluid, which transports tumor cells throughout the peritoneal cavity (3–5). These cells then implant on the surfaces of the cavity wall and abdominal organs. The omentum, a fat pad that extends from the stomach and suspends over the bowel, is the most frequently involved site (3–5). Seeding of the peritoneal cavity with tumor cells is often associated with ascites. It is increasingly recognized that progression of virtually all types of tumors is dynamically controlled by cross-talk between tumor cells and stromal cells (6, 7). As discussed below, the peritoneal cavity is a conducive environment for carcinomatosis, and the receptors and ligands that mediate interactions between ovarian cancer cells and stromal cells are candidate targets for new-generation therapies. This article is not intended as an exhaustive review of therapies, but provides an overview of the major cellular constituents of the ovarian tumor microenvironment, the complexity of their regulation, and focal points for therapeutic intervention.

Abbreviations: αSMA, α-smooth muscle actin; CAFs, cancer-associated fibroblasts; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; ECM, extracellular matrix; EGF, epidermal growth factor; FABP4, fatty acid-binding protein 4; FAP, fibroblast activation protein; FGF, fibroblast growth factor; GFP, green fluorescent protein; HA, hyaluronic acid; IL, interleukin; LIF, leukemia inhibitory factor; mAb, monoclonal antibody; M-CSF, macrophage colony stimulating factor; MMP, matrix metalloproteinase; MSCs, mesenchymal stem cells; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PLD, pegylated liposomal doxorubicin; TAMs, tumor-associated macrophages; TGF-β, transforming growth factor-β; TGFβRI, transforming growth factor-β type I receptor; TKI, tyrosine kinase inhibitor; Treg, T regulatory; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

MESOTHELIAL CELLS

Mesothelial cells are of mesodermal origin and form a protective monolayer that lines peritoneal, pleural, and pericardial surfaces (8). Interactions between ovarian cancer cells and peritoneal mesothelial cells are mediated by a variety of cell surface molecules (**Figure 1**). The ovarian cancer biomarker CA125 has been implicated in facilitating tumor cell implantation by its ability to bind mesothelin that is expressed by mesothelial cells (9). Gonadotropin-releasing hormone receptor signaling stimulates ovarian cancer cell attachment to mesothelial cells in part by inducing P-cadherin that is expressed in ovarian cancer cells and in mesothelial cells (10). Several integrins mediate attachment of ovarian cancer cells to mesothelial cells and/or facilitate tumor cell interactions with the submesothelial extracellular matrix (ECM) (11–15). Iwanicki and colleagues identified that spheroids of ovarian cancer cells displace mesothelial cells to gain access to the underlying stroma by using myosin-generated mechanical force that is dependent on α5β1 integrin and talin I (16). Mesothelial breach has also been found to be facilitated by

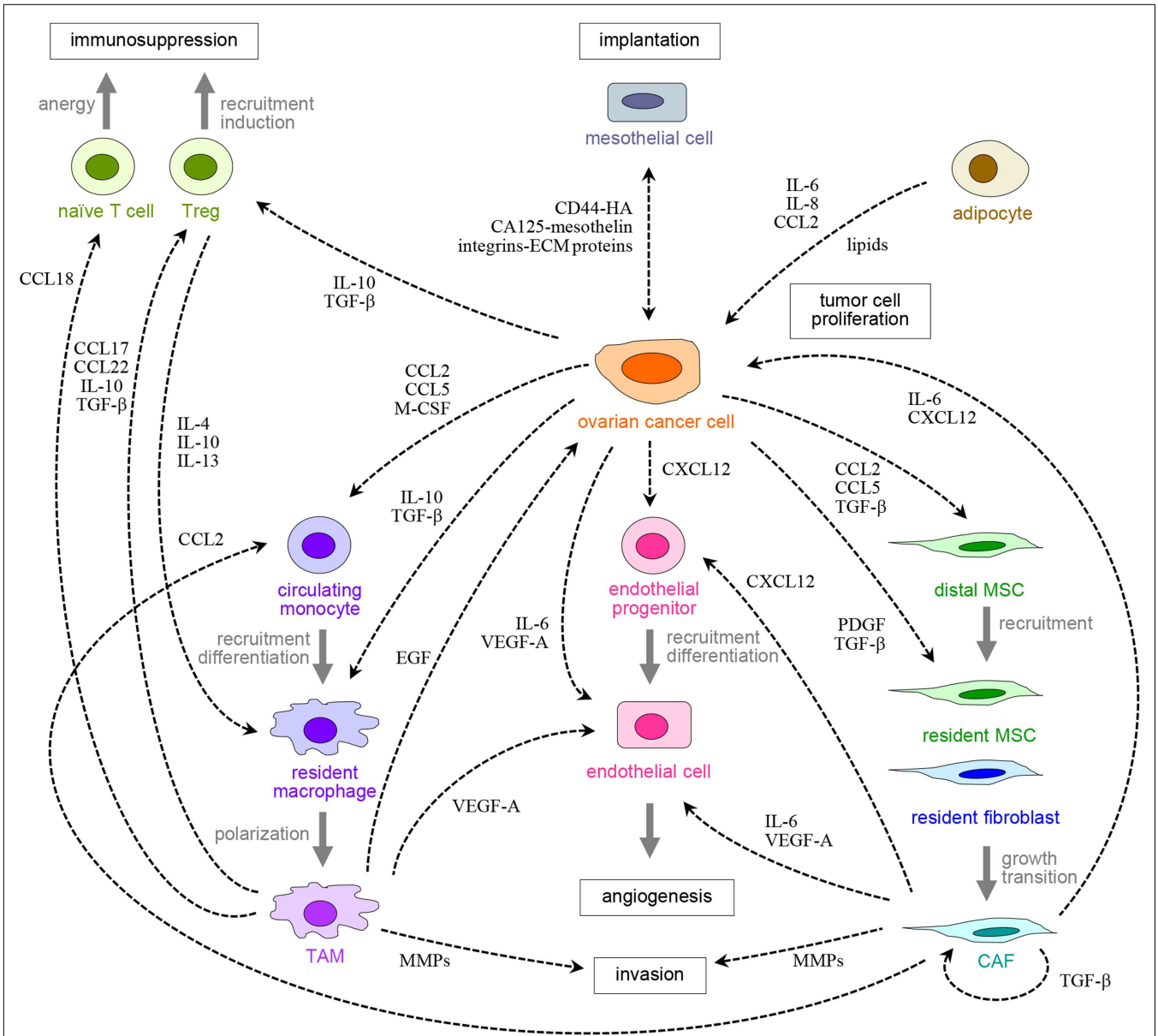

FIGURE 1 | Peritoneal carcinomatosis is orchestrated by cross-talk between ovarian cancer cells, resident peritoneal cells, and other host cells that are recruited to tumors. Examples of receptors and ligands that facilitate these reciprocal cellular interactions are shown.

CD157, a glycoprotein that is expressed in normal mesothelium and in 93% (82/88 cases) of ovarian cancers (17).

Because the mesothelium is the first point-of-contact for floating ovarian cancer cells at distal sites, targeting molecules that promote tumor–mesothelial interactions is a potential strategy to impede disease progression. Studies of the glycoprotein CD44 highlight several limitations of this approach. CD44 is expressed in ovarian cancers and binds hyaluronic acid (HA), a glycosaminoglycan that is synthesized by mesothelial cells (18). Strobel and colleagues found that treatment with neutralizing monoclonal antibody (mAb) to CD44 inhibited the number of peritoneal implants by 70% in ovarian cancer xenograft models, but did not reduce growth rates of tumors (19). Blocking tumor cell implantation alone might therefore not be therapeutically efficacious. Furthermore, neutralization of CD44 did not completely block implantation (19). Other studies have also shown that interactions between ovarian cancer cells and mesothelial cells are only partially inhibited by mAbs to a single adhesion molecule (13–16). In a study by Cannistra and colleagues, CD44 was detected in 94% (15/16 cases) of solid ovarian tumor tissues but in only 25% (2/8 cases) of ascitic tumor cells (18). To effectively block tumor cell implantation, it is likely that multiple adhesion molecules need to be targeted and these molecules need to be highly expressed on free-floating tumor cells.

ENDOTHELIAL CELLS

Tumor growth depends on the development of a neovasculature that supplies oxygen, nutrients, and growth factors. Increased angiogenesis as manifested by high tumor microvessel density has been found by several studies to be predictive of poor outcomes in ovarian cancer patients (20–22). Angiogenesis is a dynamic process orchestrated by pro- and anti-angiogenic factors that control recruitment of endothelial progenitors, growth and maturation of endothelial cells, and organization of endothelial cells into tubular structures (23, 24). Ovarian cancers express a variety of pro-angiogenic factors including the vascular endothelial growth factors (VEGF), fibroblast growth factor (FGF)-2, interleukin (IL)-6, IL-8, angiopoietin, and platelet-derived growth factor (PDGF) (25). Stromal fibroblasts and macrophages are also rich sources of pro-angiogenic factors (**Figure 1**). VEGF-A has emerged as the predominant pro-angiogenic factor in ovarian cancer (25) and is also the causative factor of ascites formation (26).

Agents that target VEGF signaling have been the focus of intensive clinical investigation in ovarian cancer. One major class of agents includes ligand inhibitors. Aflibercept is a fusion protein that combines the Fc portion of human IgG1 with the principal ligand-binding domains of VEGF receptor (VEGFR)-1 and VEGFR-2 (27). Aflibercept is generally well-tolerated, but the endpoint of a >5% response rate was not reached in a Phase II study of aflibercept in patients with recurrent ovarian cancer (28). Bevacizumab is a humanized mAb that neutralizes all forms of VEGF. Two phase II studies (GOG 170D and AVF 2949g) evaluated bevacizumab as a single agent in patients with recurrent ovarian cancer and reported response rates of 21.0 and 15.9%, respectively (29, 30). Combining bevacizumab with carboplatin and paclitaxel increased progression-free survival (PFS) by ~3.6 months as compared to standard chemotherapy alone in two phase III studies of patients with recurrent ovarian cancer (31, 32). Tyrosine kinase inhibitors (TKIs) are another class of agents that has attracted substantial interest. Sorafenib inhibits several receptor tyrosine kinases including VEGFR-2, VEGFR-3, PDGF receptor (PDGFR)-β, c-kit and Flt-3, and also RAF serine/threonine kinases (33). In a phase II trial of sorafenib, only 2 of 59 evaluable ovarian cancer patients had partial responses (34). Several TKIs that inhibit all three VEGFRs, both PDGFRs and also the FGF receptor have been undergoing clinical trials in ovarian cancer patients and are discussed in several recent articles (35–37).

ADIPOCYTES

Omental, mesenteric, and gonadal tissues are major repositories of visceral white adipose tissues and are frequently colonized by ovarian cancer cells (3, 4). Adipocytes (fat cells) are the predominant component of adipose tissue. Adipocytes promote proliferation of breast, colon, and prostate cancer cells and this stimulatory effect is mediated in part by the adipokine leptin (38–40). Leptin also stimulates ovarian cancer cell growth (41). The mechanism by which adipocytes promote ovarian cancer growth is a relatively new area of investigation. Nieman and colleagues identified that omental adipocytes secrete IL-6, IL-8, chemokine (C-C motif) ligand 2 (CCL2), and tissue inhibitor of metalloproteinases-1, and that mAbs to each of these factors inhibited chemotaxis of ovarian cancer cells toward adipocytes by at least 50% (42). Using co-cultures of omental adipocytes and ovarian cancer cells, the authors found that adipocytes stimulate tumor cell proliferation by directly transferring lipids to tumor cells (42). They also identified that fatty acid-binding protein 4 (FABP4), a lipid transporter, is more highly expressed in omental metastases than in primary ovarian tumors (42). Furthermore, the number of metastatic nodules that developed in a *Fabp4*-deficient orthotopic model of ovarian cancer was only 2% of the number of metastatic nodules that developed in the wild-type model (42). This elegant study demonstrated that adipocytes recruit ovarian cancer cells and support tumor growth through provision of energy (**Figure 1**), and raises the possibility that targeting lipid metabolism and/or trafficking could be a strategy to impede peritoneal growth and spread of ovarian cancer.

CANCER-ASSOCIATED FIBROBLASTS

Cancer-associated fibroblasts (CAFs) are a predominant component of the tumor stroma and have a profoundly negative impact on outcomes of cancer patients (7, 43). CAFs are often distinguished from normal fibroblasts by their expression of markers of myofibroblasts and activated fibroblasts such as α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP) (7, 43). CAFs derive from various cell types. Endothelial-to-mesenchymal transition has been identified as a source of CAFs in mouse models of melanoma and pancreatic cancer (44). CAFs can also derive from breast cancer cells that have undergone epithelial-to-mesenchymal transition (45). A study in which xenografts were generated from green fluorescent protein (GFP)-transfected ovarian cancer cells found that virtually all αSMA+ stromal cells lacked GFP, suggesting that CAFs did not derive from ovarian cancer cells (46). Tissue-resident fibroblasts are a major source of CAFs (43, 47) (**Figure 1**). Ko and colleagues demonstrated that ovarian cancer cells induce normal omental fibroblasts to express CAF markers and mitogenic factors such as IL-6 and chemokine (C-X-C motif) ligand 12 (CXCL12) that stimulated tumor cell proliferation (46). Overexpression of the patterning gene *HOXA9* increased the CAF-promoting ability of ovarian cancer cells by activating the expression of transforming growth factor-β2 (TGF-β2). In turn, TGF-β2 acted in a paracrine manner on omental fibroblasts and stimulated a TGF-β auto-regulatory loop in the stroma (46). Inhibition of ovarian cancer cell-derived TGF-β2 in xenograft models reduced the number of αSMA+ stromal cells in omental implants by 90% and the tumor mitotic activity by 75% (46). These findings support a model in which ovarian cancer cells "educate" omental fibroblasts to become permissive for tumor growth. Studies of Mitra and colleagues indicate that this programing is controlled in part by specific microRNAs. These authors identified differences in microRNA expression patterns in normal omental fibroblasts and in CAFs isolated from omental tumors, and demonstrated that altering expression of three microRNAs (miR-31, miR-155, miR-214) induces normal fibroblasts into CAFs (48).

Mesenchymal stem cells (MSCs) are adult stem cells that can differentiate into the osteogenic, myogenic, chondrogenic, and adipogenic lineages, and are another source of CAFs. Studies using animal models of ovarian cancer and other solid tumors have shown that bone marrow-derived MSCs home to tumors and transition into CAFs (49–51). White adipose tissues contain abundant MSCs that have multi-potency comparable to that of bone

marrow MSCs (52). Ovarian cancer cells induce normal adipose MSCs to acquire features of CAFs (46). Lysophosphatidic acid is abundant in ovarian cancer ascites and induces CAF features in adipose MSCs by stimulating TGF-β signaling (53). Because of the propensity of ovarian cancer to involve adipose tissue-rich peritoneal sites, adipose MSCs could be a significant source of CAFs in this disease. Normal cells that express CAF markers have been detected in omental tissues of ovarian cancer patients without overt omental metastasis (54). This raises the intriguing possibility that tumor-derived factors fertilize the omental "soil" before tumor cells implant.

Cancer-associated fibroblasts express many pro-angiogenic growth factors, ECM molecules, and matrix metallo-proteinases (MMPs) (7, 43). CAFs stimulate ovarian cancer cell invasiveness and the abundance of CAFs in ovarian cancers correlates with microvessel density (54). Omental fibroblasts that are stimulated by ovarian cancer cells have been found to secrete levels of VEGF-A and IL-6 that are, respectively, 5- and 10-fold higher than the levels secreted by unstimulated fibroblasts (46). A study by McLean and colleagues revealed that CAFs might drive ovarian tumor progression by expanding the cancer stem cell pool. These authors identified that propagating ovarian cancer cells with MSCs isolated from ovarian tumor tissues increased the number of cancer stem cells and that this enhancement was due in part to MSC-derived bone morphogenetic protein 2 (55).

Because CAFs express growth factors that stimulate tumor cell proliferation, metastasis, and angiogenesis (**Figure 1**), one strategy to inhibit the tumor-promoting ability of CAFs is to use agents that neutralize these growth factors. Another approach is to prevent normal fibroblasts and MSCs from transitioning into CAFs by inhibiting TGF-β signaling. A number of TGF-β inhibitors, such as ligand traps, antisense oligonucleotides, and TGF-β type I receptor (TGFβRI) kinase inhibitors, have been evaluated in pre-clinical and clinical studies (56, 57). Cai and colleagues found that treating mice with the TGFβRI inhibitor A83-01 reduced the abundance of αSMA+ stromal cells in ovarian tumor xenografts by 50% but did not increase survival (58). CAFs express PDGFRs (43) and could be inhibited by TKIs that target these receptors. Several studies have targeted the serine protease FAP. Depletion of FAP inhibited stromagenesis, tumor growth, and angiogenesis in mouse models of lung and colon cancers (59). A FAP mAb has been found to be well-tolerated but failed to show efficacy in a clinical trial of patients with colorectal cancer (60). A prodrug that consists of a FAP-specific peptide coupled to a cytotoxic analog of thapsigargin, induced stromal cell death in prostate and breast tumor xenografts and decreased tumor volumes by ~70% (61).

TUMOR-ASSOCIATED MACROPHAGES AND OTHER IMMUNE CELLS

Tumor-associated macrophages (TAMs) are the major immune component of the tumor stroma and derive from monocyte precursors that are recruited to tumors (6, 62–64). Ovarian cancer cells express factors that stimulate monocyte chemotaxis and maturation such as CCL2 and macrophage colony stimulating factor (M-CSF) (65, 66). Analogous to the Th1/Th2 dichotomy of T cell responses, macrophages exhibit polarized phenotypes in response to different signals. Stimulation of macrophages with microbial agents or interferon-γ induces an M1 phenotype that is characterized by expression of immunostimulatory cytokines. In contrast, stimulation with IL-4, IL-10, or IL-13 induces an M2 phenotype that is characterized by the expression of immunosuppressive cytokines (62, 63). It is widely recognized that TAMs exhibit an M2 phenotype and that normal macrophages are "educated" by tumor cells to transition into TAMs (62–64) (**Figure 1**). Macrophages polarize toward an M2 phenotype when stimulated with ovarian cancer ascites (67, 68). This polarization was initially attributed to IL-10 because ascites contain only low levels of IL-4 and IL-13 (62). However, IL-6 and leukemia inhibitory factor (LIF) are present at high levels in patient ascites and also induce differentiation of monocytes into TAMs (67). It has also been recently shown that ovarian tumor-derived TGF-β2 and CCL2 stimulate normal peritoneal macrophages to acquire features of TAMs (69).

TAMs are strongly associated with poor outcomes in cancer patients (64). Studies of breast cancer have revealed that TAMs are rich sources of epidermal growth factor (EGF), MMPs, and pro-angiogenic factors such as VEGF-A (70, 71). An important mechanism by which TAMs promote tumor progression is by suppressing adaptive immunity. TAMs have poor antigen presentation capability and highly express IL-10, TGF-β, CCL17, CCL18, and CCL22 (62, 63). IL-10 and TGF-β inhibit dendritic cell maturation and T cell proliferation (62, 63). CCL18 induces naïve T cell anergy and has been found to be the most abundant chemokine present in ovarian cancer ascites (72). CCL17 and CCL22 skew T cells toward a Th2 direction (62, 63). In a study of ovarian cancers, Curiel and colleagues identified that TAMs and also tumor cells produce CCL22, which mediated the recruitment of T regulatory (Treg) cells to tumors (73). Treg cells were found to contribute to ovarian tumor growth by suppressing tumor-specific T cell immunity and to be predictive of poor patient survival (73). Reciprocally, Treg cells can promote TAMs as Treg cells express IL-4, IL-10, and IL-13 that induce M2 polarization of macrophages (74) (**Figure 1**).

Targeting of TAMs is still in its infancy, but has a strong application to ovarian cancer because macrophages are abundant in ascites. One potential strategy is to "re-educate" TAMs toward a tumoridical M1 phenotype. Inhibition of NF-κB signaling in TAMs has been found to induce an M2-to-M1 switch and lead to regression of ovarian tumor xenografts (75). Another possibility is to inhibit Stat3, which is activated in macrophages that are polarized toward an M2 phenotype by ovarian cancer ascites (68). Because of its ability to stimulate monocyte chemotaxis and M2 polarization, CCL2 is an attractive target. Treatment of mice bearing metastatic prostate cancer with CCL2 mAb has been reported to inhibit the overall tumor burden by 96% (76). Trabectedin is a DNA-damaging alkaloid that has been found to also inhibit CCL2 and IL-6 production and to inhibit differentiation of monocytes into macrophages (77). Selective toxicity of trabectedin for TAMs has been demonstrated in ovarian cancer xenograft models and in patient specimens (77, 78). Trabectedin in combination with pegylated liposomal doxorubicin (PLD) has been approved in Europe for treatment of platinum-sensitive recurrent ovarian cancer. In a pivotal Phase III trial (OVA-301), the combination of trabectedin and PLD was found to be more effective than PLD alone for patients with platinum-sensitive recurrent disease, with a higher

response rate (35.3 vs. 22.6%) and increased PFS (median PFS 9.2 vs. 7.5 months) (79).

CONCLUSION

The studies to date have revealed that the peritoneal cavity is a highly receptive environment for carcinomatosis, and that progression of ovarian cancer is dynamically orchestrated by a complex network of receptor/ligand-mediated interactions between tumor cells, resident peritoneal cells, and other host cells that are recruited to tumors. Several of these receptors and ligands are targeted by agents that are in clinical use, while others are under clinical development. Because many of the ligands stimulate multiple cell types, a priority for future studies is to delineate the impact on different cell populations of neutralizing these ligands. In addition, the effects of inhibitory agents on ovarian cancer cells need to be evaluated in solid tumor tissues and also in free-floating tumor cells. Furthermore, determining the optimal combinations of stromal-targeting agents with conventional chemotherapy or other targeted therapies and the appropriate clinical setting for their use are key priorities for future studies.

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Tumor-associated macrophages contribute to tumor progression in ovarian cancer

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Ovarian cancer is the leading cause of death in women with gynecological malignancy and improvements in current treatments are needed. As with many other solid cancers, the ovarian tumor microenvironment is emerging as a key player in tumor progression and a potential therapeutic target. The tumor microenvironment contains several non-malignant cell types that are known to contribute to tumor progression and metastasis. Included in this population of non-malignant cells are several different types of immune cells, of which tumor-associated macrophages (TAMs) are the most abundant. An increasing amount of evidence is emerging to suggest that TAMs display a unique activation profile in ovarian tumors and are able to create an immunosuppressive microenvironment, allowing tumors to evade immune detection and promoting tumor progression. Therefore, an increased understanding of how these immune cells interact with tumor cells and the microenvironment will greatly benefit the development of more effective immunotherapies to treat ovarian cancer. This review focuses on the role of TAMs in the ovarian tumor microenvironment and how they promote tumor progression.

Keywords: ovarian cancer, tumor microenvironment, tumor-associated macrophages, macrophages in ovarian cancer

INTRODUCTION

Epithelial ovarian cancer (EOC) represents the leading cause of cancer mortality in women with gynecological malignancy (1). The overall 5-year survival rate for EOC patients is approximately 40%, although in the majority of patients diagnosed with advanced disease, the survival rate is significantly less (2, 3). Current standard treatment for EOC involves surgical debulking followed by platinum-based chemotherapy. Although initial response to chemotherapy is high, recurrence of chemoresistant disease is common and is a major contributor to the poor prognosis of EOC.

While much effort has gone into uncovering the genetic drivers responsible for EOC initiation and progression, the tumor microenvironment is now increasingly recognized to play an important role in EOC. The tumor microenvironment consists of several different cell types that interact with tumor cells, and with each other, to influence tumor initiation, growth, and metastasis. Immune cells represent a major component of the tumor microenvironment and allow tumor cells to evade immune destruction.

Evidence suggests that ovarian tumors, like many solid tumors, are immunogenic, containing tumor infiltrating lymphocytes that indicate an interaction between tumor cells and the host's immune system. In a seminal paper published by Zhang et al., the presence of CD3+ infiltrating T cells in tumors was shown to significantly increase long term survival in patients with advanced ovarian cancer (4). Five-year overall survival rates were 38% in patients that contained infiltrating T cells in their tumors compared to <5% in patients that contained no T cells. Furthermore, multivariate analysis showed that the presence of intratumoral T cells was an independent prognostic factor. Since then, several studies have confirmed the positive association between the presence of tumor infiltrating T cells and patient survival (5–8). The influence of intratumoral T cells on patient outcome indicates the immune system may play an antitumor role in ovarian cancer; however spontaneous regression of tumors through immune destruction is rare. In addition to cytotoxic T cells that display antitumor characteristics, ovarian tumors contain a plethora of other immune cell types that create an immunosuppressive tumor microenvironment. These include regulatory T cells (T_{regs}), dendritic cells, myeloid-derived suppressor cells, and tumor-associated macrophages (TAMs). TAMs represent the most abundant immune cell type in the ovarian tumor microenvironment and play several roles in promoting tumor progression. While all of these cell types have been shown to play an important role in ovarian cancer, some of which have been reviewed elsewhere (9, 10), this review focuses on the characteristics of ovarian TAMs and their role in ovarian cancer.

TUMOR-ASSOCIATED MACROPHAGES

Macrophages are phagocytic cells of the immune system that are derived from circulating monocytic precursors, which extravasate into tissues and differentiate in response to local signals. They represent a heterogeneous population of cells that can function to stimulate the immune system or to suppress it. This heterogeneity has been simplified to group macrophages broadly as either "classically activated" or "alternatively activated" (11). Classically activated macrophages, also known as M1-polarized macrophages, are activated by cytokines such as interferon-γ (IFNγ) and produce

pro-inflammatory and immunostimulatory cytokines (e.g., IL-12 and IL-23), and are involved in Th1 responses to infection. In contrast, alternatively activated, or M2-polarized macrophages, are activated by Th2 cytokines (e.g., IL-4, IL-10, and IL-13) are immunosuppressive, and are involved in scavenging cellular debris and tissue repair. In general, TAMs are thought to more closely resemble the M2-polarized phenotype (12). The function of TAMs has been extensively studied in many cancer types and in addition to playing an immunosuppressive role in the tumor microenvironment, TAMs have been shown to promote tumor invasion, growth, angiogenesis, and metastasis (13, 14). Given the variety of roles that TAMs play in tumor progression, it is not surprising that their presence in many tumor types is often associated with poor prognosis (15–17).

RECRUITMENT AND CHARACTERISTICS OF TAMs IN OVARIAN CANCER

Tumor-associated macrophages represent the most abundant infiltrating immune population in human ovarian tumors and ascites (18). Ovarian tumors recruit circulating monocytes and induce differentiation into TAMs via expression of factors such as CCL2, also known as monocyte chemotactic protein-1 (MCP-1), and macrophage colony stimulating factor-1 (M-CSF or CSF-1). CCL2 is overexpressed in ovarian tumor cells and cell lines, but not in TAMs (19). Interestingly, expression of its receptor, CCR2 is defective in TAMs derived from ovarian cancer patients (20). This may reflect a mechanism by which ovarian tumors retain recruited macrophages in the tumor microenvironment. CSF-1 is a cytokine considered to induce differentiation of macrophages to an M2 phenotype (12) and is overexpressed in human ovarian cancers (21, 22). Expression of CSF-1 is higher in malignant ovarian tumors compared to borderline and benign tumors (23).

Initial studies characterizing TAMs in ovarian cancer demonstrate that TAMs most closely resemble M2-polarized macrophages and express M2 markers such as CD163, CD204, CD206 (Mannose Receptor), and IL-10 (23–26). Ovarian TAMs also express the immunosuppressive chemokines CCL18, which is found in high levels in ascites from ovarian cancer patients (27) and CCL22 (28). More recently, genome-wide expression profiling has been used to investigate the polarization of TAMs in patients with high-grade serous ovarian cancer. The transcriptome of 17 human ovarian TAM samples was compared to non-polarized (M0) macrophages and identified differential expression of 1275 genes. Further analysis of these genes revealed that ovarian TAMs display a mixed-polarization phenotype. TAMs displayed upregulated expression of typical M2 markers such as CD163 and IL-10, while other M2 markers were downregulated. Similarly, some M1 markers were upregulated in ovarian TAMs, such as CD86 and TNF. This mixed-polarization phenotype has also been described in other tumor types (29–32), and suggests that TAMs most closely resemble macrophages involved in developmental processes.

TAMs AND PROGNOSIS IN OVARIAN CANCER

Studies investigating the presence of TAMs in ovarian cancer demonstrate a significant increase in the number of TAMs in malignant ovarian tumors compared to benign and borderline tumors (23, 33, 34). However, their presence as determined by staining for the macrophage marker CD68 does not influence patient outcome (33, 35–37). Expression of specific M2-associated markers in ovarian cancer indicates that certain subsets of ovarian cancer TAMs can indeed predict patient prognosis. In addition to CD68+ cells, Lan et al. also analyzed 110 advanced stage ovarian cancers for the M2 marker CD163 and demonstrated that both progression free survival and overall survival were significantly reduced in patients with high numbers of CD163+ cells (37). Serum levels of CD163 have also been shown to predict poor prognosis in patients with ovarian cancer (38). Similarly, another M2-associated marker was associated with poor prognosis in ovarian cancer. While absolute densities of CD206+ cells were not prognostic, a high CD206/CD68 ratio was strongly associated with worse progression free survival, and there was also a trend toward poorer overall survival (35). Recent studies examining markers of both M1 (HLA-DR, iNOS) and M2-polarization (CD163, VEGF) in ovarian cancer patients have demonstrated that an increased M1/M2 ratio was associated with improved patient survival (39, 40). One study quantified the M1/M2 ratio in the tumor and the stroma and found that only the M1/M2 ratio of overall tumor macrophages or macrophages present intratumorally were prognostic, the M1/M2 ratio in tumor stroma was not predictive of improved survival (39), indicating that macrophages infiltrating tumor cells may play a more important role in tumor progression. Finally, expression of B7-H4 on the surface of ovarian TAMs, but not expression in ovarian tumor cells, was associated with reduced survival and the number of B7-H4+ macrophages was significantly increased in advanced disease (41). These studies demonstrate that while total numbers of CD68+ macrophages present in ovarian tumors do not influence patient outcome, there is strong evidence for specific subsets of TAMs as prognostic factors in ovarian cancer.

In addition to surface markers present on TAMs, cytokines that are important in TAM function are also elevated in human ovarian cancers and associated with reduced survival. IL-6, which is present at high level in ovarian cancer ascites and associated with the generation of TAMs (42), is associated poor prognosis and chemoresistance (43–45). Similarly, IL-10, produced by TAMs, is increased in ovarian cancer and correlated with higher tumor grade and poor patient outcome (45–48). High levels of CSF-1 have also been shown to be a poor prognostic factor in ovarian cancer, when expressed in the tumor epithelium (21). Expression of specific markers of ovarian TAMs, as well as cytokines that are important in TAM function and recruitment acting as prognostic factors in human ovarian cancer provide strong support for the function of TAMs in ovarian cancer progression.

INTERACTIONS BETWEEN TAMs AND OVARIAN TUMOR CELLS

Ovarian cancer cells produce a variety of factors that influence TAM function and vice versa. Co-culture experiments using ovarian cancer cell lines and macrophages have revealed much about the interactions between these two cell types. Ovarian cancer cells have been shown to recruit and induce differentiation of macrophages that have tumor-promoting functions. **Figure 1** depicts some of the important interactions between TAMs and ovarian cancer cells.

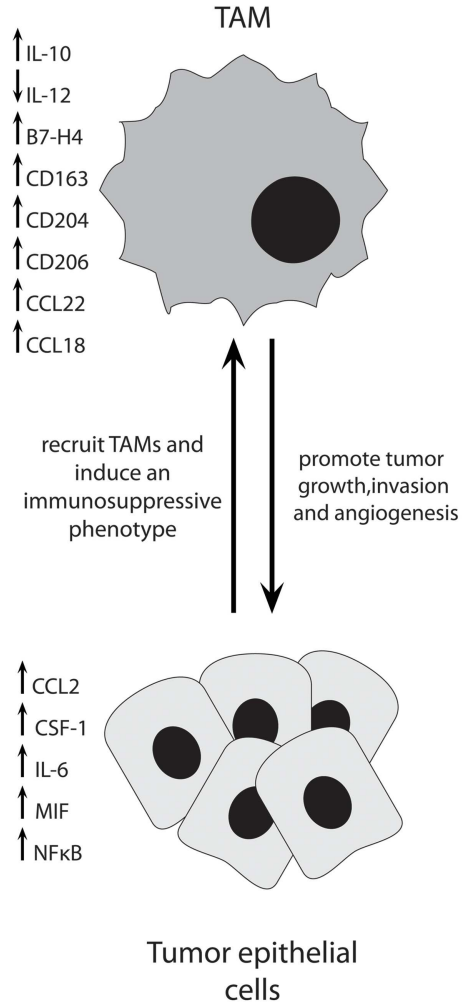

FIGURE 1 | Ovarian cancer cells produce multiple factors to recruit immunosuppressive TAMs into the tumor, where they act to promote tumor progression through multiple mechanisms.

Following co-culture with ovarian cancer cells, macrophages develop a cell-surface phenotype similar to TAMs isolated from human ovarian tumors and a significant increase in genes such as *CCL2*, *CCL22*, *TNF*α, *TGF*β*1*, and *VEGF* (49). In addition, co-culture with cancer cells upregulated the M2-associated Mannose Receptor (CD206) and Scavenger Receptor-A (SR-A, CD204), which was not seen when macrophages were co-cultured with normal ovarian surface epithelial cells (49). The induction of SR-A on macrophages was dependent on the presence of TNFα. Expression of macrophage migratory inhibitory factor (MIF) and extracellular matrix metalloprotease inhibitory factor (EMMPRIN) by ovarian cancer cells induces an increase of MMP secretion by macrophages (50), supporting a role for TAMs in tumor cell invasion and angiogenesis. Downregulation of MIF in ovarian cancer cells led to a decrease in the production of cytokines important in TAM recruitment such as CCL2 and CCL22 *in vitro* and an increase in survival and decrease in ascites *in vivo* (51). Furthermore, inhibition of MIF in ovarian tumor-bearing mice resulted in a decrease in proliferation, an increase in tumor cell apoptosis, and a decrease in the expression of angiogenic factors such as VEGF by tumors. Importantly, there was also a significant decrease in macrophage infiltration in the ascites, as well as a decrease in IL-6 and TNFα, and an increase in M1-associated IL-12 (51). Ovarian cancer cell lines and tumor biopsies have been shown to express elevated levels of IL-6, TNF, CXCL12, and its receptor CXCR4, and expression of these is co-regulated (52, 53). Decreasing the levels of all of these cytokines and chemokines in ovarian cancer cells was achieved by knocking down CXCR4 (52). When injected into mice, CXCR4 knock-down cells produced a decrease in tumor growth and an increase in survival. A significant decrease in the number of macrophages in tumors was also seen. Similar results were achieved when mice were treated with an anti-TNF antibody (52). These studies demonstrate that ovarian tumor cells employ several methods to recruit and induce TAMs to an immunosuppressive phenotype in the tumor microenvironment.

Due to the association between increased levels of IL-6 in ovarian cancer patients and the development of chemoresistance (44), Dijkgraaf et al. investigated the effect of platinum-based chemotherapy on the differentiation of macrophages *in vitro*. Treatment of ovarian cancer cell lines with cisplatin or carboplatin led to an increased ability of some cell lines to induce differentiation of monocytes to an M2-like phenotype (54). The underlying mechanism behind this was due to chemotherapy-induced activation of the NFκB pathway, which resulted in an increase in IL-6 and prostaglandin E_2 by cancer cells that promoted M2-polarization of macrophages. These results indicate that therapeutically inhibiting this effect, for example, by blocking the IL-6 receptor may increase the antitumor effects of platinum-based chemotherapy.

Ovarian tumor cells are a heterogeneous population in which Alvero et al. have identified two distinct subpopulations that have different stemness, inflammatory, and cytokine profiles (55–57). Interestingly, these two populations of cells were found to have unique effects on the differentiation of macrophages (58). Monocytes cultured in Type I EOC cell (CD44+/MyD88+, cancer stem cells) conditioned media demonstrated increased levels of scavenger receptors and cytokines important in tissue repair such as CCL5, whereas those cultures in Type II cell conditioned media demonstrated increases in IL-10, IL-8, and G-CSF and are more likely to play an immunosuppressive role in the tumor microenvironment (58). Nonetheless, both tumor cell populations differentiated monocytes into macrophages with tumor supportive, rather than antitumor properties, and these results add another level of complexity to interactions between ovarian tumor cells and their microenvironment.

Tumor-associated macrophages have been found to promote the invasiveness of ovarian tumor cells through multiple mechanisms. Co-culture of macrophages with human ovarian cancer cell lines increases the invasiveness of tumor cells through TNFα-dependent activation of JNK and NFκB signaling pathways (50). Inhibition of IKKβ, a major activator of NFκB signaling, in ovarian TAMs prevented tumor cell invasion as well as decreased TAM production of M2 immunosuppressive cytokines and increased production of M1-associated IL-12 and NOS2 (59). Adoptive transfer of IKKβ-targeted TAMs into ovarian tumor-bearing mice

resulted in a significant decrease in tumor burden and a switch to an antitumor TAM profile (59). The ability for TAMs to promote tumor cell invasion is also dependent on expression of SR-A. SR-A$^{-/-}$ macrophages displayed a reduced ability to promote invasion of ovarian cancer cells *in vitro* and slowed tumor progression *in vivo* (60). Importantly, this study also demonstrated that targeting SR-A therapeutically with a small molecule inhibitor can prevent tumor progression *in vivo*. Another study also demonstrated the important role macrophages play in ovarian tumor progression by chemically depleting macrophages *in vivo* with clodronate, which dramatically decreased tumor dissemination and the development of ascites in mice injected intraperitoneally with ovarian cancer cells, potentially due to a decrease in VEGF production (61). These studies demonstrate that TAMs promote ovarian tumor progression by employing several different strategies.

Tumor-associated macrophages foster an immunosuppressive microenvironment to promote the survival of tumor cells. Monocytes and macrophages derived from peripheral blood and ascites of ovarian cancer patients were found to be increased in number and to display a less differentiated phenotype compared to cells derived from healthy donors (62). They were also shown to have impaired antitumor activity due to defective cytotoxicity and phagocytic abilities. Another mechanism by which TAMs promote immunosuppression is via secretion of CCL22, which mediates T_{reg} cell trafficking to the tumor (28). B7-H4, which is expressed on the surface of ovarian TAMs, also contributes to immunosuppression in the tumor microenvironment. B7-H4 expression is induced by IL-6 and IL-10 and selectively blocking B7-H4 in macrophages significantly increased T-cell proliferation, whereas ectopic expression of B7-H4 in macrophages inhibited T-cell proliferation (63). In addition, the mannose receptor (CD206), which is expressed on TAMs, has been shown to contribute to the immunosuppressive function of TAMs by binding tumor mucins such as CA125, which increases the levels of IL-10 and decreases levels of the T-cell chemo-attractant CCL3 (24). Treatment of ovarian TAMs with IFNγ is able to reduce TAM secretion of CCL18 and VEGF and switch TAMs from an immunosuppressive to an immunostimulatory phenotype (64). Inducing an M1 phenotype in ovarian TAMs, for example, via treatment with IFNγ or targeting B7-H4 may prove useful in encouraging immune destruction in patients with ovarian cancer.

As has been demonstrated in other cancers, TAMs are beginning to emerge as promoters of angiogenesis in ovarian cancer. Co-culturing of TAMs with ovarian cancer cell lines led to an increase in expression of the pro-angiogenic cytokine IL-8. The conditioned media from these co-cultures significantly increased the migration and tube formation of endothelial cells compared to conditioned media from tumor cells or TAMs alone (65), indicating interactions between tumor cells and TAMs are important for promoting angiogenesis rather than direct interactions between TAMs and endothelial cells. TAMs also promote lymphangiogenesis in ovarian cancer. An increased TAM density was found to be significantly associated with an increased lymphatic vessel density in ovarian cancer patients and TAMs were shown to promote lymphatic endothelial cell proliferation, migration, and tube formation *in vitro* (66). Further studies are required to identify the mechanisms employed by ovarian TAMs in inducing ovarian tumor angiogenesis.

CONCLUDING REMARKS

Tumor-associated macrophages have been shown to play a key role in creating an immunosuppressive tumor microenvironment, as well as promote tumor growth, progression, metastasis, and angiogenesis. The studies mentioned in this review highlight several targets through which TAMs may be targeted therapeutically such as CSF-1, IL-6, NFκB and suggest that depletion of TAMs, or re-education to an immunostimulatory phenotype, may result in a decrease in tumor growth and spread as well as enhance response to chemotherapy. In ovarian cancer, much of the research into TAMs has so far been limited to immunohistochemical characterization in human patient samples and *in vitro* evaluation of their effects on ovarian tumor cells. Compared to other tumor types, a relatively limited number of *in vivo* studies have been performed using xenograft and syngeneic mouse models. Results from these models are potentially confounded by anti-tumorgraft reactions present in syngeneic models and biased immune interactions in xenograft models that use immunodeficient mice. The best assessment of anti-TAM treatment strategies in ovarian cancer would come from the use of spontaneous tumor models, however there are few spontaneous ovarian cancer models available (67, 68). Additionally, whether the role of TAMs varies between each of the histopathological subtypes has not been thoroughly investigated and requires further study. Nonetheless, the work summarized in this review demonstrate that TAMs represent an important component of the ovarian tumor microenvironment, and further studies will assist in evaluating this cell type as a potential therapeutic target.

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