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## The promise and challenge of ovarian cancer models

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### Abstract

The complexity and heterogeneity of ovarian cancer cases are difficult to reproduce in *in vitro* studies, which cannot adequately elucidate the molecular events involved in tumor initiation and disease metastasis. It has now become clear that, although the multiple histological subtypes of ovarian cancer are being treated with similar surgical and therapeutic approaches, they are in fact characterized by distinct phenotypes, cell of origin, and underlying key genetic and genomic alterations. Consequently, the development of more personalized treatment methodologies, which are aimed at improving patient care and prognosis, will greatly benefit from a better understanding of the key differences between various subtypes. To accomplish this, animal models of all histotypes need to be generated in order to provide accurate *in vivo* platforms for research and the testing of targeted treatments and immune therapies. Both genetically engineered mouse models (GEMMs) and xenograft models have the ability to further our understanding of key mechanisms facilitating tumorigenesis, and at the same time offer insight into enhanced imaging and treatment modalities. While genetic models may be better suited to examine oncogenic functions and interactions during tumorigenesis, patient-derived xenografts (PDXs) are likely a superior model to assess drug efficacy, especially in concurrent clinical trials, due to their similarity to the tumors from which they are derived. Genetic and avatar models possess great clinical utility and have both benefits and limitations. Additionally, the laying hen model, which spontaneously develops ovarian tumors, has inherent advantages for the study of epithelial ovarian cancer (EOC) and recent work champions this model especially when assessing chemoprevention strategies. While high-grade ovarian serous tumors are the most prevalent form of EOC, rarer ovarian cancer variants, such as small cell ovarian carcinoma of the hypercalcemic type and transitional cell carcinoma, or non-epithelial tumors, including germ cell tumors, will also benefit from the generation of improved models to advance our understanding of tumorigenic mechanisms and the development of selective therapeutic options.

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## Keywords

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## Introduction

Ovarian cancer claims approximately 140,200 lives each year, with an additional 225,500 patients being diagnosed annually (1). In spite of current chemotherapeutic and surgical options, this high lethality can be attributed to multiple factors, including a late stage presentation by which point the vast majority of patients have widely metastatic disease (2). This is largely due to a lack of effective early screening and detection methods. Consequently, treatment options for late stage disease are limited and patients become increasingly resistant to chemotherapy (3). It is clear that there is an urgent need for personalized therapies to improve overall survival (OS) and life quality while in treatment. As the predominance of ovarian carcinomas is histologically serous (80–85%), there is a greater research emphasis focused on this particular subtype. In North America, endometrioid tumors account for approximately 10% of ovarian carcinomas, while clear cell (5%) and mucinous (3%) carcinomas are more rare (4). In order to optimize treatment, it is important to recognize that ovarian cancer is composed of several different histotypes with unique molecular aberrations, cell of origin, and causal events. An enhanced understanding of the genomic and epigenomic landscape of these subtypes can aid in the development of new targeted agents and immunotherapeutic approaches (3,5).

Tumor-derived cell lines can play a critical role in facilitating cancer biology in *in vitro* studies; however, *in vivo* animal models can more accurately recapitulate molecular characteristics of primary tumors, and as such, be a more pertinent pre-clinical testing platform (6). The development of peritoneal metastasis and ascites in addition to the distinct tumor microenvironment are crucial elements for a model to accurately recapitulate the progression of human disease (2). Two types of mouse models, human tumor xenografts and genetically engineered mouse models (GEMMs), have the potential to significantly expand our understanding of the disease by creating *in vivo* platforms for investigation of tumorigenic mechanisms and the testing of novel therapies. Murine xenografts have typically been generated by isolating tumor cells from patients, establishing tumor cell lines *in vitro*, and then injecting established tumor lines into mice that display a suppressed immune system (7), such as thymus-deficient “nude” or severe combined immunodeficient (SCID) mice. While this method can better reflect the genomic alterations potentially seen in patients by using actual human cancer cells rather than a *de novo* murine cancer (8), one downside is that the use of an established cell line can result in a population that is not truly representative of the original tumor and will therefore produces a different response to therapy compared to those seen in patients (9). Indeed, the usefulness of the traditional xenograft models has historically been debated due to their overall low predictive rate of clinical response (10).

In spite of this, the use of xenografts derived from patients fills a pressing need for preclinical models that recapitulate aspects of the tumors found in patients, which, if optimized, can lead to a higher rate of success in transitioning drug trials from preclinical models to clinic. In an attempt to conquer some of the limitations of the xenograft system, a number of advances have been made in this technology since its inception. To account for the homogenizing effects of establishing a cell line, patient tumor cells can be directly transferred into immunodeficient mice (a process referred to as “direct transfer xenografts”, “explant xenografts”, or “tumorgrafts”), which subsequently retain the natural heterogeneity as well as the relative cell proportions of the original tumor (11). An advantage of using this method is that in addition to performing intraperitoneal or subcutaneous dispersal of tumor cells used to create traditional xenografts, multiple pieces of patient tumor gathered from a biopsy can be orthotopically implanted at clinically-relevant sites to mirror their original location in the patient and their effect on the tumor microenvironment (12). The creation of a living model, which contains a microcosm of a specific patient’s cancer, has obvious utility in assessing treatment options clinically for that particular patient. Thus, therapeutic efficacy can be determined well in advance of the treatment for individual patients, without additional risk for them and without altering the makeup of their disease. These patient-derived xenograft (PDX) models, which are tailored patient stand-ins, have been coined “avatar mice” (13) and found to have better rates of prognostic success for a variety of cancers, including epithelial ovarian cancer (EOC) (14–21).

GEMMs are a more recent type of *in vivo* platform. Animal transgenesis was first made possible in the early eighties (22) and has since made considerable progress. GEMMs enable the management and control of previously introduced transgenes or gene mutations (23). With the advent of transgenesis and enhanced gene targeting through conditional expression tools, a variety of animal models can be generated to mirror disease progression and physiologic states. Two prominent examples include the tetracycline inducible system and Cre/ loxP recombinase system (23). These systems allow for *in vivo* gene induction and/or inactivation in a tissue specific manner at temporally regulated points during either development or adulthood (24). Since advances in novel imaging technologies and early detection methods are critical to improve patient outcome, GEMM are instrumental in that regard. In addition, GEMMs are well suited for studying disease pathogenesis and investigating key genetic factors *in vivo* (12). However, there are advantages and limitations for all models. For example, a number of limitations for GEMMs require their careful consideration prior to use in preclinical or co-clinical setting, as most GEMMs cannot entirely mirror a patient’s particular disease on the molecular level. The diversity of the genomic landscape, which is typically found within human tumors (15), may be incomplete in GEM models engineered with putative “key” gene alterations; additionally, even those particular alterations may not be completely expressed or evident in all tissue types as found in the patient (25). Conversely, one major shortcoming of the traditional xenograft system compared to GEMMs is the absence of a functional immune system. While this still allows the testing of cytotoxic therapies, there has been an increased recent focus on the role of the tumor microenvironment and the immune response in treatment efficacy, particularly when treating drug-resistant or refractory disease (26,27). One such method is the use of therapies aimed at boosting the natural immune system in fighting the disease (28). Other methods

involve using human-specific antibodies to directly target tumor anti-immune mechanisms (29), which cannot be evaluated in GEMMs despite their functional (murine) immune system (30). In order to truly reflect what is seen in the patient and to be an effective testing platform, a mouse model must find a way to incorporate the immune characteristics of the human patient. Xenografts are able to accomplish this goal thanks to the recent development of humanized mice, such as the MI(S)TRG models, which have the capability to receive implanted human cancer cells and also express genes that encode human cytokines, leading to the development of a human innate immune system in the mouse (31). Dr. Jianzhu Chen and collaborators recently pioneered a humanized xenograft mouse model of chemoresistant B cell lymphoma/leukemia by injecting engineered human hematopoietic stem cells into traditional non-obese diabetic SCID (NOD-scid) immunodeficient mice (30,32). This model was responsive to human antibody therapies and led to the discovery of an effective treatment (30), which would not have been possible with the use of traditional xenograft or GEM models. As mentioned above, avatar PDX mice are an ideal option for preclinical and especially co-clinical trials as investigators can “experiment” on an exact patient’s cancer population in a living model, in advance of administering therapy to that particular patient. Humanized xenograft models can now improve the avatar platform for use in immune-related trials as well. A variety of PDXs have been developed for the various subtypes of ovarian cancer. Xenograft and GEM models complement each other by addressing various aspects of disease management from facilitating basic cancer research to providing an avenue for drug testing.

### **Animal models of ovarian cancer highlighting each histological subtype**

The site of origin and mechanisms implicated in ovarian cancer development are not entirely defined because of the histological complexity of the disease and unique causal factors involved. Therefore, in order to gain a better understanding of ovarian cancer pathogenesis it is critical to develop models specific for each histotype.

### **Serous tumor models**

The majority of EOCs are of the serous subtype (33). Furthermore, an overwhelming majority (90%) of serous EOC (SEOC) are high-grade, contributing to a high lethality for this subtype of ovarian cancer (33). There are a number of ways that mouse models can be parlayed into reducing the stagnant and bleak survival rate by improving our understanding of the disease phenotype and response to treatment. Due to the higher prevalence of serous and endometrioid carcinomas, a larger number of animal models are available for these histotypes compared to mucinous ovarian carcinoma (MOC) and clear cell carcinomas (CCCs) (34). Previously, the ovarian surface epithelium (OSE) was postulated to be a primary cell of origin for high grade serous carcinoma (HGSC); however, more recent preclinical and clinical data have converged along the hypothesis of the fallopian tube as a major tumor initiation site (35–37). Thus, Sherman-Baust *et al.* utilized GEMMs (36) to display progression from untransformed tubal epithelium to invasive ovarian HGSC. This animal model expresses the SV40 large T-antigen (TAg), which blocks activation of Tp53 and Rb pathways, under the control of the mullerian-specific Ovgp-1 promoter (mogp). These GEMMs were dubbed “mogp-TAg”. Mice in this system develop lesions that are

morphologically and immunohistochemically reminiscent of neoplastic precursor ovarian HGSC lesions, including serous tubal intraepithelial carcinoma (STIC) and ovarian invasion (36). With the stated goal of earlier tumor detection and improving therapy, Perets *et al.* developed a genetic model of HGSC arising from the fallopian tube (35). The Pax8 promoter, which is selectively expressed in fallopian tubal secretory cells (FTSECs) but not the ovary or OSE, was used to conditionally inactivate key HGSC drivers, including *Brcal*, *Brcal2*, *Tp53*, and the phosphatase and tensin homolog (*Pten*) tumor suppressor genes. This Pax8-mediated silencing led to the formation of preneoplastic HGSC lesions (STICs) in the fallopian tube STICs and HGSC metastasis to ovary and peritoneum, closely mimicking human disease progression (35).

Another mouse model confirming the tubal origin hypothesis for HGSC utilizes *Dicer*; a gene critical for mRNA synthesis (37). When the combination of *Dicer* and *Pten* genes was inhibited via anti-Mullerian hormone receptor type 2-directed Cre (*Amhr2-Cre*)-mediated recombination. This mechanism utilizes the Cre recombinase enzyme to facilitate loxP site-specific DNA recombination. The double knockout mice developed HGSC originating in the fallopian tube and not the ovary. These tumors later metastasized to the abdominal cavity and gave rise to ascites. Upon histologic analysis, certain key features of these tumors including papillary tumor morphology, as well as nuclear and mitotic activity strongly resembled HGSC in patients (37). Microarray gene expression profiles and gene set enrichment analysis (GSEA) indicated that gene expression profiles of murine tumors closely resemble human HGSC (35–37). In addition to *TP53* and *RB*, The Cancer Genome Atlas (TCGA) conducted a large-scale study revealing that aberrations in the *BRCA1* and *BRCA2* genes are present in approximately 20% of high-grade ovarian carcinomas, while 96% of SEOC show alterations in the TP53 pathway (5,38). Previous GEM models for serous carcinoma have been established through combined inactivation of both *Rb* and *Tp53*, as well as *Brcal* and *Tp53*, yet much remains to be learned regarding SEOC tumorigenesis (39). A triple mutant *Brcal* (2); *Tp53*; *Rb* GEM model resulted in tumors resembling the genomic profile of human SEOC, which were then used to study pathway interactions and the initiation and progression of EOC. Interestingly, conditional loss of *Brcal* or *Brcal2* alone was not sufficient to induce transformation; however, when it was coupled with loss of *Tp53* and *Rb* function, tumors characteristic of SEOC developed (40). The same group later adapted these models as tumor donors for serial xenograft studies. After inducing the original triple mutant GEMM via intrabursal injection of adenovirus expressing Cre recombinase (Adeno-Cre viral injection), tumor pieces were orthotopically transplanted under the bursa of inbred FVB mice (FVB/NCr or FVB/NJ lines), noted for their large litter sizes. While the implantation failure rate was found to be initially high and tumors developed with a long latency, subsequent passages reduced both. This attribute, combined with the recapitulation of clinically relevant phenotypes, supports the use of these orthotopic transplants to determine the efficacy of putative SEOC therapies, including PARP and immune therapies in immunocompetent mice (41).

While these studies provided mechanistic insight into tumor initiation and progression, they did not specifically investigate the contribution of the immune system to tumor development. For example, Mucin 1 (MUC1), a tumor-associated antigen, is a possible

target for immunotherapy as MUC-1 expression is high in ovarian cancer, including HGSC and ovarian endometrioid tumors, and correlates with EOC progression (42,43). Preclinical models for MUC1-positive ovarian tumors have been created which can be used for this purpose: *MUC1/Kras/Pten* triple transgenic mice overexpress human MUC1 as a transgene, carry a conditional *K-ras*<sup>G12D</sup> oncogenic mutation and *Pten* loss of function (42,43). Initial studies using ovarian intrabursal delivery of Adeno-Cre have indicated that *MUC1/K-ras/Pten* mice develop metastatic tumors congruent with the human ovarian endometrioid histotype (43), as previously shown (44). Interestingly, Adeno-Cre delivery to the fallopian tube results in high-grade tumors but the endometrioid histotype is preserved, suggesting that the genetic combination dictates the histotype independent of the tumor cell of origin. The *Kras/Pten* GEMM expressing human MUC1 as a self-antigen closely mirrored the local and systemic tumor immune responses seen in patients by triggering the development of *de novo* MUC1 antibodies in tumor bearing hosts and demonstrate the potential for testing the efficacy of immunotherapies in GEMMs (43).

Access to specific diagnostic and prognostic biomarkers can be of great help in matching patients with optimal therapies in the earliest stages of the disease (45). In an attempt to identify new prognostic indicators for high-grade serous (HGS) patients, nude mouse xenografts were used to validate an *in vitro* analysis of gene expression patient profiles. Mice were injected with A2780 ovarian tumor cells, a serous cell line selected for high levels of *COL11A1* expression, a gene found in many HGSC patients with poor prognosis and whose levels increase during disease progression, or a version of the same line in which short hairpin RNAs (shRNAs) was used to inhibit this gene via RNA interference (RNAi). Interestingly, the sh-*COL11A1* was found to have an inhibiting effect on tumor growth consistent with the hypothesis that *COL11A1* correlates with disease severity in patients (46). Xenograft models can additionally prove helpful for examining the beneficial and detrimental effects of drug interactions on patient tissue without exposing patients to increased risk. Paclitaxel (PTX) is a useful therapy for ovarian cancer patients, and dexamethasone (DEX) is a complimentary medication used to decrease the chance of serious reactions and preventing hypersensitivity to PTX infusion (47). However, a recently identified potential side effect of DEX administration is the apparent strengthening of the cancer against PTX's antitumor effects in breast cancer cell lines (48). To examine the mechanism of this effect in ovarian cancer, SKOV-3 tumor cells were injected subcutaneously in BALB/c nude (nu/nu) mice. By examining the monotherapies and combination treatments, it was confirmed that DEX did inhibit PTX's effects and a possible mechanism was identified (49), providing oncologists with crucial information about the treatments they are administering. Ovarian cancer comes in a range of sensitivities to conventional therapies, such as platinum agents, and progression often corresponds with acquired resistance (50). Xenografts can be useful platforms for not only identifying which patients are sensitive and resistant prior to treatment, but also by predicting acquired resistances as treatment continues (51). Notch is one of the most altered pathways in serous carcinoma and has been found to play a key role in cancer stem cells and tumor chemoresistance. Targeting the Notch pathway via inhibitors resensitized resistant disease to platinum therapy (52), which can reopen tried and tested therapies in chemoresistant patients with the worst outcomes. By using a previously-described model of serous xenografts



created by implanting primary tumors and ascites derived from patients into NOD-scid mice (53), it was found that a combination treatment of the Notch inhibitor, MRK-003, and PTX had an enhanced antitumor effect vs. either monotherapy (54).

As SEOC is the most common histological subtype, it can be useful to contrast with other subtypes in order to determine notable differences in therapeutic responses and best meet patients' treatment needs. CCC, for instance, is treated similarly to serous carcinoma despite therapy resistance (55). A comprehensive genomic, *in vitro*, and *in vivo* comparison between CCC and serous carcinoma found myriad differences between the two subtypes. Cell lines of both types were examined in the absence of oxygen and glucose and CCC was found to be more resilient than serous. Mechanistic pathways were identified, and to confirm this effect in a living system, the ES2 CCC cell line was injected into nude mice and inhibition of those key pathways led to inhibition of tumor progression (56). In addition, an anti-angiogenesis therapy, sunitinib, was tested in NOD-scid mice bearing xenografts from the serous tumor tissue lines LTL237, LTL247 and LTL259, as well as the CCC line LTL175. Samples of these lines were embedded under the renal capsule, with the therapy being delivered orally. It was found that sunitinib disproportionally increased CCC apoptosis, but not that of the serous xenografts, which demonstrates that there are fundamental differences in expression between the two subtypes which must be accounted for in treatment (56). Examining molecular pathway differences between subtypes can be critically important in understanding how they differ and what treatments they respond to. In examining the function of the WNT/ $\beta$ -catenin pathway in ovarian carcinomas, the WNT7A ligand was detected exclusively in serous epithelial tissue, and not in the endometrioid subtype. To observe the effect of loss- and gain-of-function of this ligand, nude mice were subcutaneously or intraperitoneally injected with either SKOV3.ip1 (57), in which shRNA inhibited WNT7A, or control SKOV3 cells that overexpressed it. Knockdowns had reduced growth, and higher expression correlated to increased tumor growth, indicating the ligand as a tumor promoter, prognostic predictor, and putative therapeutic target for EOC (58).

When determining if a targeted treatment method is appropriate for multiple subtypes of cancer, xenografts can be used to quickly run panels of tests in a wide cross-section of potential patient types (59,60). While human epidermal growth factor receptor 2 (HER2/*neu*)- and estrogen receptor (ER)-targeted therapies have been successful in breast cancer (61,62), their usefulness in ovarian cancer continues to be assessed. To do so, a variety of tumor fragments derived from SKOV3 as well as five primary ovarian cancer and ascites lines were implanted into CD-1 nude mice. These lines consisted of HOX493 and OV1002 (serous), HOX516 and HOX486 (mixed serous/endometrioid), and HOX424 (mixed clear cell/ endometrioid). HOX424 was the most platinum resistant line consistent with its histological subtype but was also the most responsive to the HER2 therapy. Using genomic analysis, it was found that the treatment reduced expression of most genes that are notably expressed in CCC over other subtypes. Conversely, genes of lower expression levels in CCC were expressed at higher levels post-therapy. This not only demonstrates that each subtype needs unique treatment considerations due to underlying molecular differences, but also that the treatments administered can dramatically alter gene expression in the tumor population.

Molecular analysis performed post-therapy could be a powerful tool to allow oncologists to adjust treatment in order to react to the changed tumor makeup (63).

It is worth noting that a recent analysis of the genomic profiles of a broad variety of ovarian cancer cell lines determined that many of the most commonly used and cited lines, such as the previously mentioned SKOV3 and A2780 lines, are not representative of HGSC (64). The study elaborates on more appropriate lines to use for specific studies, based on their genomic similarities to the typical HGSC copy number aberrations (64).

Considering these confounding issues that can be found in established cell lines, PDX models show promise in accurately recapitulating the primary disease. Recently, a panel of xenografts spanning a variety of EOC subtypes was derived from 138 patient samples (65). The samples were grafted into nude NCr-nu/nu mice according to sample type; solid tumors were grafted subcutaneously while ascites samples were dispersed intraperitoneally. A total of only 34 successful grafts were developed due to an overall low take rate of 25%. The tumor grafts were analyzed and compared favorably to patient histotypes from which they were derived. Of the 34 tumor grafts developed, 16 were serous and 3 were mixed serous-endometrioid. While dissemination was not correlated with histotype, two of the HGSC grafts, as expected, were found to have the highest potential of invasion and metastasis. Overall, this study found recapitulation of the patient phenotype, including histotype and response to chemotherapy. However, a point of concern was that the genomic landscape of the tumor grafts did not entirely recapitulate the original tumor and, surprisingly, a higher level of genomic variability was seen in the PDX models compared to patient tumors.

Scott *et al.* recently summarized 11 groups who have successfully developed PDX models for HGSC along with their engraftment methodology (66). The factor that is highlighted as of primary importance is annotation of the patient-derived lines. As the important role of the tumor microenvironment is increasingly recognized, specifics about tumor cell preparation and engraftment method are crucial. Additionally, molecular and genomic profiling of PDX tumors can specifically identify key genetic changes and the particular histotype of the grafts for a direct comparison to the original patient diagnosis. This way, molecular therapies can be efficiently tested against their intended target genes and biomarkers for response to a particular therapy, which could be identified for specific histotypes. In a follow-up analysis by the same group, they identified the use of identical frontline treatments across all EOCs as an issue which more advanced PDX modeling could help resolve (67). This study in particular highlights the flexibility of the PDX platform in terms of the variety of graft types possible and the questions they can help address. For example, in terms of disease progression, PDX were used to examine the invasion potential of various phenotypes and, interestingly, the success of engraftment was noted as a potential prognostic indicator of aggressive disease. In regards to therapy, the high similarity seen between the PDXs and their corresponding patient tumors can inform therapy selection and predict response, as well as determine molecular changes post-therapy. While the lead-time required to generate PDX and the low rate of tumor engraftment may limit their use as a co-clinical platform, there are contexts, such as in advance of recurrence, where the timeline is expanded enough to accommodate model development.



A large-scale clinical trial is underway at the Mayo Clinic to assess the predictive accuracy of PDX models in determining patient response to therapy, with a special focus on platinum resistant patients (68). Dr. Paul Haluska, the leader of this study, is proposing to implant mice with tumor fragments immediately after they are removed from patients and administer standard platinum chemotherapy to mice on a similar dosing schedule as the corresponding patients. PDX mice will further receive a variety of treatments that will be used to guide the course of action if a patient is found to be platinum resistant. Genomic data collected from this trial are expected to identify specific molecular signatures that correlate with a particular therapeutic response (68).

## Endometrioid models

Endometrioid ovarian cancer is the second-most common subtype of EOC, and it has distinct differences in treatment response compared to serous EOC. Characteristic features of endometrioid carcinomas include glandular formations, squamous differentiation, and presentation typically involves a large ovarian tumor during early stages of the disease (4). Oncogenesis for this subtype is associated with endometriosis (69). GEMMs for the endometrioid subtype of ovarian cancer have been successfully exploited to resolve the site of origin and pathogenesis for this particular subtype (44,70,71).

Endometriosis has long been suggested as the initiating event in endometrioid ovarian carcinoma; however, the mechanism through which this occurs has remained unknown for a long time. Our studies in animal models have uncovered the first genetic link between endometriosis and endometrioid ovarian cancer, which could help explain their frequent association in women. Novel GEM models using an oncogenic *K-ras*<sup>G12D</sup> allele or/and a conditional *Pten* deletion resulted in endometriosis and endometrioid ovarian adenocarcinoma with widespread metastases, respectively. Adeno-Cre injection into the ovarian bursal cavity was used to achieve transformation with a high degree of phenotypic similarity to human endometrioid ovarian carcinoma (44). These models introduced a previously unstudied combination of genes while accurately replicating the tumor morphology and metastatic potential characteristic of human endometrioid ovarian carcinoma. Interestingly, our molecular genetic evidence that endometriosis is a precursor for endometrioid ovarian cancer has now been validated in clinical studies (72–79).

Similarly, mutations of the *ARID1A* tumor suppressor gene are present in a significant proportion of endometriosis-associated endometrioid ovarian carcinomas (80). In addition, TCGA data found that 49% of ovarian endometrioid carcinomas harboring mutations in the *ARID1A* gene also show changes in *PTEN* (81). Consequently, *Arid1a* and *Pten* conditional double knockout mice were recently generated (82) via the same method of intrabursal Adeno-Cre delivery described earlier (44). Thirteen of the 22 mice developed ovarian tumors, five of which were endometrioid and contained within the ovary, while eight were undifferentiated and metastasized to the peritoneal cavity. In agreement with the histomorphology of human endometrioid ovarian carcinomas, all of the murine tumors displayed squamous differentiation. This model has also proven accurate in recapitulating poor prognostic patient tumors as the majority of undifferentiated human ovarian carcinomas are aggressive and have a fast rate of metastasis. Of the 52 mice, only double

knockouts for *Arid1a* and *Pten* developed tumors, highlighting a cooperating role for *Arid1a* and *Pten* in the development of endometrioid ovarian carcinoma. Interestingly, both *Arid1a* and *Pten* are also frequently mutated in clear cell ovarian cancer; however, no clear cell ovarian tumors were identified, suggesting that the OSE, which is activated via intrabursal Adeno-Cre delivery, is not likely the cell of origin for this particular histotype.

Similar to GEMMs, endometrioid tumor xenografts were also generated to investigate the potential role of oncogenes and tumor suppressor genes in the pathogenesis of endometrioid tumors. Thus, an investigation into the distinct molecular mechanisms found in ovarian cancer subtypes led to the identification of a specific miRNA, miR-370, which is downregulated in endometrioid ovarian cancer (83) and may act as a tumor suppressor during pathogenesis. In order to test this hypothesis, two variants of the IGROV1 endometrioid cell line, with and without overexpression of miR-370, were injected into the axillary fossae of nude mice. It was found that the presence of miR-370 did indeed suppress tumor growth and promote platinum sensitivity (84). Similarly, the OVTW59 endometrioid cell line was generated and injected into SCID mice, and tumor xenografts were selected for increased invasive potential. This allowed for the identification of gene expression profiles that correlated with an aggressive phenotype. It was found that the gene which encodes for the insulin-like growth factor-binding protein 3 (*IGFBP-3*) was expressed at lower levels in invasive tumors, indicating potential function as a suppressor of invasion and metastasis in ovarian endometrioid cancer (85). Avatar endometrioid models have also been successfully developed as described by Ricci *et al.*: five were endometrioid, three were mixed serous-endometrioid, and one was mixed endometrioid. The phenotype of the endometrioid xenografts was found in general to resemble that of the patient tumors they are derived from, although a mixed serous-endometrioid graft was one of the only mice tested whose chemotherapy profile differed from the original tumor (65).

## Mucinous models

MOC is a rare histotype with a significant resistance in advanced stages to typical platinum and taxane compounds. The cell of origin for MOC is elusive and neoplasms tend to be large, with a mean size of 18 cm at diagnosis. The sheer mass of these tumors may occasionally serve as an indication of MOC (86). Comprising roughly 2–10% of EOCs, advanced stage MOC generally result in a poor outcome, suggesting an urgent need for the development of clinically relevant models and novel therapeutics for this disease (86–88). In a large clinical trial comprised of MOC patients with advanced stage disease who were administered standard chemotherapy consisting of combination carboplatin and paclitaxel, there was an overall decreased sensitivity to chemotherapy coupled with a decreased progression free survival (PFS) and OS when compared to SEOC patients (89). A second study corroborated this information, assigning women with advanced MOC to the study group and women with SEOC as controls. Again, PFS and OS were diminished for the study group when compared with SEOC patients. 57.9% of MOC patients exhibited sensitivity to platinum compounds whereas 70.8% platinum sensitivity was observed for the SEOC control group (90). These studies indicate a need for improved therapeutic options for the MOC subtype. However, GEMMs do not yet exist for the mucinous subtype of ovarian

cancer, as it is difficult to establish a cell of origin and because MOC presents with unique clinical features when compared to other EOCs (86–88).

The laying hen model offers a promising alternative *in vivo* system to explore the mechanisms involved in EOC initiation and progression. This model has received praise and attention due to the absence of genetic and chemical manipulation involved in inducing ovarian carcinogenesis while maintaining congruous histology and pathogenesis of advanced stage human MOC as demonstrated by the formation of ascites and peritoneal metastases (2,91). In a study comprised of 26 hen tumors (18 well differentiated and 8 poorly differentiated), 5 well differentiated mucinous carcinomas developed in addition to 4 moderate to poorly differentiated mucinous carcinomas (91). Key characteristics of the mucinous tumors include glandular formations clustered together and surrounded by cytoplasmic mucin, analogous to human MOC. Although a limited number of transgenic chicken models have been pioneered, novel strategies are being investigated to reproducibly deliver genes to transgenic hen models. Notably, a gelatin nanoparticle containing plasmid DNA and expressing enhanced green fluorescent protein (EGFP) has been reported to be a safe and efficient delivery tool for gene transfer via egg injection (92). Despite the lack of transgenic models available for the laying hen, this spontaneous EOC model has shown merit in an interventional setting, especially in testing chemoprevention strategies. Notably, a flaxseed enriched-diet was able to reduce ovarian cancer severity, suggesting the need for clinical trials evaluating dietary prevention methods, flaxseed in particular, for ovarian carcinoma (93). Characterization of ascites collected from chicken ovarian tumors uncovered evidence for E-cadherin upregulation, thus identifying another potential therapeutic target and gene network for ovarian cancer research (94). Due to the comparable histology, etiology, and disease staging between tumors of the laying hen and human MOC, this model shows good potential in advancing ovarian cancer research. While genetic manipulation has yet to be achieved on a large scale, the laying hen can be used to examine therapeutic strategies and facilitate interventional studies.

Based on successful *in vitro* studies examining a novel Src inhibitor KX-01, a MOC orthotopic xenograft model was created to verify these effects *in vivo*. The RMUG-S and RMUG-L cell lines, originally isolated from women with MOC tumors (95,96), were injected intraperitoneally into nude mice. KX-01 was evaluated alone and in combination with oxaliplatin, demonstrating a notable therapeutic effect in both cases but particularly when combined. Similarly, the epidermal growth factor receptor (EGFR), a poor prognostic factor in multiple cancers and expressed in 48% of MOC, is a potential therapeutic target. To examine the effect of the anti-EGFR antibody cetuximab, the mucinous lines RMUG-L and MCAS were grafted into BALB/ c nude mice via subcutaneous injection. This xenograft study confirmed the *in vitro* results observed, which was that cetuximab partially reduced tumor growth if the tumor had a *KRAS* mutation but completely inhibited growth in those with increased EGFR expression and without a *KRAS* mutation (97). This demonstrates that even within a histological subtype, treatment tailored to individual patients can have a big impact on prognosis (97). The phosphoinositide-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway is activated in a variety of cancers and plays a key role in tumor initiation and progression (98). The pathway is

activated in 70% of ovarian cancers and as a result it is a promising therapeutic target (99). To assess the dual inhibition of PI3K and mTOR in MOC lines, seven lines were tested in nude mice MOC xenografts. Of the seven lines tested, five showed synergy between the dual inhibitor and traditional paclitaxel/cisplatin treatments and two lines (OMC-1 and RMUG-S) showed suppressed tumor growth without adverse side effects (100). Ricci *et al.* successfully generated two MOC avatar xenografts, both of which were somewhat responsive to PTX while only one of the mice was responsive to cyclophosphamide chemotherapy (65). Patient tumor histology was recapitulated with a high degree of similarity with the corresponding PDX xenograft, indicating the clinical utility of this *in vivo* platform (65).

## Clear cell models

CCC and endometrioid ovarian carcinoma are believed to originate from endometriosis. This distinct histotype is associated with chemoresistance and a poor patient outcome, thus demonstrating the need for improved therapeutic options (101). Clear cell tumors share similarity in their clinical presentation with endometrioid ovarian neoplasms and the majority of patients are diagnosed at early-stage when the disease has not metastasized (102). Similar to advanced stage MOC tumors, a hallmark of CCC is chemoresistance. It is clear that *in vivo* models of CCC are crucial for determining alternate therapy solutions and there are ongoing investigations into similar avenues as with MOC.

As is the case with MOC, no GEMMs for CCC have been engineered. However, treatments aimed at targeting specific genes are beginning to garner more attention. About 46–57% of CCCs harbor mutations in the *ARID1A* gene, which encodes the BAF250a protein, another key player in ovarian clear cell carcinogenesis. As demonstrated in xenograft models, mutations of the PI3K/AKT/mTOR signaling pathway are common in CCC, suggesting that inhibitors of this pathway may bear clinical utility. While there is a paucity of *in vivo* mouse models for CCC, an early-phase clinical trial is currently under way to examine the combination of an mTOR inhibitor temsirolimus, carboplatin, and paclitaxel as first-line therapy in patients suffering from stage III-IV CCC (103). In addition to gene therapy, targeting angiogenesis may provide treatment options for CCC. High expression of vascular endothelial growth factor (VEGF) in CCC tumors is correlated with shorter OS, and thus merits investigation as a treatment target for CCC. VEGF can be inhibited through the use of the monoclonal antibody bevacizumab (101,104) *in vivo*. Patients treated with bevacizumab exhibited drastic growth inhibition of platinum-refractory CCC tumors (104).

Multiple groups were successful in generating models using nude mice and the cell line RMG-I, an ovarian CCC line isolated from patient ascites (105). This line overexpresses EGFR and was chosen to validate the hypothesis that inhibition of EGFR-ERK could inhibit tumor progression. RMG-I tumor cells successfully engrafted in all mice, resulting in tumors as early as day 5 (106). An inhibitor of the mammalian checkpoint kinases Chk1 and Chk2 delivered in parallel with traditional cisplatin treatments was tested in a similar model and resulted in suppressed tumor growth (107). RMG-I was also used in a subcutaneous xenograft model along with the TOV-21 and ES-2 CCC lines to investigate the therapeutic efficacy of dual PI3K and mTOR inhibitors; the study revealed tumor growth suppression

with no substantial adverse effects (108). Of the two CCC avatar mice Ricci *et al.* generated, one mouse developed a higher level of peritoneal dissemination and subsequently formed ascites (65). Both CCC avatars were somewhat responsive to PTX, while one of the two was highly sensitive to cyclophosphamide and one was completely resistant, demonstrating the need for more tailored therapeutic options even within a particular subtype. Interestingly, transcriptomic profiling revealed that the CCC avatar with greater peritoneal metastasis harbored *PIK3CA* and *TP53* mutations while the other mouse was wild type for both (65).

While this review mainly focuses on the main subtypes of EOCs, rarer OC variants, including small cell ovarian carcinoma of the hypercalcemic type (SCCOHT) and transitional cell tumors merit investigation as well. SCCOHT is an incredibly rare and aggressive neoplasm that is histopathologically distinct from ovarian epithelial, transitional cell, and germ cell tumors. This rare form of ovarian cancer primarily affects women between ages of 9 and 43 and has a one year survival rate of 50% and a 5-year survival rate of 10% (109–111). Both *in vitro* and *in vivo* models are needed to develop a tailored therapeutic regimen that will improve prognosis. In an effort to characterize the disease, resected patient tumor samples were propagated in nude mice for six generations. Consistent morphology was achieved between primary patient tumors and murine xenografts. Tumor fragments were subsequently analyzed through comparative genomic hybridization (CGH), electron microscopy, histology, and serum calcium levels, which revealed that SCCOHT was indeed heterogeneous and distinguishable from both germ cell tumors and EOC (112). Otte *et al.* successfully created a patient-derived cell line and performed a comprehensive morphologic, cytogenetic, and immunohistochemical analysis of tumor cells. In addition, a xenograft model of SCCOHT was generated using NOD-scid mice and the murine tumor phenotype matched the original patient tumor. Scanning electron and transmission electron microscopy revealed rounded and rapidly dividing cells in early stages of differentiation. Notably, vimentin; an intermediate filament, was expressed in the vast majority of SCCOHT-1 cells, implicating a role for intracellular and matrix interactions. This patient-derived cell line and xenograft allowed for more in-depth analysis of the properties and signaling pathways involved in SCCOHT (109–111). Otte *et al.* later elaborated on these findings to optimize treatment for this disease. SCCOHT-1 cells were found to be highly chemoresistant *in vitro* but showed sensitivity to epothilone B (113). Tumor xenografts exhibited diminished tumor size following treatment, thus mimicking the cytotoxic effects *in vitro*. Interestingly, xenograft mice treated with a combination of calcium and epothilone B achieved normal calcium serum levels in contrast with mice treated with only one of the two agents. The research conducted by Otte *et al.* demonstrates the critical role of xenograft mouse models as a testing platform for targeted SCCOHT therapy. More recently, SCCOHT tumor sequencing and immunohistochemical analysis have highlighted the function of *SMARCA4*, with aberrations of this gene largely responsible for disease pathogenesis. This finding will be used to guide novel therapies for this aggressive tumor, in addition to emphasizing the potential for genetic counseling as a means of disease management and prevention (114).

## Conclusions

Serous, endometrioid, mucinous, and clear cell EOC subtypes present with distinct histopathological and molecular characteristics that pose unique therapeutic challenges. In order to improve patient outcome and effectively treat these different diseases, treatments need to be personalized; thus, it becomes critical to investigate tumorigenic mechanisms and conduct large-scale drug screening studies for each subtype. These unique attributes and challenges can be addressed through the use of both xenograft and GEM mouse models. In addition, the distinct features of the laying hen model of spontaneous cancer, which confers certain benefits that murine xenograft models and GEMMs do not offer, makes it an additional valuable tool for EOC research. Emphasis should be placed on improving chicken transgenesis and employing the natural advantages inherent in this model to examine alternative treatment and prevention strategies. While most research studies focus on HGSC tumors, rarer ovarian cancer variants, such as small cell ovarian carcinoma of the hypercalcemic type and transitional cell carcinoma, or non-epithelial tumors, including germ cell tumors, will also benefit from improved animal models and drug testing platforms. These studies highlight the combined role for both xenograft and GEM models in cancer research. Certain challenges and advantages are inherent to both strategies. GEMMs allow for the analysis of specific roles and interactions of oncogenes and tumor suppressors during disease progression in a replicable efficient system (115). Xenografts and especially avator PDX models have unique attributes that make them particularly well suited for therapeutic analyses, including concurrent human and murine clinical trials (6).

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