Epithelial Ovarian Cancer Experimental Models

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Abstract

Epithelial ovarian cancer (OvCa) is associated with high mortality and, as the majority (>75%) of women with OvCa have metastatic disease at the time of diagnosis, rates of survival have not changed appreciably over 30 years. A mechanistic understanding of OvCa initiation and progression is hindered by the complexity of genetic and/or environmental initiating events and lack of clarity regarding the cell(s) or tissue(s) of origin. Metastasis of OvCa involves direct extension or exfoliation of cells and cellular aggregates into the peritoneal cavity, survival of matrix-detached cells in a complex ascites fluid phase, and subsequent adhesion to the mesothelium lining covering abdominal organs to establish secondary lesions containing host stromal and inflammatory components. Development of experimental models to recapitulate this unique mechanism of metastasis presents a remarkable scientific challenge and many approaches used to study other solid tumors (lung, colon, and breast, for example) are not transferable to OvCa research given the distinct metastasis pattern and unique tumor microenvironment. This review will discuss recent progress in the development and refinement of experimental models to study OvCa. Novel cellular, three-dimensional organotypic, and *ex vivo* models are considered and the current *in vivo* models summarized. The review critically evaluates currently available genetic mouse models of OvCa, the emergence of xenopatients, and the utility of the hen model to study OvCa prevention, tumorigenesis, metastasis, and chemoresistance. As these new approaches more accurately recapitulate the complex tumor microenvironment, it is predicted that new opportunities for enhanced understanding of disease progression, metastasis and therapeutic response will emerge.

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Keywords

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1 INTRODUCTION

Ovarian cancer (OvCa) continues to be a poorly understood disease with an extremely poor prognosis. Its cell of origin is still unknown and researchers have only begun to elucidate its molecular mechanisms. Several molecular changes have been confirmed in human tumors and functionally characterized in cell and animal models (e.g. p53, PI3 kinase, ARID1, K-ras, BRCA1, 2).[1–6] However, no genetic alteration has, as yet, been found to be a clinically "actionable" genetic change. One reason for the slow progress made in understanding the biology of OvCa and translating that knowledge into substantial clinical benefits has been a lack of clinically representative model systems that mimic the progression of the human disease. Since the majority of women diagnosed with epithelial OvCa (>75%) have intra-abdominal metastasis at the time of diagnosis, models that replicate the molecular events that underlie dissemination are a crucial tool to gain the knowledge necessary for the development of new treatments.

Several excellent reviews discuss the current knowledge of OvCa biology.[1–3,7] This review offers a comprehensive overview of experimental models available for the study of ovarian cancer. By reviewing the current state of model systems, the hope is to stimulate discussions on how these models may be improved, as well as provide a needed reference. The biology of OvCa poses special challenges to the development of model systems. The disease commonly referred to as 'ovarian cancer' is not a single entity, but consists of several histological subtypes (serous, endometrioid, clear cell, and mucinous) with distinct molecular aberrations.[1,3] The site of origin for high-grade serous ovarian cancer, the major histological subtype, is still unknown and is intensely debated. Possible sites include the abdominal peritoneum and the fallopian tube fimbriae, as well as the ovarian surface epithelium.[7,8] Moreover, OvCa is distinct from most other solid tumors in that hematogenous metastasis is rare.[9] The initial early event in epithelial OvCa metastasis is proteinase-mediated shedding into the peritoneal cavity.[10–12] Intra-peritoneal metastatic implants are initiated by single cells and spheroids adhering to peritoneal mesothelial cells and anchoring in the sub-mesothelial matrix to establish secondary lesions.[13–15] Accumulated peritoneal ascites (often > 500ml) contains a unique non-adherent population of individual tumor cells and multicellular aggregates (spheroids) exposed to a set of microenvironmental cues that promote metastatic implantation.[1,13,14] Since the mortality of OvCa is directly attributed to disseminated peritoneal metastasis, this distinctive microenvironment provides a marked set of therapeutic challenges and necessitates research that makes use of a suite of model systems to recapitulate specific cellular and molecular events in metastasis.

2 THREE-DIMENSIONAL CULTURE MODELS OF OVARIAN CANCER TO RECAPITULATE THE TUMOR MICROENVIRONMENT

Ovarian tumors are not a solid matrix of epithelial cells, but rather a mixture of epithelial, stromal, immune, and endothelial cells.[1,3,9] This complex tumor microenvironment (TME) includes (a) primary tumor with tumor associated stromal cells (fibroblasts, mesothelial cells) and inflammatory cells (macrophages, leukocytes); (b) non-adherent cells suspended in ascites with inflammatory and mesothelial cells; and (c) metastases to various parts of peritoneal cavity that contact mesothelial cells, adipocytes, and fibroblasts at the metastatic site.[9] This heterogeneity of cell types likely impacts tumor histology, growth potential and ability to evade chemotherapy. Optimal treatment for ovarian cancer patients consists of tumor debulking (removing all tumors >1mm) followed by chemotherapy. As ovarian cancer is often widely disseminated with many microscopic implants that cannot be surgically removed, leading to disease recurrence within the first 2–3 years, there is a critical need for a more detailed understanding of early events in (re)colonization of metastatic implants. These experimental questions require new model systems that have been designed to recapitulate events occurring in the OvCa TME.

While our knowledge of OvCa biology has largely been derived from experiments using cancer cells grown in two-dimensions on plastic, studying OvCa initiation, progression and metastasis in a three-dimensional (3D) system has many advantages. In particular, epithelial cells grown in 3D with extracellular and/or cellular components of the TME exhibit a number of features similar to those of cells in the clinical microenvironment. Therefore, 3D models that incorporate extracellular matrices (ECM) and stromal cells provide an opportunity for understanding the crosstalk between OvCa and stromal cells; for detailed analysis of the contribution of stromal elements to cell adhesion, invasion, and nutrient access; and to assess the influence of the TME on tumor cell survival, proliferation and differentiation.

2.1 Ovarian cancer cells and three-dimensional matrices

Initial strategies for creating 3D cultures[16,17] began with mixing OvCa cell lines or primary cells with different forms of ECM. The ECM used was typically either a purified series of proteins, such as collagen, or an extract derived from a more complex mixture, such as Matrigel®. The early events that follow sub-mesothelial matrix contact are modeled by culturing cells on top of 3D collagen gels, allowing the evaluation of changes in gene expression that accompany metastatic anchoring [Figure 1Aa–c].[2,18] Matrix penetration, survival and proliferation within the mechanically constrained 3D matrix environment can be modeled by seeding single cells within 3D collagen gels [Figure 1Ad–e]. Indeed, successful growth of a metastatic aggregate requires proliferation within a mechanically constrained matrix microenvironment, a process facilitated by the expression of matrix-degrading proteinases such as membrane type 1 matrix metalloproteinase (MT1-MMP) that promotes proliferation within an interstitial collagen mileu.[19,20] OvCa cells grown in 3D culture on growth factor-reduced (GFR)-Matrigel exhibit a central hypoxic region, leading to the decreased diffusion and perfusion of drugs.[21] The GFR-Matrigel OvCa model has

been used to screen for drugs that are able to penetrate deep into the hypoxic and acidic region of the 3D culture.[22]

2.2 Spheroids or multi-cellular aggregates

Ovarian cancer spheroids that range in size from 30–200 μm can be isolated from patient-ascites[23] or produced by growing OvCa cells on non-adherent plates[24], spinner-flasks,[25] or by using the hanging drop culture method [Figure 1B].[26] Multi-cellular aggregate formation may facilitate metastasis, since spheroids resist anoikis and can adhere to both omentum and exposed ECM.[13,14] Adhesion of spheroids initiates a transition from a floating cell population to a peritoneally-anchored metastatic lesion. Following adhesion to a collagen surface, the cells acquire unique mRNA profiles that more closely resemble *in vivo* tumor gene expression profiles.[26]

2.3 Co-culture of mesothelial with ovarian cancer cells

The main sites of OvCa metastasis are mesothelial cell-lined peritoneal surfaces, including the peritoneum, omentum and pleural surface.[15] Notably, in 1985 the first 3D peritoneal culture was used to investigate the adhesion of primary human OvCa cells to primary human mesothelial cells grown on a bovine corneal endothelial cell-derived extracellular matrix.[27] Other 3D OvCa co-cultures followed, with cancer cell lines or primary cells grown as monolayers on plastic or as tissue-like multi-cellular aggregates [Figure 1B], which were co-cultured with mesothelial cell monolayers.[28–33] A derivation of this strategy was the utilization of conditioned medium secreted by mesothelial cells to monitor the influence of secreted factors on cancer cell invasion through ECM.[34] Following adhesion of tumor cells to peritoneal mesothelium, rapid mesothelial cell retraction is initiated, resulting in exposure of the interstitial collagen-rich sub-mesothelial matrix to which OvCa cells avidly adhere via integrin-mediated interactions [Figure 1A]. [33,35,36] The resulting integrin engagement and clustering modify gene expression profiles, altering the expression of genes that potentiate matrix penetration, anchoring, and proliferation.[2,18]

2.4 Complex three-dimensional organotypic models with multiple cell types and extracellular matrices

A recent model of OvCa metastasis provides an environment that more closely mimics the *in vivo* human peritoneal microenvironment, as it includes the superficial layer of the peritoneum ("mesothelium") to include both mesothelial cells and the sub-mesothelial ECM interspersed with primary human peritoneal fibroblasts.[15] The 3D organotypic model uses tumor-derived primary human OvCa cells or cancer cell lines cultured with omentum-derived primary human mesothelial cells, primary human fibroblasts and patient-derived extracellular matrix [Figure 1C].[37] This model recapitulates key events in OvCa metastasis including adhesion, proliferation, and invasion, thereby re-establishing morphological and functional features of the corresponding tissue *in vivo.*[37] By using fluorescently labeled cells and fluorescence-activated cell sorting, changes in gene or protein levels and post-translational modification can be individually investigated in the cancer cells, mesothelial cells or fibroblasts after co-culture. Thus far, the 3D "mesothelium-mimetic" organotypic culture has contributed to our understanding of the role of c-Met[38], MMP-2[39], HOXA9[40], E-

cadherin[12], β_3-integrin[41] and α_5-integrin[12] in early OvCa metastasis. The 3D organotypic culture of peritoneal metastasis is particularly suited for use as a predictive preclinical model for drug testing, since drug sensitivity in *ex vivo* and *in vivo* studies can be recapitulated in the 3D model.[38,39] Furthermore, this model can be expanded to use other cell types (e.g. adipocytes[42], macrophages) and provides a system to test the effects of treatments on both normal peritoneal cells and tumor cells, and may be modified for use in high-throughput screening (HAK, EL unpublished).

2.5 Peritoneal and organ explants

Three dimensional systems using *ex vivo* human or mouse peritoneal or omental explants are used to evaluate OvCa tumor cell interactions with intact peritoneal tissue (MSS, unpublished).[39,43] Dissected peritoneal explants can be pinned to optically clear silastic resin and incubated with OvCa cells or multi-cellular aggregates [Figure 1D]. Use of fluorescently-tagged OvCa cells enables optical monitoring of adherent cells as well as visualization by confocal microscopy. Scanning electron microscopy can also be used to visualize the morphology of early heterogeneous cell-cell adhesive interactions. Furthermore, explants can be histologically sectioned and used to quantify depth of tumor cell penetration under various conditions. This model confers the ability to monitor the extent and kinetics of adhesion, observe early events in peritoneal anchoring, and test potential anti-adhesive therapeutics. Similarly, tissue explants can also be used to evaluate pre-neoplastic changes in potential progenitors from the ovary and fallopian tube. For example 3D alginate scaffolds have been used to support the *ex vivo* growth of ovarian surface cells, normal mouse oviducts and baboon fimbria.[44–46]

In the current iteration, these models are limited by the lack of vasculature, immune and endothelial cells, and other extracellular components present *in vivo* as well as by the ability to provide reproducible conditions only for a short period of time (<1 week). Nevertheless, the development and use of advanced 3D and *ex vivo* OvCa models can bridge the gap between *in vitro* studies, OvCa mouse models, and the *in vivo* human disease. The choice of a model system for a given experiment should reflect the experimental question to be addressed and the stage of OvCa metastasis under investigation.

3 CELL LINES AND XENOGRAFTS

Although OvCa cell lines and primary tissues that recapitulate the molecular diversity, cellular heterogeneity, and histology seen in patient tumors are available, there is no uniform collection or structured overview of these valuable reagents. Here we summarize the most frequently used OvCa cell lines, including those used as xenografts (Supplementary Table S1). The positive attributes of cell culture include ease of propagation, relatively low cost, ability to control culture conditions, less heterogeneous cell populations, and the presence of side population cells which represent a renewable source of OvCa stem cells. Cell culture is limited, however, by the absence of the tumor microenvironment, artifacts from prolonged culture conditions in unphysiological culture medium on plastic, high passage number, and the lack of patient-matched normal cell counterparts. When these cells are used to generate xenografts, positive attributes include recapitulation of tumor heterogeneity with recruitment of host stromal cells and maintenance of both tumor stroma and tumor/non-tumor

vasculature interactions. This is balanced by the time and expense of these models and the maintenance of only a small portion of niche cells which do not represent the entire tumor.

3.1 Serous ovarian cancer epithelial cell lines and intraperitoneally derived xenografts

Most high-grade serous OvCa cell lines (Supplementary Table S1) have been derived from ascites of patients ranging from chemotherapy naïve to heavily treated, with various stage malignancies[47]. Cell lines established from patient whole tumor tissue (rather than ascites), that are considered platinum-sensitive, include HEY[48], derived from the peritoneal deposit of a moderately differentiated papillary cystadenocarcinoma[49], and the cisplatin-resistant derivations HeyA8 and HeyC2, both generated by passaging HEY in athymic nude mice.[48] OVCAR-4[50] was developed from a patient refractory to platinum-based chemotherapy, and Caov-3[51] from the solid components of a serous OvCa prior to treatment with cyclophosphamide, adriamycin, and 5-fluorouracil. Given that taxanes are the second cornerstone of OvCa treatment, a paclitaxel-resistant subline (SKOV3TR) was derived from SKOV-3 cells through selection in incrementally increasing paclitaxel concentrations.[52,53]

Some cell lines have been treated with chemotherapeutic agents to produce resistant sub-lines.[54] Drug resistant sub-lines of A2780, which are all cisplatin resistant at the corresponding micro molar values, include CP70, C30, and C200. An adriamycin-resistant A2780 cell line, A2780ADR (100nM resistance and cross-resistant to melphalan and vinblastine)[55] and paclitaxel-resistant A2780 sub-lines, PTX10 and PTX22 (5ng/mL and 15ng/mL, respectively)[56], are also available. The IGROV-1 cell line was derived from the ovarian tumor of a patient with stage III human ovarian adenocarcinoma who had not received chemotherapy. By subjecting the chemotherapy-naïve cells to 0.5μg/mL and 1.0μg/mL cisplatin, IGROV-1/Pt 0.5 and IGROV-1/Pt 1 sub-lines were developed. The chemo-resistant 2008 cell line was established from a serous cystadenocarcinoma of the ovary. Monthly treatment of 2008 with 1μM cisplatin with subsequent selection resulted in the 2008 *C13 subline[57]. Deep sequencing data for IGROV-1 cells are available[58] and the line is also included in the NCI 60 cell line panel. Indeed, 6 OvCa cell lines (OVCAR-3, OVCAR-4, OVCAR-5, OVCAR8, IGROV-1, SKOV3) are included in the NCI 60 cell lines and are used by the NCI for *in vitro* drug screening. Therefore, they can be considered as the most extensively characterized set of OvCa cell lines, with drug sensitivity, microRNA, mRNA and proteomic profiling data available.[59] Most importantly, their genetic identity can be verified through DNA fingerprinting[60], allowing reliable confirmation of their identity.

A number of the cell lines are suitable for development as murine xenografts (Supplementary Table S1, Figure 2A–B). OVCAR-3 has been successfully passaged in mice and developed as an i.p. xenograft that retains many characteristics of its human tumor counterpart with histology similar to a serous OvCa.[61] SKOV3[62] and PEO23[63] were collected on relapse after cisplatin and chlorambucil treatment and represent well differentiated serous adenocarcinoma cell lines that form xenograft tumors with a histology that is also very similar to a human serous cancer. Passaging the SKOV3 cells in nude mice allowed the development of several sublines (SKOV3ip.1[64], SKOV3x[65]) that grow as disseminated disease in the abdomens of nude mice. This mouse model is characterized by numerous (100–200) small nodules on the surface of the peritoneum, bowel mesentery, and

diaphragm and the transformation of the mouse omentum into a larger tumor[39,64,65], mimicking human advanced stage serous-papillary OvCa [Figure 2A]. When gene expression in SKOV3 was compared to that of SKOV3ip.1, the latter showed upregulation of v-erb-b2, FGFR1, MMP-19 and downregulation of nm23B[66], possibly explaining the rapid growth of these cells as xenografts. OVCAR-3[67] and OVCAR-5 [Figure 2Ba] have a similar widely disseminated xenograft growth pattern. In contrast, intraperitoneal injection of HeyA8 cells yields a few large tumors that grow in the ovaries and mouse omentum without peritoneal dissemination [Figure 2Bb], mimicking a distinct human OvCa growth pattern.[68] Most OvCa cell lines can be successfully transfected or transduced to express fluorescent or bioluminescent vectors to enable longitudinal optical imaging of tumor growth [Figure 2Ab-c, 2Bc]. Furthermore, the i.p. and s.c. take rate of human OvCa cell lines can be improved by mixing them before injection with Matrigel.[16]

3.2 Clear cell, endometrioid, and mucinous ovarian cancer cell lines

Clear cell carcinoma, which is by definition a poorly differentiated high grade tumor, is represented by several OvCa cell lines [Supplementary Table S1], including ES-2[69], TOV-21G[70], OVISE and OVTOKO[71], and TYK-nu and OVMANA.[72] ES-2 cells exhibit low to moderate resistance to a number of chemotherapeutic agents including doxorubicin, cisplatin, and etoposide. OVISE and OVTOKO were established from metastatic tumors of two patients after treatment with five to six courses of chemotherapy. Both lines are insensitive to cisplatin, doxorubicin, cyclophosphamide, and etoposide. Interestingly, i.p. injection of the OVTOKO cells yields peritoneal implantation and distant metastasis in a mouse model, whereas OVISE cells showed no dissemination and metastasis. OVISE contains a homozygous ARID1A mutation often observed in human clear cell cancers.[6] Both TYK-nu and OVMANA have been fully sequenced and a PIK3CA mutation in OVMANA was identified[72] that recapitulates the genetic changes observed in human clear cell OvCa.[5]

Endometrioid OvCa is represented by several cell lines [Supplementary Table S1]. The first endometrioid cell line was MDAH2774, developed from cells in the ascitic fluid from a patient with endometrioid OvCa and forms tumors in nude mice.[73] 59M was established from the ascitic fluid sample of patient who previously presented with a synchronous stage IA carcinoma of the ovary and an *in situ* carcinoma of the endometrium.[74] COV362 was established from a pleural effusion, and like its sub-clone COV362.4, shows anchorage-independent growth in agar.[75] TOV-112D was derived from a Grade 3, stage FIGO IIIC endometrioid OvCa.[70] A mouse endometrioid cancer cell line[76] has been established from the LSL-KRas^{G12D};Pten$^{fl/fl}$ mouse model.[77] EFO-27, representing a mucinous papillary adenocarcinoma, was derived from a solid omental metastasis.[47]

3.3 Primary ovarian and fallopian tube cells, immortalized lines, and establishing lines from primary ovarian tumors

Establishing primary OvCa cells has remained a challenge as the optimal cell culture medium and ECM to successfully and reproducibly culture primary OvCa has remained elusive even though various methods have been published. During the initial process of establishing a cell line from a patient tumor rapidly growing contaminating cells, such as

fibroblastic and stromal cells, can out-compete the cancer cells in culture. Precautions to avoid non-cancer cell contamination include Percoll gradient separation and isolation with CD45 antibodies.[78,79] Other methods to isolate primary OvCa cells from ascites and solid tumor tissue include enriching tumor cells using immunomagnetic beads coupled to EpCAM antibodies[80] or filtration through a nylon cell strainer[81] and the use of optimized media previously shown to enhance the growth of breast cancer cells.[82] The primary cells may be characterized by EpCAM and HE4 protein expression and CK7 mRNA expression.[81]

Since the OvCa cell of origin may be the abdominal peritoneum, the surface epithelium of the ovary[7] or the fimbriated end of the fallopian tube[83], culturing the primary epithelial cells from these sites is crucial to identifying the pathways involved in the neoplastic transformation of serous OvCa. However, when attempting to propagate these primary epithelia *in vitro*, culture conditions induce senescence and differentiation. Propagation of human immortalized ovarian surface cells (IOSE)[84] and fallopian tube fimbria has been made possible by stable transfection with SV40 T antigen.[85,86] For example, IOSE-29 does not form tumors, while its neoplastic counterpart IOSE29EC forms s.c. and i.p. tumors in SCID mice.[87] Immortalized fallopian tube cell lines used for i.p. injection retain gross, histological, immunophenotypical and genomic characteristics similar to human high grade OvCa.[8,88] When fimbrial cells are cultured on collagen-coated transwells, cell polarization is enhanced, which allows the investigation of DNA damage from chemotherapy and other sources.[88] The use of a 3D alginate scaffold also supports the growth of normal mouse oviducts and ovarian surface cells, as well as baboon fimbria.[44–46] A human fallopian tube cell line has also been established from tumor tissue.[89]

3.4 Mouse and rat ovarian cancer cell lines

The syngeneic mouse model allows OvCa initiation directly from mouse OSE (MOSE). MOSE are scraped from the mouse bursa and are passaged in culture on plastic until phenotypic changes occur, such as the loss of cell contact inhibition, which results in cellular mounds and changes in cell morphology.[90] During neoplastic progression in the cell culture dish, the tumor-suppressor proteins E-cadherin and connexin-43 are lost, distinguishing early from late MOSE.[91] When MOSE are injected i.p. into C57BL6 mice, ascites and metastatic tumors form by about 90 days[92], while s.c. injected cells cause solid tumor formation confined to the injection area. When non-transformed cells are injected i.p. and s.c., no tumor formation is observed. The most commonly used mouse OvCa cell line is ID8, developed *in vitro* from ovaries of the C57BL6 mouse strain.[90] The MOSE cells IG10 and IF5 are cognate tumor-inducing clonal cell lines which *in vivo* have very different survival times and tumor loads (IF5: 182 ±3 days, IG10 72±2 days).

Although the transformation into MOSE occurs *in vitro*, the MOSE system may to some extent recapitulate progression of normal OSE to an epithelial OvCa. A rat ovarian orthotopic xenograft variant has been reported that uses the spontaneously immortalized rat surface ovarian epithelial cell line NuTu-19 in Fisher 344 immunocompetent rat hosts. In this model, development of pelvic extension and peritoneal metastases were dependent upon the number of injected cells.[93] The syngeneic mouse or rat orthotopic models are very useful

for studies requiring the preservation of an intact immune system and can be used to confirm results obtained with xenograft models.

3.5 Cell line authentication

Good cell culture practices and the observation, monitoring, and documentation of cell morphology, behavior, and growth rates will reduce the frequency of cell misidentification. Changes may indicate microbial contamination, genotypic drift due to high passage number, or cross-contamination with another cell line. Given these daily risks and the tremendous implications of using the wrong cell line for experiments, the authentication of cell lines is now being emphasized by scientific journals and granting agencies. Human cell lines should be regularly authenticated and identified using DNA short-tandem repeat (STR) profiling.[94] Although OvCa cell lines derived from a multitude of sub-types are available, and multiple OvCa cell lines have been assessed in a single study, availability of a collection of regularly authenticated, mycoplasma tested, and well characterized epithelial OvCa cell lines would be optimal, a need in the field highlighted by a recent report on the extent of OvCa cell line misidentification [95]. Ideally, the cell lines would be accompanied by extensive genetic/epigenetic and other molecular information.

3.6 Ovarian cancer stem cells

Ovarian cancer stem cells have now been isolated from a number of sources, including primary tumors, ascites, and established OvCa cell lines.[53,96–99] The first report of the isolation and identification of stem cells from OvCa patients described two ascites-derived clones able to form multicellular-aggregate, anchorage-independent spheres in culture, and serially propagate xenograft tumors in nude mice that were histopathologically similar to their parental tumors.[96] A number of surface markers have been used to isolate stem cells from primary patient OvCa (Supplementary Table S2). In addition, subpopulations of tumorigenic stem-like "side populations" cells in cultures of OVCAR3, IGROV-1, and SK-OV-3 have been reported.[98,100] Since these represent a small population of chemoresistant cells in a tumor, the development of model systems that include stem cells may lead to a better understanding of tumorigenesis and drug resistance in OvCa.

3.7 Orthotopic implantation of cell lines and whole tissue tumor grafts ("Xenopatients")

Early events in metastasis cannot be fully appreciated when i.p. xenografts are used, since they rely on the artificial dispersion of single cell suspensions of cancer cells in the peritoneal cavity, rather than dissociation of metastatic cells from an intact primary tumor tissue. Orthotopic ovarian xenografts may mimic more closely the process of dissemination from the primary ovarian tumor relative to the i.p. xenografts discussed above and are therefore well suited for studying metastasis. However, there are significant anatomic differences between rodents, which have a closed bursa at the end of a long and coiled fallopian tube with longitudinal folds enclosing the ovary[101], and humans where the tube is straight, short, and the epithelium is exposed to the peritoneal cavity. Several approaches have been utilized for orthotopic mouse models: injection of cancer cell lines under the ovarian bursa, implantation of human or murine tumor fragments adjacent to the ovary or under the renal capsule, and i.p. injection of minced human tumor.[102–105] Critical steps in the metastatic process are invasion through the ovarian bursa followed by peritoneal spread

with colonization and invasion of the organs in the peritoneal cavity. Intrabursal injection of OvCa cells causes tumor formation in the ovary, but peritoneal dissemination may not occur. Moreover, only a small volume (>5µl) can be injected under the fat pad without causing mechanical disruption of the bursa and peritoneal spillage.

Implantation of whole human OvCa tissue fragments under or adjacent to the ovarian bursa of SCID mice generates ovarian tumors that histologically resemble the tumor of origin.[102] In this model, tumor take rate is approximately 65% at 16 weeks and production of malignant ascites has been recorded, although metastases occurred infrequently. However, in one study using initial implantation of intact human ovarian tumor fragments, metastatic dissemination initiated from orthotopic ovarian tumors was reported following serial passage of human xenografts in the ovarian fat pad.[106] After several passages, peritoneal spread was recorded, resembling the clinical features of the human disease. Higher tumor take (~95%) is observed when fragments of human ovarian tumors are implanted under the renal capsule of SCID mice[104], perhaps reflecting a more permissive host milieu facilitated by the rich kidney vasculature. While tumor growth was generally slow and observed metastases were rare, the histological and immunophenotypic characteristics of the original tumor are preserved in the tumor graft. Growth of less aggressive tumors, such as benign, borderline, and granulosa tumors is also possible when fragments are implanted under the renal capsule.

An alternative method to implantation of full tissue fragments, which requires mouse surgery, is to inject a finely minced primary human tumor slurry directly i.p in mice.[107] Mice are then monitored for engraftment using manual palpation and small animal ultrasound. This methodology has led to a high engraftment rate (~74%) from over 200 consecutive patients with untreated ovarian, primary peritoneal and fallopian tube cancers.[105] The "xenopatients" recapitulated the histological [Figure 2C], molecular and platinum-sensitivity characteristics of the source patient tumors. Thus, "xenopatients" could be useful models for assessing response to therapeutic interventions or biomarker discovery in a manner reflecting the behavior of the original tumor. Indeed, response to platinum chemotherapy was recorded in an orthotopic serous fallopian tube explant, mirroring the clinical response of the donor patient.[108] These data suggest that these models, albeit very labor-intensive, may be useful tools for individualizing patient therapy.

4 GENETICALLY ENGINEERED MOUSE MODELS OF OVARIAN CANCER

Genetically engineered mouse models (GEMMs) of OvCa, which can closely recapitulate human disease, have the potential to greatly improve our understanding of OvCa biology.[109] These models, involving genes expressed or deleted specifically in the ovary or the fallopian tube, allow us to evaluate the physiological relevance of defined genetic changes in OvCa and to better understand the mechanistic significance of genetic lesions revealed in human tumors. Such pre-clinical models are clearly also suited for testing new compounds for cancer prevention[76] and therapy[110,111], since they can be used to model drug resistance as well as contribute to the discovery and validation of novel serum biomarkers. GEMMs of OvCa are summarized in Supplementary Table S3.

4.1 GEMM using various combinations of p53, RB, Myc, Akt and Pten

Generating GEMMs of OvCa in which the fallopian tube or ovarian surface epithelium (OSE) is specifically targeted has proven difficult due to the lack of defined transcriptional promoters active specifically in these cell types. One of the first animal models of OvCa used the TVA receptor system driven by the epithelial specific cytokeratin 5 (CK5) promoter to delete the *p53* tumor suppressor in isolated ovarian surface epithelia *in vitro*.[112] OSE cells in which *p53* had been deleted in this manner were then infected *in vitro* with retroviruses expressing combinations of relevant oncogenes (c-*myc*, K-*ras*, and/or *Akt).* The cells, now with altered gene expression, were transplanted into host mice. This process ensured that tumors were unlikely to arise from tissues other than OSE that express CK5. These mice developed OvCa with papillary structures consistent with serous OvCa and using these mice it was shown that rapamycin inhibits the growth of tumors depending on Akt activity.[110] Subsequent work showed that a single oncogene, in this case c-Myc, was sufficient to drive OSE tumorigenesis when both *p53* and *BRCA1* were deleted using the TVA system.[113] Clearly, this model showed that the ovarian surface is capable of forming tumors when the correct oncogene and tumor suppressor gene mutations are introduced.

The lack of defined cell type specific promoters continues to hinder more conventional transgenic approaches to driving the deletion or activation of relevant tumor suppressors and oncogenes in the OSE. Since the promoters used to express genes in OSE are also active in other tissues of Müllerian origin (fallopian tube, uterus, cervix, ovarian granulosa cells), the origin of emerging "ovarian" tumors in the mouse model is open to question. One of the most effective promoters used to drive Cre recombinase or other transgenes has been the promoter of the Müllerian Inhibiting Substance II Receptor (MISRII) gene, also known as the Anti-Müllerian Hormone Type II Receptor (AMHRII). The MISRII promoter primarily drives transgene expression in the ovarian surface and ovarian granulosa cells, but it also expressed the transgene in the mesenchyme of the oviduct. The first transgenic mouse model to utilize the MISRII promoter used it to drive expression of the SV40 large T antigen (SV40-Tag), a viral oncogene that binds to and inactivates both p53 and all members of the retinoblastoma (Rb) pocket protein family of cell cycle regulators. As a result, approximately 50% of the animals developed large undifferentiated ovarian adenocarcinomas by 90–100 days.[114] In order to more effectively control the timing of oncogene expression, a Lox-STOP-Lox (LSL) cassette was inserted between the *SV40-Tag* and the ubiquitous CAG promoter. Oncogene expression was then induced following direct intrabursal injection of adenovirus expressing Cre-recombinase, which, theoretically, only reached oviductal and ovarian surface epithelial cells.[115] Approximately 89% of the *pCAG-LSL-SV40-Tag* mice injected in this way developed poorly differentiated tumors with metastasis throughout the abdominal cavity, similar to the tumor type and incidence observed when SV40-Tag expression was driven by the MISRII promoter.[114,115] Thus, intrabursal injection and MISRII both effectively target the ovarian surface, while SV40-Tag expression in the oviductal mesenchyme does not induce oviductal tumors.

Cre-Lox technology has been used more extensively to induce targeted deletion of the *Pten* tumor suppressor and to activate the *K-Ras* oncogene. In two separate models, *LSL-KRas^{G12D};Pten$^{fl/fl}$* mice were induced to develop ovarian tumors either through adenoviral

delivery of Cre[77] or through expression of a Cre transgene under the control of the aforementioned MISRII promoter.[116] The *MISRII-Cre; LSL-KRas^{G12D}; Pten^{fl/fl}* mice develop a form of low grade epithelial serous cancer derived from the ovarian surface with 100% penetrance. This is currently the only transgenic mouse that models a low grade serous OvCa.[116] In contrast, when tumors were induced in *LSL-KRas^{G12D};Pten^{fl/fl}* mice by infection of the OSE with adenovirus expressing Cre-recombinase, the mice developed endometrioid or poorly differentiated carcinomas [Figure 2D].[77] In this model, adenoviral vectors that express Cre recombinase were used to selectively express or delete genes in the ovary. These vectors are injected into the ovarian bursa, theoretically only reaching oviductal, ovarian surface epithelial, and bursa cells. However, in practice they sometimes leak out through the injection site and reach uterine tissue. The difference between these two models, both of which target *K-Ras* and *Pten,* is the manner in which Cre is delivered. [77,116] This illustrates the importance of defining the target tissue and developmental timing for deletion/activation of the critical oncogenes and tumor suppressors. For example, follicular depletion and a loss of circulating ovarian hormones such as estrogen are evident in the *MISRII-Cre; LSL-KRas^{G12D};Pten^{fl/fl}* mice[116] but not in the *Ad-Cre; LSL-KRas^{G12D};Pten^{fl/fl}* mice.[117] To what extent such developmental and follicular effects of transgene expression play a role in tumorigenesis in these mice is not clear.

The *Pten* tumor suppressor has also been deleted in mice in combination with the *Apc* tumor suppressor, a signaling molecule in the Wnt/β-catenin pathway.[118] The combined deletion of *Pten* and *Apc* by intrabursal injection of Cre-recombinase expressing adenovirus resulted in the formation of the endometrioid sub-type OvCa in all injected animals[118], similar to that seen in the *Ad-Cre; LSL-KRas^{G12D};Pten^{fl/fl}* mice.[77] These animals develop a more aggressive phenotype when additional mutations in p53 and in PI3Kca are introduced.[119] Based on the observation that the AKT pathway is up-regulated in human OvCa due to the mutation or amplification of the *PIK3CA* gene[5], a mouse model has also been designed with an activating mutation in PI3K (*PIK3CA^{H1047R}*) that, when combined with deletion of *Pten,* led to robust activation of the Akt pathway. After intrabursal adenoviral Cre-recombinase injections, the *Ad-Cre; PIK3CA^{H1047R};Pten^{fl/fl}* mice formed ovarian serous adenocarcinomas or granulosa cell tumors.[111] This model has been used to test the efficacy of a PI3K/mTOR pathway inhibitor which was initially effective in inhibiting tumor growth, although the tumors eventually became resistant.[111] These distinct mouse models of OvCa in which *Pten* is deleted demonstrate that *Pten* deletion alone is not sufficient for tumor formation, but the specific context of *Pten* deletion in the OSE or in the OSE and granulosa cells impacts the histotype and grade of the cancer formed.

4.2 GEMM of BRCA mutations

The *BRCA1* and 2 genes are either mutated or silenced by methylation in approximately 20% of all high grade OvCa.[4] Several conditional *BRCA1* and *BRCA2* mouse models have been generated. In these, the *BRCA* allele, flanked by LoxP sites, is deleted either by Cre-recombinase expression driven by the MISRII or FSHR promoter, or by adenoviral injection.[117,120–122] Targeted deletion of only *BRCA1* or *BRCA2* in the ovaries is generally not sufficient to induce malignancy, although FSHR promoter driven deletion of only *BRCA1* did generate cystic tumors in one model.[117] The prolonged estrous phase and

enhanced estrogen signaling in this model may account for this phenotype, since FSHR is expressed in granulosa, ovarian surface, and oviductal cells.[117] Tumor formation may be influenced either by loss of *BRCA1* in cells other than those of the ovarian surface or oviduct or by the effects of increased estrogen signaling in cells without *BRCA1*. Conditional loss of *BRCA1* alone (Cre-recombinase injection into the bursa) was insufficient to generate tumors and resulted in hyperplasia of the OSE. Combining loss of *BRCA1* with loss of *p53* resulted in either leiomyosarcomas or no tumors at all, unless the Rb family was also inactivated.[120] Loss of BRCA1 in combination with p53 (BRCA1$^{LoxP/LoxP}$ p53$^{LoxP/LoxP}$) led to development of a leiomyosarcoma but not a serous OvCa.[120–122] In a recently reported mouse model[122] the key event initiating OvCa was the inactivation of the Rb tumor suppressor and related p107/p130 pocket proteins through targeted expression of a mutant form of the SV40-Tag that cannot inactivate p53. This, when combined with the inactivation of *p53* and *BRCA1* deletion, caused progression to advanced serous OvCa. Similarly, combined loss of *p53* and Rb function led to tumor formation.[123]

4.3 GEMM of fallopian tube cancer

Since the emergence of the hypothesis that the fallopian tubes may be the primary site of origin of high grade serous carcinoma[124], a few models have been introduced to help explain potential genetic causes of tubal cancers[109]. When the oviductal glycoprotein (OVGP) gene promoter was used to drive SV40-Tag expression, mice developed oviductal, uterine and vaginal tumors.[125] Tumor cells were occasionally found in the ovaries, but it was not determined if these represented the primary lesion or a metastasis. Recently, *MISRII-Cre; Pten$^{fl/fl}$;Dicer$^{fl/fl}$* mice have been generated in which both the *Pten* and the *Dicer* gene, required for efficient micro-RNA processing, were deleted in all tissues of Müllerian duct origin. These mice developed tumors resembling high-grade serous OvCa in the mesenchyme of the oviduct that metastasized to the peritoneum, the diaphragm, and formed ascites.[126] Histologic analysis and gene expression profiling suggested that these tumors resembled high-grade serous cancer in humans; however, the tumors did not have mutations in *BRCA*, *p53* or deficiencies in homologous repair.[126] This GEMM consistently develops high-grade serous cancer, which metastasizes within the abdominal cavity in a very similar fashion to human OvCa. Interestingly, a recent abstract suggests that a *Pten$^{fl/fl}$; Dicer$^{fl/fl}$/p53LSL$^{R172H/+}$* triple knock out develops both tubal and ovarian tumors.[127] Thus, it remains to be determined whether the epithelial expression of the "p53 signature" in *BRCA*-mutant human OvCa and the formation of serous cancers from the stroma in this mouse model[126] can be reconciled in terms of human disease origin. Lastly, very recent reports suggest that an inducible Pax8-Tet^{ON} system can generate a high grade serous cancer from the fallopian tube epithelium when *p53* and *BRCA1* or *BRCA2* are simultaneously deleted.[128] The tumors were more likely to be found in the proximal rather than the distal portion of the oviduct. It is the distal portion that is anatomically comparable to the fimbriae of the human fallopian tube and is the more likely site of OvCa formation. This discrepancy in tumor localization when compared to human disease may be explained by the relative strength of the Pax8 promoter in transgenic mice.

GEMMs offer the potential to introduce specific modifications to pathways in a time and tissue dependent manner that can help to confirm or deny the importance of genetic

mutations in disease onset and chemoresistance. Other advantages of GEMMs are that OvCa develop disease in a shorter time period than non-human primates and chickens allowing studies to be completed quickly. With the use of viral delivery to introduce cre-recombinase, mutations can be turned on and off in different stages of tissue development. Criticism of GEMMs were raised due to concerns that the existing GEMMs do not employ genetic lesions relevant to human high grade serous cancers and, in many instances, may be driven by inappropriate regulatory sequences and promoters, which may explain, in part, their failure to recapitulate the peritoneal dissemination clinically observed in human OvCa. Unfortunately, OSE do not express a tissue specific promoter and a promoter only expressed in specific epithelial cells of the fallopian tube (e.g. secretory cells[8]) has not yet been uncovered. Another drawback of GEMMs is that they can produce different phenotypes due to the background strain of the mouse model.[120,123] Since human OvCa is genetically complex, it is likely that genetic mouse models represent only one particular molecular subtype of OvCa, rather than the entire spectrum of the corresponding human disease.[4]

5 THE LAYING HEN MODEL OF EPITHELIAL OVARIAN CANCER

One of the major obstacles to progress in OvCa prevention and treatment is a dearth of spontaneous disease models. The primary animal model available for the study of spontaneous epithelial OvCa is the laying hen.[129] As early as the 1960s, reports identified domestic egg-laying hens as probable surrogates for human OvCa and suggested that they be investigated as potential models.[130,131] A landmark large scale epidemiological study documented an age dependent onset of the disease, with the first occurrence of OvCa observed in hens of 2–2.5 years of age with an incidence of 4%, while in hens aged from 4–6 years the incidence was 30–50%.[132] These findings have been confirmed in a recent study demonstrating that the first observable incidence of spontaneous OvCa was in hens aged between 2–2.5 years with an incidence of epithelial OvCa of all stages that reached a peak of nearly 60% at 4 years.[133] A distinct advantage of pre-clinical studies in laying hens is that large-scale studies with hundreds of animals, conducted over extended periods of time, can be performed at relatively little cost. After a hen has completed her 2nd year of laying, producing from 300 to 500 eggs, she has ovulated approximately the same number of times as a woman who has undergone menopause, when the risk of OvCa increases dramatically.[134] A considerable body of epidemiological data supports the link between the number of lifetime ovulations and OvCa risk. Specifically studies using the domestic hen, support the "incessant ovulation" hypothesis by showing that when ovulation is arrested in hens, either (i) pharmacologically by dosing with progestins[135], (ii) using a model of restricted ovulation through nutrient deprivation, or (iii) via genetic selection for low-egg laying variants the incidence of OvCa is lower.[135–137] The high rate of OvCa in egg-laying hens could be the consequence of genomic damage to the ovarian surface epithelium associated with incessant ovulations or may indirectly result from facilitated genetic changes or impaired DNA repair which ultimately lead to the development of OvCa in the hen. A direct relationship between ovulation and OvCa remains to be determined.[129]

5.1 Description of the hen model

Epithelial OvCa in the hen presents with many features of the human disease [Figure 3A, B] including heterogeneity with at least four clearly distinguishable histological subtypes. In contrast to human OvCa, in which serous disease represents 70% of late stage disease, 50% of late stage tumors in the hen have histologic features of endometrioid carcinoma in the primary tumor and corresponding peritoneal metastases.[138,139] However, similar to their human counterparts, hens exhibit clear cell, endometrioid, mucinous and serous histological subtypes [Figure 3C]. The clinical presentation in hens is similar to that in women, in that hens develop substantial volumes of ascites fluid, peritoneal dissemination of metastases, and pulmonary emboli. Hens develop solid tumors in the lung parenchyma, which is rare in women with advanced serous OvCa, but may be observed in human endometrioid or clear cell cancers.[140]

Certain molecular markers of human OvCa are reproduced in the hen including early expression of E-cadherin [Figure 3D][139], up-regulation of COX-1[141], alterations in p53, K-Ras and Her2/neu[142]; expression of CA125[143], and several surrogate immunohistochemical markers, including PCNA, p27, TGFβ1, cytokeratin AE1/AE3, pan cytokeratin, EGFR, Lewis Y, CEA, Tag 72, and erbB-2.[144] Gene profiling studies and bioinformatics comparisons between laying hen and human cancer gene expression patterns have further demonstrated the conservation of genetic lesions associated with OvCa and histotype-specific mutations.[142,145,146] Because of the importance of the chicken as a meat-animal and its extensive use as a model for developmental studies, robust genomic resources for the chicken have been developed, including cDNA, long-oligo and GeneChip™ genome arrays.[147,148] The chicken epi-genome has also been mapped.[149] Extensive chicken genomic resources are available via the NCBI[150], the Genome Institute at Washington University, and in a recently published collection of chicken genomic resources.[151]

5.2 Prevention

The first pre-clinical intervention study in the laying hen evaluated the efficacy of medroxyprogesterone acetate (Depo-Provera®) in reducing the frequency of spontaneously developing reproductive tract adenocarcinoma, showing a 15% risk reduction in animals treated with the progestin.[135] The authors observed that the high rate of reproductive tract adenocarcinoma further supported the use of the laying hen model of spontaneous ovarian carcinogenesis to test chemoprevention strategies. A recent chemoprevention trial in hens studied treatment with progestin alone, estrogen alone and progestin plus estrogen.[152] Results show that treatment with progestin alone and in combination with estrogen decreased the prevalence of OvCa with a concomitant reduction in egg laying.[135,152] Both groups concluded that their findings were consistent with the theory that a reduction of ovulatory events may prevent the development of ovarian adenocarcinoma.[135,152] The utility of the hen model for pre-clinical evaluation of therapeutic modalities for the prevention of OvCa (reviewed by Rodriguez[153]) has been demonstrated in studies testing the efficacy of progestins[135,152], anti-inflammatory NSAIDs[154]; and dietary intervention with flaxseed.[155]

5.3 Studies of early ovarian cancer in hens

A distinct advantage of the hen model of OvCa is that the disease arises spontaneously in chickens and therefore presents investigators with the opportunity to study the initial events leading to OvCa, identify targets for prevention, and devise and test interventions. One example of an early event in OvCa development that has been observed in hens and corroborated in human clinical specimens is the presence of anti-ovarian auto antibodies in the serum of hens with early stage OvCa.[156,157] Another example is the increased expression of E-cadherin in hen OvCa, which is clearly seen at the point of neoplastic transformation where normal surface epithelium transitions morphologically into precursor lesions which develop into early primary tumor sites (as shown in Figure 3Da,c).[139] Similar patterns of increased E-cadherin in inclusion cysts of human ovarian tumors have been described.[7] In addition, the pattern of expression of tubal genes in primary ovarian tumors of the hen [158] and repetition of the "p53-signature" in pre-neoplastic lesions of the tubal and ovarian surface epithelium of the hen [141] are conserved in the hen analogous to humans.

5.4 Imaging in hens

Doppler ultrasonography has been employed to analyze the kinetics of an ultrasound contrast agent used to identify and follow ovarian tumor-associated neo-angiogenesis in early-stage OvCa in the hen. Findings were corroborated on necropsy.[155,159] The kinetics of the ultrasound contrast agent was shown to be indicative of ovarian tumor-associated neo-angiogenesis in early stage OvCa. The use of contrast-enhanced ultrasound for the detection of early ovarian tumors demonstrates the potential for the development of new clinical diagnostics tools using the hen model. Moreover, the hen model may also be useful for testing the efficacy of different contrast agents in a preclinical setting.

5.5 Limitations and challenges

There has been very slow progress in the development of stable cell lines derived from chicken OvCa or ascites for mechanistic studies in hens. Ovarian surface epithelial cells, tumor cells from primary cancer, and tumor cells isolated from ascites fluid from hens have been described, but as yet these studies have been limited to primary cultures or cell cultures with low passage numbers.[160] Similarly, there has been very little progress in the development of transgenic chicken models for the manipulation of targeted genes by over-expression, mutation, ablation, and silencing. Promising studies, however, have shown that chick embryos can be genetically manipulated, offering hope for the eventual development of transgenic chicken models of OvCa.[161,162]

Another challenge of working with hens is the limited availability of chicken specific reagents, such as antibodies for immunohistochemical analyses. A panel of commercially available surrogate cancer antigen antibodies has been examined for their cross-reactivity in hens[144] and successfully adapted for the use with hen tumor tissue using robust antigen-retrieval procedures. However, despite the clearly demonstrated power of the hen model and its potential for evaluating therapeutics and preventatives, only a handful of investigators have taken advantage of the model. Access to experimental poultry facilities and lack of capacity for chickens in standard biomedical vivarium facilities limit more widespread adoption of the hen as the standard animal model for OvCa research.

6 SUMMARY

Within the last five years, significant advancement has been made in developing a more comprehensive suite of tools with which to experimentally address the pathobiologic mechanisms that underlie the poor survival of OvCa patients. Despite this progress, the conduct of experimental translational research in OvCa remains limited by substantial challenges. For example, a large number of OvCa cell lines have been derived and maintained in culture, some of which exhibit a chemoresistant phenotype. The value of this resource would be substantially enhanced by the establishment of a "biobase" collection of regularly authenticated, well characterized, and well annotated cell lines for distribution to the research community, similar to the resource available to the breast cancer research community.[163] Access to patient-matched normal and tumor cells, normal epithelial and mesenchymal cells from other primary sources (for example, fallopian tube), and enhanced access to well-annotated patient tissue would also facilitate research progress. Several complex 3D and organotypic models of OvCa metastasis have been developed that take into account cell and tissue architecture and the contributions of extracellular matrix as well as stromal and inflammatory cells in the tumor microenvironment. Lacking, however, are models that contribute to our understanding of the very earliest events in OvCa metastasis; the shedding of cells from the primary tumor. Use of these models to elucidate molecular events that initiate and regulate the "terminal transition" from free-floating ascitic cell or multi-cellular aggregate to peritoneally anchored metastatic lesion may identify novel therapeutic targets to block metastasis. A challenge will be adaptation of these 3D and organotypic models to a high throughput format to facilitate drug screening. The use of murine OvCa models with orthotopic injection into the peritoneal cavity, ovarian bursa, or kidney capsule has proven utility to address a number of important research questions. However, evolution in our understanding of OvCa initiation, progression and early stages of metastasis is hampered by the need for improved genetic models. Identification of more precise tissue-specific promoters to target genetic lesions (or sets of mutations) identified in human OvCa tumors to specific tissues in the reproductive tract (ovarian epithelium, fimbriae of fallopian tube, etc.) would promote progress. In this regard, the laying hen model of spontaneous OvCa holds promise and the development of additional research tools, reagents, and approaches for use in this model system is warranted. Lastly, as we realize that "ovarian cancer" is many diseases, sub-type specific models will need to be developed and tested. With careful calibration of reagents and experimental models, together with enhanced collaboration, we are poised for significant advancement of our goal to identify novel, effective, and personalized approaches to detect and treat ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

AMHRII	Anti-Müllerian Hormone Type II Receptor
CK5	Cytokeratin 5
ECM	Extracellular Matrix
GEMM	Genetically Engineered Mouse Model
GFR	Growth factor-reduced
IOSE	Immortalized Ovarian Surface Epithelium
i.p	intraperitoneal
LSL	Lox-STOP-Lox
MISRII	Müllerian Inhibiting Substance II Receptor
MOSE	Mouse Ovarian Surface Epithelium
MT1-MMP	Membrane Type 1 Matrix Metalloproteinase
OvCa	Ovarian Cancer
RB	Retinoblastoma
s.c	subcutaneous
STR	Short-Tandem Repeat (STR) profiling
TME	Tumor Microenvironment

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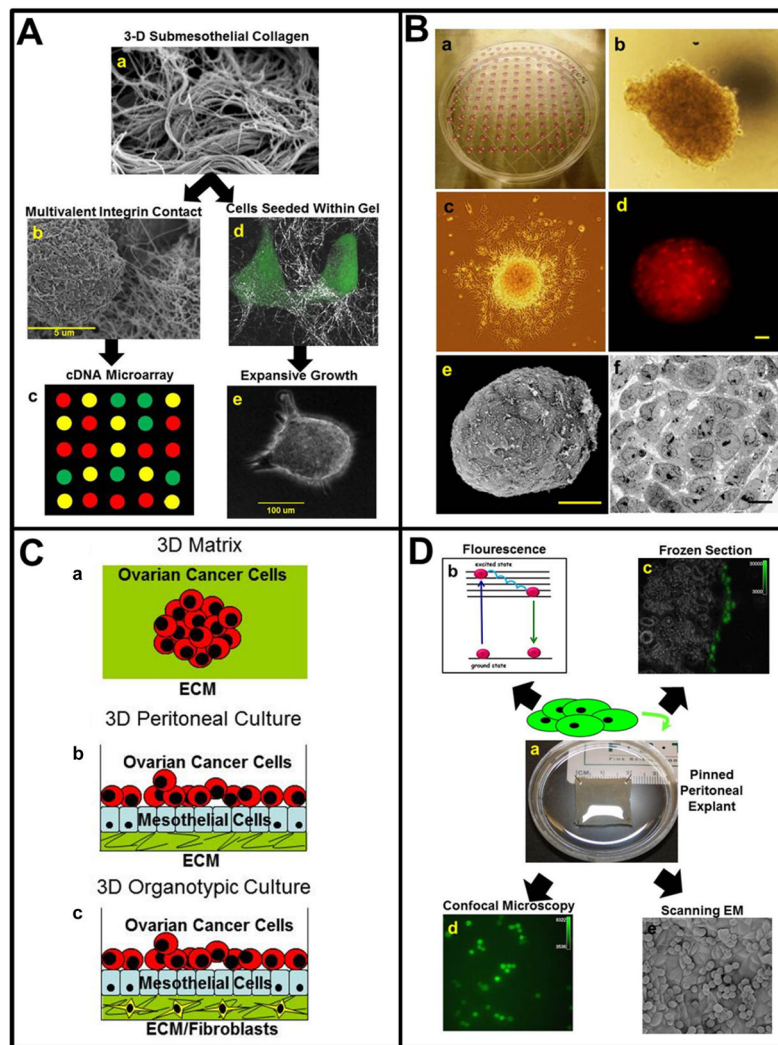
FIGURE 1. Three-dimensional organotypic and *ex vivo* models of ovarian cancer metastasis
A) Three-dimensional (3D) culture models of cellular contact with sub-mesothelial collagen. (**a**) Following initial intra-peritoneal adhesion, mesothelial cell retraction exposes the sub-mesothelial interstitial collagen-rich matrix to which ovarian cancer (OvCa) cells avidly adhere. Scanning electron micrograph of murine submesothelial collagen matrix. (**b**) Initial multivalent cell-matrix contact of OvCa cell (left) with collagen (right) is visualized by SEM. (**c**) Culturing cells atop 3D collagen (as in b) followed by cDNA microarray analysis can reveal changes in gene expression that result from the initial adhesive contact. (**d**) Metastasizing cells migrate into the 3D collagen matrix to anchor metastatic lesions. Confocal reflectance microscopy overlaid with fluorescence microscopy visualizes cells within the 3D matrix. (**e**) Cells seeded into a 3D matrix proliferate in a matrix metalloproteinase-dependent manner to form expansive matrix-anchored multicellular aggregates. Micrographs shown in a, b are courtesy of Yueying Liu, University of Notre Dame and Dr. Katarina Wolf, Radboud University Nijmegen, The Netherlands. Photographs and micrographs shown in e are courtesy of Dr. Natalie Moss, Northwestern University.

B) Multicellular aggregate (MCA) cultures mimic non-adherent OvCa cells in ascites.
(**a**) To generate MCA cultures, cells are suspended at a concentration of 100,000 cells/ml and seeded as 20 μl droplets on the inner surface of a tissue culture dish lid. After addition of PBS to the culture dish to maintain humidity, the lid is gently inverted. Following aggregation, individual MCAs may be subcultured for use in additional assays. (**b**) Light micrograph of individual MCA generated using DOV13 cells. (**c**) Dispersal of MCA generated from DOV13 cells. An individual MCA was subcultured onto a coverslip coated with type I collagen and photographed after 12 hours. Dispersal can be quantified by DAPI staining and measuring inter-nuclear distance.[2] (**d**) Fluorescence micrograph of individual MCA generated using CellTracker Red-labeled DOV13 cells. (Scale bar 30 μm) (**e**) Scanning electron micrograph of MCA generated using DOV13 cells. MCAs were placed in primary fixative (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M Cacodylate buffer pH 7.35) followed by fixation with osmium tetroxide, dehydration in ethanol, and critical point drying. Platinum coated samples were examined using a Hitachi S-4700 Field Emission Scanning Electron Microscope (scale bar 50 μm). (**f**) Transmission electron micrograph of MCA generated using DOV13 cells. Following primary fixation as described in e, samples were encapsulated in HistoGel, fixed with osmium tetroxide, dehydrated in ethanol and acetone, infiltrated with Epon/Spurr's resin and cut into ultrathin sections. Sections were mounted on nickel grids, stained with uranyl acetate and Sato's Triple Lead stain and examined using a JEOL 1400 transmission electron microscope (scale bar 10 μm). Micrographs shown are courtesy of Yuliya Klymenko, University of Notre Dame.

C) Ovarian cancer *in vitro* models recapitulate peritoneal metastasis. (**a**) Ovarian cancer growing as spheroids in a 3D matrix. (**b**) A 3D peritoneal culture using primary human peritoneal mesothelial cells and extracellular matrix to investigate OvCa cell adhesion. (**c**) A 3D organotypic "meso-mimetic" culture of the peritoneal cavity. Primary human omental fibroblasts are embedded in extracellular matrix and a layer of primary human omental mesothelial cells plated on top. Ovarian cancer cells are added to the culture and adhesion, invasion, and proliferation of the cancer cells is investigated. Using fluorescently-labeled OvCa cells, changes in gene and protein expression can be individually evaluated in the cancer and stromal cells.

D) *Ex vivo* peritoneal explant model of early events in intra-peritoneal adhesion. (**a**) Optically clear silastic resin is generated using a Sylgard® 184 silicone elastomer kit (Fisher), using approximately 6 ml per well of a 6-well culture plate. Dissected murine peritoneal tissue obtained from a midline incision is pinned mesothelial-side up to the silastic resin and immersed in PBS. Tissue integrity is maintained for up to 48 h. Addition of fluorescently tagged tumor cells to the explant enables monitoring by (**b**) relative fluorescence of cell lysate, (**c**) fluorescence microscopy of frozen sections, (**d**) confocal microscopy, or (**e**) scanning electron microscopy. Beneath the tumor cells in (**e**, round), the cobblestone mesothelium is visible. Note that images in panels (**b–d**) are representative of a 2 h time point following addition of OvCa cells to the explant. Photographs and micrographs shown are courtesy of Yueying Liu, University of Notre Dame.
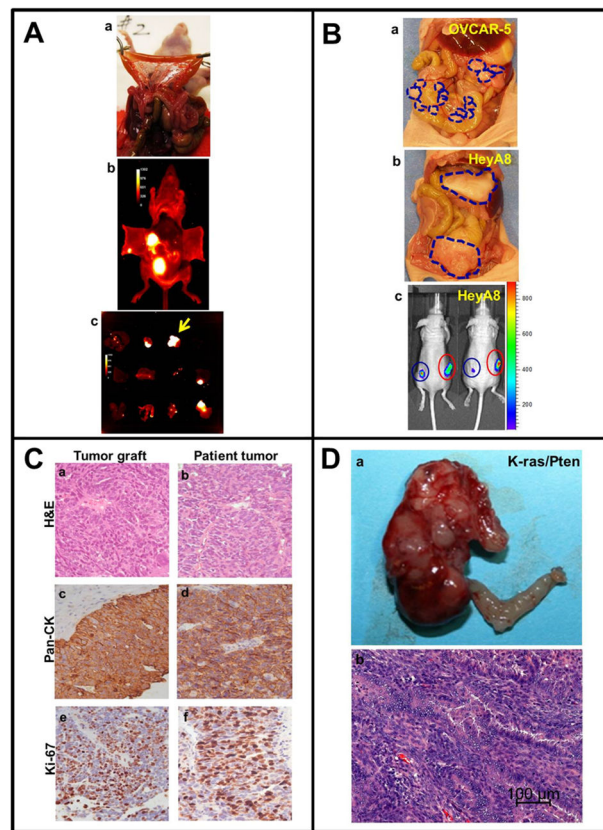
**FIGURE 2. Cell line-based and patient-derived *in vivo* xenograft models**
A) Optical imaging of disseminated i.p. metastasis. (**a**) SKOV3ip.1 cells were transduced using a lentiviral vector to express red fluorescent protein (RFP), followed by i.p. injection (5×10^6 cells) into nude mice. After 24 days, mice were sacrificed, the peritoneal cavity opened and metastatic nodules visually enumerated. (**b**) Fluorescent images of peritoneal organs *in situ* were taken using a Xenogen IVIS Lumina imaging system. (**c**) Dissected organs were imaged using a Xenogen IVIS Lumina system. Yellow arrow indicates dissected omentum. Photographs shown are courtesy of Yueying Liu, University of Notre Dame.

B) Distinct *in vivo* growth patterns of ovarian cancer cells mimic human metastatic dissemination. (**a**) OVCAR-5 cells (1×10^6) were injected i.p. into nude mice. After 45 days, mice were sacrificed. Blue lines outline widely disseminated growth of the xenograft as small peritoneal nodules. (**b**) HeyA8 cells (1×10^6) were injected i.p. into nude mice. After 28 days, mice were sacrificed. Blue lines outline the growth of large tumors. (**c**) HeyA8 cells (0.5×10^5) expressing luciferase were injected s.c. into the mouse flank in the absence (blue circle) or presence (red circle) of carcinoma-associated fibroblasts (CAFs, 1×10^5). After 14 days, mice were subjected to longitudinal *in vivo* bioluminescence imaging using the Xenogen IVIS 200 Imaging System. Photographs shown are courtesy of Dr. Marion Zillhardt, University of Chicago.

C) Histological comparison of patient tumor and "xenopatient" graft in the mouse. Following surgical removal of a serous ovarian tumor, a 1 cm fragment was finely minced and injected i.p. into SCID/Beige mice. At about 20 weeks, mice were sacrificed and tumors

collected. Mouse tumor grafts (**a,c,e**) and patient tumors (**b,d,f**) were subjected to staining with hematoxylin and eosin (H&E, **a,b**), pan-cytokeratin (**c,d**), or Ki67 (**e,f**), as indicated. Photographs shown are courtesy of Saravut John Weroha, Mayo Clinic.

D) Formation of ovarian tumors in *LSL-KRas^{G12D}; Ptenfl$^{/fl}$* mice. Tumors were initiated by injection of adenovirus expressing cre recombinase in the right ovarian bursa. The left ovary was not injected and served as an internal control. Mice were sacrificed after 8 weeks and the primary tumor was excised (**a**), embedded in paraffin, and subjected to staining with H&E (**b**). Photographs shown are courtesy of Dr. Iris Romero, University of Chicago.
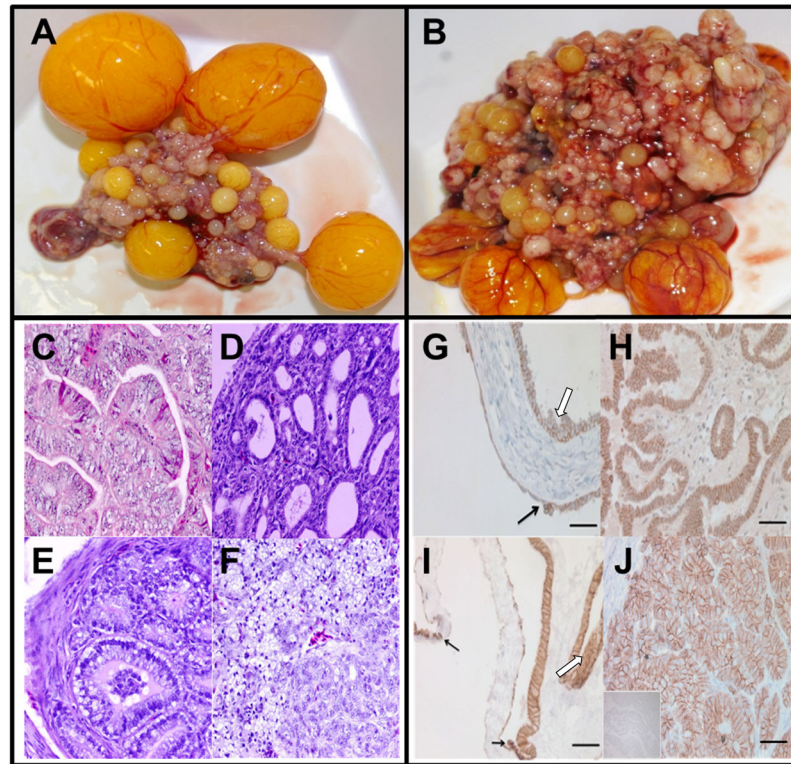
FIGURE 3. The domestic egg laying hen model of ovarian cancer

A) Normal ovary of a domestic laying hen. The ovary contains a set of 4 large pre-ovulatory hierarchical follicles, small developing follicles, and a post-ovulatory follicle.

B) Primary malignant ovarian tumor in laying hen with stage IV OvCa. The tumor was classified as a serous OvCa on an endometrioid background. The tumor has metastasized to distant organs with profuse ascites and had an endometrioid histotype. Multiple solid tumor masses are seen.

C) Histological types of malignant ovarian tumors in hens. (**a**) Ovarian serous carcinoma showing sheets of lacelike papillary folding and cells with large pleomorphic nuclei with mitotic bodies. (**b**) Ovarian endometrioid carcinoma with confluent back-to-back glands. Glands contain a single layer of epithelial cells with sharp luminal margin. (**c**) Ovarian mucinous carcinoma with crowded glands in clusters without intervening stroma surrounded by a fibromuscular layer. The epithelium contains columnar and intercalated ciliated goblet cells. The nuclei are separated from the basement membrane and have moved toward the apical surface with occasional stratification and luminal secretion. (**d**) Poorly differentiated ovarian clear cell carcinoma showing vacuolated cells containing high-grade nuclear atypia that invade the stroma and theca layer of stromal follicles. Deposition of eosinophilic hyalinized matrix in the stroma and necrotic bodies are also seen. Hematoxylin and eosin staining (original magnification 40X).

D) Immunohistochemical localization of E-cadherin expression in human (a,b) and laying hen (c,d) normal ovarian and cancerous tissue. The uninvolved epithelial cells in normal human ovarian tissue (**a**) and in normal laying hen ovarian tissue (**c**) lack E-cadherin, while early neoplastic transformation (black arrow) of adjacent 'normal' ovarian surface

epithelial cells and mild to moderate dysplastic cells (white arrow) are marked by an increase in E-cadherin expression. Tumor cells of a human endometrioid type tumor (**b**) and a primary hen tumor (**d**) express high E-cadherin levels in distinct patterns throughout the tissue (bar= 50µm).