Molecular and Cellular Pathobiology

Perturbation of Rb, p53, and Brca1 or Brca2 Cooperate in Inducing Metastatic Serous Epithelial Ovarian Cancer

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Abstract

The majority of human high-grade serous epithelial ovarian cancer (SEOC) is characterized by frequent mutations in p53 and alterations in the RB and FOXM1 pathways. A subset of human SEOC harbors a combination of germline and somatic mutations as well as epigenetic dysfunction for BRCA1/2. Using Cre-conditional alleles and intrabursal induction by Cre-expressing adenovirus in genetically engineered mice, we analyzed the roles of pathway perturbations in epithelial ovarian cancer initiation and progression. Inactivation of RB-mediated tumor suppression induced surface epithelial proliferation with progression to stage I carcinoma. Additional biallelic inactivation and/or missense p53 mutation in the presence or absence of Brca1/2 caused progression to stage IV disease. As in human SEOC, mice developed peritoneal carcinomatosis, ascites, and distant metastases. Unbiased gene expression and metabolomic profiling confirmed that Rb, p53, and Brca1/2-triple mutant tumors aligned with human SEOC, and not with other intraperitoneal cancers. Together, our findings provide a novel resource for evaluating disease etiology and biomarkers, therapeutic evaluation, and improved imaging strategies in epithelial ovarian cancer. Cancer Res; 72(16); 4141–53. ©2012 AACR.

Introduction

Ovarian cancer is the fifth deadliest disease among American women (1), with more than 50% presenting with serous papillary histology. High-grade serous epithelial ovarian cancer (SEOC) is associated with intraperitoneal spreading (carcinomatosis) and distant metastases. Standard treatment is aggressive surgical resection followed by platinum-taxane chemotherapy. Platinum-resistant cancer recurs in approximately 25% of patients within 6 months (2), and the overall 5-year survival probability is 31% (3). Current methods for early detection, such as transvaginal ultrasound and blood cancer antigen 125 (CA125) levels have shown limited effectiveness (4, 5).

The Cancer Genome Atlas (TCGA) study of a large high-grade SEOC cohort revealed that mutations in TP53 predominated, occurring in at least 96% of tumors (6). Brca1 and Brca2 were mutated in 22% of tumors, representing a combination of germline and somatic mutations (6). Known cancer-associated pathways such as RB1 and PI3K/RAS were deregulated in 67% and 45% of cases, respectively (6).

Advances in modeling cancers in genetically engineered mice (GEM) have facilitated mechanistic understanding of tumor development and applicability in preclinical studies for several cancers (reviewed in refs. 7–15). GEM models of endometrioid (10) and other epithelial ovarian cancers (11–15) have been generated based on alterations of specific genetic pathways. While 2 studies showed that concomitant inactivation of Brca1 and p53 or Brca1 and p53 in mouse ovarian surface epithelium (OSE) cells can give rise to carcinomas with serous histology, respectively (11, 16), in 2 other studies ovary-specific conditional inactivation of Brca1 and p53 in the presence or absence of Rb, resulted in ovarian leiomyosarcomas (13, 15). Despite these extensive studies, the combination of pathway perturbations leading to consistent initiation and progression of metastatic SEOC-like disease in the mouse has been elusive. Here, we describe a comprehensive temporal study of the most common pathways altered in human SEOC. Using selective induction of individual or combinations of Brca1 or Brca2, p53 knockout and/or mutant alleles and Rb tumor suppression (Rb-TS) inactivation, we analyzed the relative contribution to disease progression from tumor initiation to invasive carcinomas with carcinomatosis and distant metastases. Genome-wide expression, serum metabolomic, and marker studies indicate that perturbing all 3 pathways yields disease arising from OSE with the characteristics of human SEOC.
Materials and Methods

Experimental animals

All experimental animals were maintained in accordance with the Institutional Animal Care and Use Committee (ACUC) and the NIH Guide for the Care and Use of Laboratory Animals. All procedures conducted on mice were approved by the ACUC of Frederick National Laboratory for Cancer Research (FNLCR), Maryland. *Brcal*Δ/Δ (*FVB;129-Brcal*tm1brn*<sup>neo</sup>*), *Brcal*<sup>2R</sup>*<sup>neo</sup> (STOCK *Brcal*tm1brn*<sup>neo</sup>*), *p53<sup>B14B</sup>* (FVB;129- *Trp53<sup>p53<sup>B14</sup>B</sup>*)<sup>neo</sup>*), and *p53<sup>534</sup>*<sup>neo</sup>* (FVB;129- *Rosa26STOPfl<sup>lox</sup>lacz*) mice were obtained from the MMHCC Mouse Repository (National Cancer Institute, Rockville, MD). Conditional *TgK18GT<sup>121</sup>*<sup><sup>fl</sup></sup>+<sup><sup>+</sup></sup> BAC transgenic mice were generated by insertion of a floxed eGFP STOP T<sub>121</sub> cassette several base pairs upstream of the first ATG start codon in exon 1 of the keratin 18 gene by recombineering (Song and colleagues; manuscript in preparation). *Rosa26STOPfl<sup>lox</sup>lacz* reporter mice (B6;129- *Gt(Rosa)26Sor<sup>p53<sup>fl</sup></sup>*) were purchased from the Jackson Laboratory.

Adenovirus administration

Recombinant adenovirus Ad5-CMV-Cre (Adeno-Cre) was purchased from the University of Iowa Transfer Vector Core at a titre of 10<sup>11</sup> to 10<sup>12</sup> infectious particles/mL. To show our capability to successfully conduct intrabursal inductions, we conducted intrabursal injections of Adeno-Cre as previously described (11) on 7- to 8-week-old female ROSA26STOP<sup>lox</sup>lacz reporter mice. The right ovary was injected with approximately 7 µL of Adeno-Cre virus into the ovarian bursa near the oviducts during surgical surgery 36 hours following super ovulation. Injected ROSA26STOP<sup>lox</sup>lacz females were euthanized 2 weeks postinjection, ovaries were removed and whole mounts stained for LacZ expression (Supplementary Materials and Methods).

Tissue collection, pathologic, and immunohistochemical analysis

Animals were euthanized by CO<sub>2</sub> inhalation, ovaries and other affected organs were fixed in 10% neutral buffered formalin, processed for paraffin embedding, and characterized by microscopic evaluation. Five micrometers serial sections were cut for hematoxylin and eosin and immunohistochemistry. Pathologic evaluation of histologic findings was carried out by a board certified veterinary pathologist (P.L. Martin) according to existing human epithelial ovarian cancer classifications [International Federation of Gynecology and Obstetrics (FIGO)].

Paraffin sections were deparaffinized in xylene, rehydrated in ethanol according to standard protocol, and subsequently used for immunohistochemical stains. Antibodies and conditions used for immunohistochemical stains are listed in Supplementary Materials and Methods. For all immunohistochemical stains 3,3'-diaminobenzidine (Sigma) substrate was used to visualize peroxidase activity followed by hematoxylin counterstain, dehydration, and mounting.

Detailed procedures and primer sequences used for genotyping and detection of recombined alleles, LacZ staining, MRI, microdissection and sequencing, metabolome profiling, and gene expression profiling can be found in Supplementary Materials and Methods.

Results

Conditional inactivation of RB tumor suppression in OSE causes stage I SEOC

On the basis of the frequency with which Rb is altered in human cancers, redundancy among the pRb family proteins appears to be more widespread in the mouse than in the human. Indeed, recent cancer genome studies indicate that several points in this pathway are altered, but in a mutually exclusive pattern for individual tumors. The sum of these lesions indicates that most human solid malignancies, including SEOC, are aberrant in RB-TS (17). Given the limitation of allele combinations possible in the GEM experimental systems and the redundancy in the pathway, we used an epithelial-specific Cre-dependent allele expressing T<sub>121</sub> to dominantly interfere with all 3 pocket proteins (pRb, p107, and p130), thus facilitating RB-TS inactivation in vivo with a single allele (18, 19). T<sub>121</sub>(the N-terminal domain of SV40 T antigen) was directed to OSE using transgenic *TgK18GT<sub>121</sub>*<sup>fl</sup>+ mice that carry a bacterial artificial chromosome (BAC) containing the mouse cytokeratin (CK) 18 gene, into which a Cre-conditional loxP-GFP-stop-loxP (LSL) T<sub>121</sub> cassette was inserted (Fig. 1A and Song and colleagues; manuscript in preparation). To selectively induce T<sub>121</sub> in OSE, adenoviral Cre (Adeno-Cre) was introduced under the bursa (Supplementary Fig. S1A, a and b). Deletion efficiency was monitored by immunohistochemistry 3 months postinduction for GFP (no deletion), T<sub>121</sub> (deletion), and CK18 (cell type; Fig. 1A and B). GFP was detected diffusely in CK18-expressing OSE cells of the uninjected and in some cells of the injected ovary (Fig. 1B, i and ii). T<sub>121</sub> was present only in OSE cells of the injected ovary (Fig. 1B, iii and iv), indicating successful recombination within the *TgK18GT<sub>121</sub>* transgene. Mice were examined at various times postinduction. All uninjected ovaries were normal up to 24 months postinduction (Fig. 1B, i, iii, and v). In contrast, 72% (16 of 22) of injected *TgK18GT<sub>121</sub>* ovaries had cytologic abnormalities in the OSE. Of these, 27% (6 of 22) developed minimal to mild atypical hyperplasia with occasional papillary epithelial projections 3 to 24 months postinduction. An additional 27% (6 of 22) of mice had OSE carcinoma in situ (CIS) with no significant invasion into the underlying ovary or overlying bursa (5 and 24 months postinduction; Fig. 1C, i and ii, and Fig. 2A). In 18% (4 of 22) of induced *TgK18GT<sub>121</sub>* mice, the ovarian tumors had progressed to SEOC pathology with early invasion into ovary and bursa (Fig. 1C, iii and iv, and Fig. 2A). Histologically, tumors were characterized by high mitotic (>12 mitoses per ten ×40 fields) and apoptotic rates (Fig. 1C, iii and iv), consistent with the acute induction of these outcomes in other cell types upon RB-TS inactivation (20). In summary, inactivation of RB-TS was sufficient to cause OSE proliferation, with progression only to stage I disease (wild-type vs. *TgK18GT<sub>121</sub>*. Mann–Whitney test, P < 0.0001).
Brca1/2 or p53 mutations/deletions alone or in combination fail to initiate OSE pathology

The current report of high frequency of TP53 mutations and involvement of Brca1 and Brca2 aberrations in human patients with SEOC (6) prompted us to examine roles for these perturbations in mouse SEOC. Because p53 missense mutations have tissue and mutant-specific functions that can differ from loss of protein (21, 22), we evaluated both conditional inactivation (p53<sup>fl/fl</sup>) and missense mutation (p53<sup>R172H</sup>) expression using the Adeno-Cre induction described earlier. The p53 DNA-binding mutation R172H is analogous to the R175H hotspot mutation frequently identified in human cancers, including

![Diagram of Brca1/2 and p53 interactions in OSE](https://example.com/diagram)

**Figure 1.** Cre-mediated induction of genetic events in OSE cells by intrabursal Adeno-Cre injection. A, scheme depicting the targeting strategy. B, expression of GFP, T121 antigen, and CK18 in serial sections from ovaries of a TgK18GT<sub>121</sub> mouse 3 months postinduction. GFP expression (i, brown, arrows) and lack of T121 expression (iii) in uninduced OSE. Occasional GFP staining (ii, arrows) and T121 expression (iv, brown, arrows) in the induced OSE. Note that the OSE is hyperplastic with increased numbers of tightly packed columnar cells. Immunohistochemical staining for CK18 (brown) depicts epithelial cells (v and vi, arrows). C, CIS (i) in the induced OSE of a TgK18GT<sub>121</sub> mouse 6 months postinduction (i). Immunohistochemistry confirming the expression of T121 (ii; brown). SEOC with minimal invasion into underlying ovary in a TgK18GT<sub>121</sub> mouse 24 months postinduction (iii). Note unaffected fimbriae and oviduct. Higher magnification of SEOC (iv). Scale bars in B represent 50 μm and in C 100 μm.
Figure 2. Histopathologic contribution of single and compound genetic events in the transformation of OSE. A, RB-TS pathway inactivation in TgK18GT121\(\text{tg}/\text{+}\) mice is sufficient to initiate surface epithelial proliferation and early SEOC. B, individual or combined inactivation of Brca1, Brca2, and/or p53 are insufficient to cause ovarian epithelial tumorigenesis. C, combination of TgK18GT121\(\text{tg}/\text{+}\) and p53 alleles causes SEOC. D, disease staging in mice with compound induction of RB-TS, p53, and Brca1 or Brca2. N, normal; HP, hyperplasia. E, statistical evaluation of disease progression based on histology score (HP = 0.25, CIS = 0.5, stage IA = 1.0, IB = 1.5, IIA = 2.0, IIB = 2.5, IIIA = 3.0, IIB = 3.5, IV = 4). Mice removed from study before the age of 3 months were not included in the statistical analysis. Bars depict group mean histology score with error bars representing SEMs. One-way ANOVA with Bonferroni multiple comparison posttest was used to test for significant differences between genotypes. F, explanation of the histopathologic staging system used. The classification was adopted from FIGO used in human ovarian carcinomas.
ovarian carcinomas (23). In addition, we generated mice with allele combinations to model the frequent loss of the remaining wild-type p53 allele observed in human tumors.

Reproductive tracts and affected organs of all single and double combinations of Brca1, Brca2, and p53 alleles were analyzed (Brca1, Brca2, p53, p53/p53, p53/p53, TgK18GT121, Brca1/p53, Brca2/p53, Brca1/Brca2, Brca1/p53/p53, Brca2/p53/p53, Brca2/Brca2/p53/p53). None of the injected or un.injected ovaries from these mice displayed pathologic changes in the OSE up to 24 months postinduction (Fig. 2B, Table 1). Brca1/fl and Brca2/fl mice developed nonovarian neoplasms at a similar frequency as wild-type mice (Supplementary Table S1). However, mice carrying the p53/fl allele, a null allele before recombination, developed an increased frequency of lymphomas and sarcomas consistent with the tumor spectrum of p53–/– mice (ref. 24; Supplementary Table S1). The failure to elicit OSE pathology alone or in combination indicates that perturbation of p53 and/or Brca1/2 cannot initiate and may instead progress disease.

Histopathologic features of SEOC in Rb, p53, and Brca1/2 compound mice parallel human disease

To model the concurrent dysregulation of RB-TS and p53 with or without Brca1 or Brca2 pathways, we generated mice carrying a range of allele combinations. The frequency of SEOC pathology development among these animals varied as follows: 72% TgK18GT121/fl, 88% TgK18GT121/fl/p53/fl, 70% TgK18GT121/fl/Brca1/fl/p53/fl, 68% TgK18GT121/fl/Brca2/fl/p53/fl, and 78% TgK18GT121/fl/Brca1/fl/p53/fl (Table 1, Fig. 2C–E). Disease progression in these mice was staged in a manner similar to the human ovarian carcinoma staging scheme (FIGO: Fig. 2F, Supplementary Fig. S1B and S1C; ref. 25).

Ovarian tumor development could be followed by MRI and resulted in large palpable masses on the injected ovary along with hemorrhagic or serous ascites (Fig. 2A–C). OSE carcinomas showed a range of morphologic patterns observed in SEOC of women, including papillary, micropapillary/figleree, microcystic, poorly differentiated adenocarcinoma, and solid/undifferentiated (Fig. 3D–I). Multiple histologic patterns were frequently observed in the same tumor. The cytology was often high-grade with prominent cellular/nuclear pleomorphism and >10 mitoses per ×40 field. Similar to SEOC in humans, the mice developed multifocal peritoneal carcinomatosis with tumor spread to all abdominal organs (Fig. 3F). Distinct metastases were also observed, and followed a pattern similar to that reported in humans (26). Metastases were observed in the pleura of 87.5% (21 of 24, Fig. 3K), in the lung of 83% (20 of 24) and in the liver of 46% (11 of 24) of mice with stage IV disease (Fig. 3L).

In addition to pathologic changes in the OSE, 2% (11 of 337) of mice had transformation of the oviduct epithelium. The oviduct lesions ranged from atypical hyperplasia (4 of 11) to carcinoma in situ (3 of 11) to adenocarcinoma (4 of 11). In contrast to the OSE lesions, which primarily had a papillary morphology, the oviduct lesions were characterized by a glandular/acinar histology (Fig. 4). On the basis of these significant histologic differences, it is considered unlikely that any of the carcinomatosis or metastasis arose from these oviduct adenocarcinomas.

Immunohistochemical analysis confirmed that all SEOC, peritoneal and distant metastases expressed T121 and CK18. Further immunohistochemistry revealed expression of CA125 and Wilms tumor 1 (WT1), markers typically expressed in human SEOC (27, 28), in OSE and carcinoma cells (Supplementary Fig. S2A). Pax8 and calretinin staining (Supplementary Fig. S2B) has been used to differentiate between human SEOC (calretinin–, Pax8+) and intraperitoneal mesothelioma (calretinin+, Pax8–; refs. 29, 30). While normal murine OSE and most SEOCs were Pax8 negative, 9% (2 of 22) SEOC and peritoneal carcinomatosis had rare (<1% of cells) Pax8 expression (Fig. 4). In contrast, all of the oviduct adenocarcinomas had strong nuclear Pax8 expression similar to the normal oviduct epithelium (Fig. 4A–F). To further distinguish SEOC from intraperitoneal malignancies, such as diffuse malignant peritoneal mesothelioma (DMPM), we determined the staining patterns for estrogen receptor (ER), progesterone receptor (PR), and cytokertatin CK5 or CK6. As in human SEOCs (29, 31–34), normal murine OSE and carcinoma cells as well as abdominal carcinomatosis and metastases were strongly ER positive with rare expression of PR and did not express CK5 or CK6 (Fig. 4G–X; Supplementary Materials and Methods). In contrast, a human DMPM was CK5/CK6+, ER+ and PR– as expected (Fig. 4F, I, L, R, X; refs. 32–34). In summary, these mice develop tumors that grossly, histologically, and immunohistochemically resemble human SEOC.

Role of Brca1/2 and p53 in tumor progression

The overall penetrance of SEOC in compound mutants containing RB-TS, biallelic p53 with or without Brca1/2 deletion was very similar (Table 1, Fig. 2E). The inactivation of Brca1 increased the incidence of stage III and IV SEOC from 8% in TgK18GT121/fl/p53/fl;121/+ mice to 16% in TgK18GT121/fl/Brca1/fl/p53/fl;121/+ mice (Table 1, Fig. 2E). However, this increase did not reach statistical significance, possibly due to the fact that the Brca1 and 2 alleles are less efficiently rearranged than p53 and TgK18GT121 alleles (Supplementary Fig. S3A–C). While it is possible that the deletion is inefficient, no progression was observed in TgK18GT121/fl/Brca1/fl compared with TgK18GT121/fl/+ mice up to nearly 2 years postinduction indicating that no deleted cells contributed to the tumor progression.

Induced TgK18GT121/fl/+; p53/fl;121/+ mice carrying one mutant p53/fl;121/+ allele developed mainly low-grade SEOC at a frequency between 21% and 42% (Table 1, Fig. 2C and D). In contrast, compound mutant mice containing biallelic p53 modification (either p53/fl;121/+ or p53/fl;121/+ p53/fl) presented with SEOC of all stages at an increased frequency of 68% to 88%, respectively (Table 1, Fig. 2E) indicating that biallelic p53 perturbation is associated with SEOC disease progression.

Consistent with other studies, mutant P53/fl;121/+ was not stably expressed in normal OSE or carcinoma cells from induced TgK18GT121/fl/+; p53/fl;121/+ mice as determined by

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Table 1. Histopathology of the OSE and SEOC stages in Adeno-Cre–injected mice

<table>
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<th>Genotype</th>
<th>Normal (%)</th>
<th>Hyperplasia (%)</th>
<th>Carcinoma in situ (%)</th>
<th>SEOC stages I–IV (%)</th>
<th>IA (%)</th>
<th>IB (%)</th>
<th>IIA (%)</th>
<th>IIB (%)</th>
<th>IIIA (%)</th>
<th>IIIB (%)</th>
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(continued)
immunohistochemistry (Supplementary Fig. S4A). However, strong P53 expression was detectable in SEOC from TgIK18GT121+/+; p53R172H/+ mice indicating that an additional event interfering with the remaining p53 wild-type allele is required for protein stabilization (Supplementary Fig. S4B and S4C). Interestingly, in 1 of 12 TgIK18GT121+/+; p53R172H/+ and 2 of 19 TgIK18GT121+/+; Brca1−/−; p53R172H/+ mice with stage III or IV SEOC (Table 1), tumor sections revealed small foci containing carcinoma cells with strong nuclear P53 expression suggesting spontaneous protein stabilization (Supplementary Fig. S4B). Microdissection and sequence analysis confirmed the loss of the p53 wild-type allele in tumor foci with increased nuclear P53 expression (Supplementary Fig. S4D).

Taken together, these data suggest that RB-TS and p53 cooperate in the development of metastatic SEOC. While only RB-TS inactivation is sufficient to initiate tumorigenesis, the mutation/loss of both p53 alleles is required for the metastatic phenotype (stage IV disease).

**Gene expression analysis of mouse ovarian tumors confirms similarity to human SEOC**

To determine whether TgIK18GT121, p53, Brca1/2 driven tumors resemble human SEOC at the molecular level, we conducted global gene expression analysis on 27 ovarian carcinomas and 3 pooled normal OSE samples (Supplementary Materials and Methods). Principle component analysis (PCA) and unsupervised hierarchical clustering revealed that the profiles of tumor samples were clearly distinct from normal OSE (Supplementary Fig. S5A). There was no obvious sub-clustering based on Brca1 or Brca2 status at the global gene expression level (Supplementary Fig. S5B) indicating that the majority of transcriptional changes are due to RB-TS and p53 pathway perturbation. Sample-level enrichment–based pathway ranking (SLEPR) analysis (35) using BioCarta terms identified commonly altered pathways in tumors compared with normal samples (Fig. 5A). As expected, RB, p53, DNA damage signaling, and repair pathway alterations were significantly changed in the majority of tumor samples (Fig. 5A, Supplementary Fig. S5C). To determine whether these changes reflect networks frequently disturbed in human high-grade SEOC, TCGA-curated pathways (6) were compiled and reproduced using mouse orthologous genes. As in the majority of human samples, the FOXM1 transcription factor network and its proliferation-related target genes were consistently overexpressed in the murine tumors (Supplementary Fig. S5C).

The comparison of 4 histologic types of human ovarian cancer (serous, endometrioid, mucinous, and clear cell ref. 10) with murine carcinoma samples revealed overlap of the murine and human SEOC samples in the space of the 3 top principal components relative to samples of other subtypes, indicating the strong similarity between these expression profiles (Fig. 5B). In addition, cluster analysis of merged mouse and human data using classifier gene sets showed that mouse samples represent all 4 subgroups of human SEOC originally derived from the TCGA study (6): differentiated (40%, 11 of 27), immunoreactive (30%, 8 of 27), proliferative (15%, 4 of 27), and mesenchymal (15%, 4 of 27; Fig. 5C) and are genetically distinct from DMPMs (Fig. 5D, Supplementary Fig. S5D; ref. 36).
Metabolic changes in blood from mouse SEOC resemble changes seen in human ovarian cancer

To determine whether these mouse models can be used for biomarker discovery, we conducted metabolomic profiling on blood from 15 controls and 15 compound mutants with either Brca1 or Brca2 deletion (Supplementary Materials and Methods) at 3 time points: before intrabursal injection (T0), after onset of disease in compound mutants (T1: 12–20 weeks postinduction), and at termination of the experiment (T2: 24–45 weeks postinduction; Fig. 6A). At 12 to 20 weeks postinduction the OSE lesions in mice ranged from hyperplasia to early carcinoma (stage IA), which had further progressed to advanced disease stages at the terminal time point (Supplementary Materials and Methods). Wild-type mice had no significant changes in the OSE at any time point. Representative MRI scans of the ovaries are depicted in Fig. 6B.

A total of 318 named biochemicals were identified comprising all major biochemical groups. Two-way ANOVA identified biochemicals that had a significant main effect ($P < 0.05$) for either genotype or time, with the greatest changes occurring at the terminal time point (Supplementary Materials and Methods). Several metabolomic changes in tumor-bearing animals overlap with previous findings obtained from tissue of human patients with metastatic ovarian cancer (37). These included increases in 2-hydroxybutyrylate, 3-hydroxybutyrate, citrate, $\alpha$-tocopherol, and short-chain acyl carnitines as well as a decrease in taurine (Fig. 6C).

In addition, blood of animals bearing metastatic ovarian cancer contained decreased citrulline and increased spermidine and putrescine. The systemic redirection of ornithine from the urea cycle to polyamine biosynthesis was expected to result in a significant nitrogen stress to the animal and additional metabolites support this assertion. Specifically, the TCA cycle associated amino acids aspartate and glutamate were both increased in the blood of animals with metastatic ovarian cancer with aspartate increasing dramatically. Besides its role in the urea cycle, aspartate serves as the predominant backbone of pyrimidines in their de novo biosynthesis. The increased blood aspartate occurred simultaneously with increased abundance of many pyrimidine pathway metabolites, including orotate, uridine, thymidine, and 2-deoxyctydine (Fig. 6D and E). Several of the metabolites revealed temporal changes during disease progression (Fig. 6F), indicating that these mouse models provide a useful platform for the identification of candidate biomarkers for earlier detection and/or for monitoring treatment response.

Discussion

Here, we report highly penetrant GEM models for epithelial ovarian cancer. By interfering with various combinations of RB, p53, and/or Brca protein functions, we dissected the relative contribution of these pathways to the development of stage IV disease. As in humans, mice developed peritoneal carcinomatosis, ascites, and distant metastases. The histologic

Figure 4. Immunophenotype of SEOC arising from the mouse ovarian surface and oviduct epithelium, as well as abdominal carcinomatosis and human peritoneal mesothelioma. A, G, M, and S, normal mouse OSE; B, H, N, and T, carcinomas arising from the OSE; C, I, O, and U, abdominal carcinomatosis D, J, P, and V, normal oviduct epithelium; E, K, Q, and W, adenocarcinomas arising from the oviduct; and F, L, R, X, human DMPM. Rare expression of CK5 indicated by arrow in W. Scale bars, 200 μm.
Morphology and immunophenotypical expression of markers, such as CA125, WT1, ER, and PR mimics human SEOC. In addition, transcriptional and metabolomic profiling confirmed that Rb, p53, Brca1/2-triple mutant tumors align with human SEOC and represent all 4 subclasses. Previous studies showed that concomitant inactivation of Brca1 and p53 or Rb1 and p53 in mouse OSE cells can give rise to advanced ovarian carcinomas with serous histology (11, 14, 16). While one study showed that intrabursal inactivation of Brca1 alone resulted in an increase in the number of preneoplastic changes in the OSE 240 days postinduction (38), in our models neither deletion/mutation of p53, Brca1, or Brca2 alone nor the combination thereof was sufficient to cause any significant histologic changes in the surface epithelium, which is consistent with other reports (11, 13, 15, 16).

These data show that of Brca1/2, p53, and RB pathway perturbation, only the latter was sufficient to acutely initiate disease. The fact that Rb deletion by itself did not result in histopathologic abnormalities in previous studies (11, 13) implies that RB family members compensate for the loss of pRB. Indeed, loss of p130 in addition to Rb deficiency previously resulted in the acceleration of tumorigenesis in mouse models of lung adenocarcinoma and small-cell lung cancer (39, 40). While we do not propose that impaired Rb-pathway function is the only possible initiating event, the present study (along with the high frequency of RB pathway aberrations in human SEOC) suggests that this is a common
Figure 6. Metabolomic profiling of blood during SEOC progression. A, experimental design. B, representative MRI scans from mice 8, 20, and 27 weeks postinduction (pi), respectively. Arrow indicates the injected ovary; T indicates the ovarian tumor. Scale bar, 1 cm. C, similar changes in human ovarian cancer tissue (38) and murine whole blood metabolome (this study). D, integrative model of metabolomic changes observed in the blood of mice with SEOC compared with their littermate controls. Metabolites whose abundance increased are shown in red, decreased in green, and unchanged or not detected in black. E, boxplots comparing the abundance distribution of select metabolites between animals with metastatic ovarian cancer and their littermate (WT, wild-type) controls at the terminal time points. F, heatmaps depicting temporal metabolite changes in SEOC compared with WT mice. The colors display each group mean level to the median for that compound, such that white represents the median, saturated red represents a 2-fold increase relative to, and saturated blue is one half the median value.
initiation, while impaired Brca1 or Brca2 and p53 facilitate SEOC progression.

The approach of targeting RB-TS inactivation specifically to epithelial cells via CK18 transcriptional regulation enabled us to minimize the appearance of ectopic tumors such as lymphomas and sarcomas. Lympathic, mesenchymal and smooth muscle cells can be transformed more readily than epithelial cells, as seen in previous studies where conditional inactivation of Brca1, p53α, and Rb by intrabursal injection of Adeno-Cre resulted in the development of leiomyosarcomas due to transformation of smooth muscle cells in the ovarian bursa adjacent to the surface epithelium (13).

Mortality in patients with SEOC is frequently due to the effects of disseminated abdominal disease on vital organs such as the gastrointestinal tract and is secondary to distant metastasis. The latter may be present at the time of diagnosis (stage IV disease) or can arise following relapse. The GEM models described here not only recapitulate intraperitoneal spreading from a primary ovarian tumor but also the most common sites of distant metastases found in humans: pleura, lung, and liver (26). Human ovarian tumors harboring mutations in both p53 alleles were more likely associated with high-grade SEOC, lymph node metastasis, and the development of distant metastasis (41). In agreement with these data, the progression to stage III/IV SEOC pathology in the GEM models was dependent on biallelic mutation/inactivation of p53. While there was no obvious difference between complete loss or stabilization of mutant p53, future studies will explore whether any difference in ethiology or response to therapy results from these 2 mechanisms.

The combination of Brca1 inactivation with an initiation event did not appear to drive progression beyond stage I disease (noninvasive carcinoma). However, in combination with p53 aberration Brca1 inactivation may increase the incidence of disease. These findings are consistent with studies of Brca1, Brca2, and Li-Fraumeni families (42), showing that mutation carriers are predisposed to cancers, but the statistics of onset imply the need for stochastic initiating event(s). Future studies examining global genome and expression profiles of Brca wild-type and mutant SEOC may indicate potential differences. In addition, these tumors may respond differently to therapeutic agents. The models described here can be used to develop cell lines and allograft models for evaluating drug potency relative to Brca mutation status.

Although the exact cellular origin(s) of SEOC in humans is unclear, the emerging hypothesis is that the fallopian tube is the likely source (43, 44). This idea is based largely on early tubal lesions detected after prophylactic surgery in BRCA1 mutation carriers (45–48). Because progression cannot be followed in these cases, it is difficult to conclude that the lesions detected will give rise to SEOC. It is also possible that SEOC can arise from multiple sites. Experimental transformation of human OSE, as well as human epithelial cells derived from the fallopian tube gave rise to high-grade SEOC, respectively (31, 49). In this study, we showed that GEM-SEOC can initiate in the OSE, based on assessment of a significant number of induced mice at distinct times during progression in which disease was limited to the ovary. In some other cases, viral leakage to the adjacent oviduct (fallopian tube), was detected, but resulted in distinct pathology. Though the vast majority of human SEOC express Pax8, which is consistent with their origin in fallopian tube epithelium, it has not yet been shown to be a defining/requisite feature of these tumors. The preponderance of the remaining evidence (morphology, other immunohistochemical markers, and gene expression profiling) suggests that the murine tumors in our study resemble SEOC rather than mesotheliomas. Many of the existing murine ovarian carcinoma models have been derived by transformation of OSE (10, 11, 16). While one study showed similarity to human endometrioid ovarian cancer based on transcriptional profiling (10), SEOC-like mouse models were characterized mainly based on histopathologic features (11, 16). The status of Pax8 expression in these models was not described. Future experiments limiting induction to tubal epithelia in these models will facilitate addressing the cell of origin paradigm.

In conclusion, we have developed GEM models of SEOC with several key histopathologic, immunophenotypical, and genetic features of human SEOC. These models will serve as a foundation for future preclinical research.

Disclosure of Potential Conflicts of Interest
JE McBunn is employed at Metabolon as associate director. No potential conflicts of interest were disclosed by the other authors.

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Perturbation of Rb, p53, and Brca1 or Brca2 Cooperate in Inducing Metastatic Serous Epithelial Ovarian Cancer

Ludmila Szabova, Chaoying Yin, Sujata Bupp, et al.


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