Loss of LKB1 and PTEN tumor suppressor genes in the ovarian surface epithelium induces papillary serous ovarian cancer

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Epithelial ovarian cancer presents mostly with serous, endometrioid or mucinous histology but is treated as a single disease. The development of histotype-specific therapy has been challenging because of the relative lack of studies attributing disrupted pathways to a distinct histotype differentiation. mTOR activation is frequently associated with poor prognosis in serous ovarian cancer, which is the most common and most deadly histotype. However, the mechanisms dysregulating mTOR in the pathogenesis of ovarian cancer are unknown. We detected copy number loss and correlated lower expression levels of LKB1, TSC1, TSC2 and PTEN tumor suppressor genes for upstream regulators of mTOR activity in up to 80% in primary ovarian serous tumor databases, with LKB1 allelic loss-predominant. Reduced LKB1 protein was usually associated with increased mTOR activity in both serous ovarian cancer cell lines and primary tumors. Conditional deletion of Lkb1 in murine ovarian surface epithelial (OSE) cells caused papillary hyperplasia and shedding but not tumors. Simultaneous deletion of Lkb1 and Pten, however, led to development of high-grade ovarian serous histotype tumors with 100% penetrance that expressed WT1, ERα, PAX8, TP53 and cytokeratin 8, typical markers used in the differential diagnosis of serous ovarian cancer. Neither hysterectomy nor salpingectomy interfered with progression of ovarian tumorigenesis, suggesting that neither uterine nor Fallopian tube epithelial cells were contributing to tumorigenesis. These results implicate LKB1 loss in the OSE in the pathogenesis of serous ovarian cancer and provide a compelling rationale for investigating the therapeutic potential of targeting LKB1 signaling in patients with this deadly disease.

Introduction

High-grade serous cystadenocarcinoma is the most common and most lethal epithelial ovarian cancer histotype, representing ~50% of all malignant ovarian tumors (1). Approximately 1–2% of women are at risk of developing this disease in their lifetime. The early events in disease progression are not well defined because ovarian cancer is usually diagnosed in advanced stages. Also unknown are the mechanisms that drive differentiation of epithelial ovarian cancers into the common histotypes that are characteristically very similar to the Müllerian duct-derived Fallopian tube (serous), uterus (endometrioid), or cervix (mucinous) (2,3). Simultaneous activation of AKT and mTOR complex 1 (mTORC1) has been observed in 87% of human ovarian carcinomas (4), and elevated mTOR signaling is associated with poor prognosis (5). Whether increased mTOR signaling is merely a reflection of the high proliferative index or dysregulated activation of mTOR signaling directly contributes to the pathogenesis of the disease is not clear. LKB1, PTEN, TSC1 and TSC2 are upstream regulators of mTOR signaling (6), and alterations in genes encoding these proteins are commonly observed in patients with the familial cancer-prone genetic disorders. For example, STK11, which encodes LKB1, is associated with PeutzJeghers syndrome (7) and some sporadic cancers (8–10), suggesting that dysregulation of mTOR by the loss of these tumor-suppressor pathways might be an important mechanism for ovarian carcinogenesis as well.

Ovarian serous cystadenocarcinomas have been traditionally thought to originate from the meso-epithelial cells covering the ovary, known as the ovarian surface epithelium (OSE) (11). However, occult tumors are often found in the distal Fallopian tube epithelium of BRCA1 patients undergoing prophylactic salpingo-oophorectomy, suggesting that Fallopian tube epithelial cells might also be an origin for serous ovarian epithelial cancer (12,13). The histological similarity between the most common subtype of epithelial ovarian cancer, serous cystadenocarcinoma, to the Müllerian duct-derived Fallopian tube lends further credibility to this hypothesis. Thus, origins of serous ovarian cancer in either the OSE or the Fallopian tube epithelium are not mutually exclusive.

In a previous study, we showed that conditional deletion of Lkb1 and Pten in female reproductive tract tissues resulted in oviductal cysts and adenomas, endometrial adenocarcinomas and adenoma malignum/minimal deviation carcinomas (14). In these reproductive tract tissues, deletion of those genes occurs only in the Müllerian duct mesenchyme-derived stroma. Thus, we hypothesized that the mutated stromal microenvironment is contributing to neoplasia and tumorigenesis in the female reproductive tract tissues by mechanisms similar to those observed with mutated stroma in other tumor settings (15). Here, we examined the ovaries in the mutant Lkb1 and Pten mice to study whether or not their deletion contributes to ovarian carcinogenesis and show that mice develop serous ovarian carcinomas with 100% penetrance, indicating that the OSE is indeed a site for serous ovarian cancer development and that the combination of disrupted LKB1 and PTEN tumor suppressor activities is important for differentiation of the serous histotype. Analyses of both human ovarian cancer cell lines and ovarian serous cystadenocarcinoma databases revealed that loss of LKB1 and other upstream modulators of mTOR activity is commonly observed in humans with the disease, as well, further supporting a critical role for these tumor suppressor genes in controlling histotype differentiation and progression.

Materials and methods

Mouse genetics and animal husbandry

All animal experimentation protocols used in this study were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital, and are in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Mice used in this study were kept in standard housing conditions and were maintained on a mixed genetic background (C57BL/6;129/SvEv). These mice strains: Amhr2-cre (16), Stk11fl/fl;Lkb1+/− (17), Tsc2−/− (18), Tsc2−/−;Tsc1−/− (19) and Pten−/− (20) were mated to produce Amhr2-cre+;Stk11−/−, Amhr2-cre+;Tsc2−/−, Amhr2-cre+;Tsc2−/− and Amhr2-cre+;Stk11−/−;Pten−/− and referred to as Lkb1−/−;Tsc2−/−;Tsc1−/−;Pten−/−, respectively. Amhr2-cre mice were crossed with Rosa26;LacZtm1Gle reporter mice as previously described (21). Tail biopsies were collected for genotyping, and PCR conditions for Lkb1, Tsc1, Tsc2 and Pten allele have been described (17,20,22,23). Ovaries were

Abbreviations: CNA, copy-number alterations; IHC, immunohistochemistry; OSE, ovarian surface epithelium.

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collected postmortem for histological examination. Hysterectomies and salpingo-oophorectomies were performed using standard laparotomy of mice at 4 weeks. The hysterectomized/salpingo-oophorectomized mice were euthanized 8 weeks post-laparotomy for ovarian analyses. Gross pictures of the animals or the tumors were taken using a Nikon SMZ1500 microscope with an attached Spot camera (Diagnostic Instruments, Sterling Heights, MI) or with a Nikon D60 digital camera and macro lens.

Primary cell isolation from ascites
Ascites fluid was collected from the peritoneal cavity of Lkb1<sup>cko</sup>/Pten<sup>cko</sup> mice with a sterile syringe and centrifuged. Cell pellets were mixed with HBSS, layered onto another tube with Lymphocyte Separation Media (Lonza, Hopkinton, MA), and centrifuged for 30 min. Cells were isolated and treated with ACK lysis buffer (Lonza, Hopkinton, MA), cultured on cell-culture slides (BD Biosciences, Franklin Lakes, NJ) and stained for cytokeratin 8 (Neomarkers, Fremont, CA).

Histology, immunohistochemistry, immunofluorescence and β-galactosidase staining
Tissues were fixed, processed and sectioned as previously described (21,22). The following primary and secondary antibodies were used for immunohistochemistry (IHC) and immunofluorescence: cytokeratin 8 (Developmental Studies HybridomaBank, IA), phospho-ribosomal S6 kinase (pS6), phospho-mTOR (pmtOR), phospho-4EBP1 (p4EBP1) (Cell Signaling Technology, Danvers, MA), Ertr (DAKO, Carpinteria, CA), PAX8 (Proteintech group, Chicago, IL), WT1, TP53 (Santa Cruz Biotechnology, Santa Cruz, CA), AlexaFluor secondary antibodies (Invitrogen, Carlsbad, CA) or biotinylated donkey anti-mouse or anti-rabbit F<sub>a</sub> (Jackson ImmunoResearch Laboratories, West Grove, PA). Photos were taken with a Nikon TE2000S with an attached Spot camera (Diagnostic Instruments, Sterling Heights, MI) or Nikon Eclipse Ni fitted with Nikon DS/F2/DS-Q1MC cameras.

Human ovarian serous cancer tissue and database analyses
Formalin-fixed, paraffin-embedded human ovarian high-grade serous carcinoma tissue samples (<i>N</i> = 19) were obtained from the Department of Pathology, Massachusetts General Hospital with Institutional Review Board approval. Hematoxylin and eosin slides and LKB1 and pS6 protein colocalization were evaluated by a gynecologic pathologist. Tissue microarrays (BC11115 and BC111109) were purchased from US Biomax, Rockville MD for LKB1 IHC. LKB1 and pS6 protein colocalization were each scored as either positive or negative staining in tumor areas. If positive, LKB1 and pS6 protein colocalization were each scored as being present diffusely, focally, or in scattered tumor cells. Data analysis to identify DNA copy-number alterations (CNAs) in serous ovarian cell lines, MGH and TCGA tumor samples was performed following the procedures previously described (24). DNA copy number and mRNA expression data for LKB1, TSC1, TSC2 and Pten genes were collected from 288 TCGA samples. Heatmaps for DNA copy number and mRNA expression were generated using the Cluster and Tree View program (25). Relative mRNA expression analyses were generated by normalizing filtered signal intensities of LKB1, TSC1, TSC2 and Pten genes with the average of three house-keeping genes (GAPDH, GUSB and ACTB). Pearson’s correlation coefficient was utilized to assess the strength of the linear relationship. The slope of the regression line represents the mean change in mRNA for a one-unit increase in CNA. The <i>P</i> value is an assessment of the hypothesis that this slope has a value of zero. A low <i>P</i> value provides evidence that the slope is not value-zero.

Western blot analyses
Protein extracts from human ovarian cancer cell lines were prepared in RIPA buffer as previously described (22). Equal amounts of protein were loaded and β-actin was used as loading control. LKB1, S6, pS6, TSC1 (Cell Signaling Technology, TSC2 (Abcam)), TP53 (Leica Biosystems, Buffalo Grove, IL), β-actin (Neomarkers) and antirabbit or antimouse, horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology or Jackson Immunoresearch Laboratories) antibodies were used.

Results
To determine whether there is a correlation between loss of upstream regulators of mTOR activity and ovarian serous cystadenocarcinomas, we analyzed deletions of the Lkb1/STK11, Pten, Tsc1 and Tsc2 genes in two published high-grade serous ovarian cancer databases, TCGA (26) and MGH (24). Allelic loss of any of the four genes was observed in 76% of the tumors in the TCGA database (Figure 1A) and in 90% of the non-overlapping MGH database (Supplementary Figure S1A, available at Carcinogenesis Online), but deletion of one or more alleles of the LKB1 gene predominated with loss in 61 or 76% of the tumors, respectively (Figure 1A and Supplementary Figure S1A, available at Carcinogenesis Online). In TCGA tumors with deleted LKB1, 73% were also deleted for one or more of the PTEN, TSC1 and/or TSC2 genes (Figure 1A and Supplementary Figure S1B, available at Carcinogenesis Online). Conversely, the majority of tumors with copy number deletions in PTEN, TSC1 or TSC2, also had deleted LKB1. Correlation of CNA with expression in the TCGA samples indicates that there is a direct relationship between DNA copy number of these four genes and their relative levels of expression (Figure 1B and Supplementary Figure S1C, available at Carcinogenesis Online). Since LKB1 was most prominently altered in the database analyses, we examined LKB1 expression by immunofluorescence in human ovarian high-grade serous carcinomas (<i>N</i> = 92) (Figure 2A and B). Human serous carcinoma tissue samples examined showed either complete loss (54%) (Figure 2B) or partial/scattered to no loss of LKB1 (46%) (Figure 2A). In control-normal postmenopausal ovaries, LKB1 was only observed in the OSE (Figure 2C). Examination of the phosphorylation level of S6 ribosomal protein (pS6), a downstream marker of mTORC1 activity, by immunofluorescence in a subset of these carcinomas (<i>N</i> = 19), showed higher levels where expression of LKB1 was absent (Figure 2D), whereas in normal OSE, their expression can be observed in the same cells (Figure 2C). We also analyzed the status of LKB1 expression by immunoblot in a panel of 17 human ovarian cancer cell lines and observed reduced LKB1 expression in 59% (<i>N</i> = 10/17) (Supplementary Figure S1D, available at Carcinogenesis Online). Examination of LKB1 in 10 different human serous ovarian cancer lines showed reduced LKB1 and simultaneously increased pS6 in 5/8 or 63% of the samples (Figure 2E) compared with T29 control human OSE cells (27). Reduced TSC1, TSC2 and/or PTEN expression was observed in the majority of human ovarian serous cancer cell lines as well. As expected, expression of PTP3 was very low in the majority of the serous cell lines.

To understand the pathophysiological consequences of LKB1 loss in human high-grade serous cancer patients, we conditionally deleted the Lkb1 gene in mouse OSE cells using the Amhr2-cre allele. Amhr2-cre (also known as Misr2-cre (16)) is expressed in both OSE and ovarian cells of the ovary (Supplementary Figure S2A, available at Carcinogenesis Online). Loss of LKB1 protein in OSE and stromal cells but not in oocytes of Lkb1<sup>cko</sup> mice ovaries confirmed deletion of Lkb1 (Supplementary Figure S2B and C, available at Carcinogenesis Online). Histological examination of adult Lkb1<sup>cko</sup> ovaries (>16 weeks and <i>N</i> = 18/22) showed surface papillary serous hyperplasia and widespread shedding of surface epithelial cells (Figure 3B, C, E and F), whereas normal OSE, comprised of a single layer of flattened cells, were observed in controls (Figure 3A and D). However, no tumor formation was observed in older Lkb1<sup>cko</sup> mice examined (<i>N</i> = 22). One Lkb1<sup>cko</sup> mouse developed a unilateral ovarian blood-filled cyst (Supplementary Figure S2D and E, available at Carcinogenesis Online).

PTEN loss (Figure 1 and Supplementary Figure S1A, available at Carcinogenesis Online) and simultaneous activation of AKT and mTOR signaling is frequently observed in human ovarian carcinomas (4). LKB1 has been shown to phosphorylate and activate PTEN, and mutations affecting this interaction are observed in Peutz-Jeghers patients (28). Combined loss of Pten and Lkb1 synergistically increases mTOR activity and induces tumorigenesis in other organ systems (29,30). Increased PTEN expression observed in Lkb1<sup>cko</sup>/Pten<sup>cko</sup>ovaries (<i>N</i> = 2/3; Supplementary Figure S3A–C, available at Carcinogenesis Online), suggested a feedback mechanism to minimize the deleterious effect of LKB1 loss. Deletion of PTEN alone does not result in ovarian carcinogenesis (22) but deletion of Pten in Lkb1<sup>cko</sup>mice caused abnormal enlargement of the abdominal area in double mutant (Lkb1<sup>cko</sup>;Pten<sup>cko</sup>) mice, usually by 16 weeks (Supplementary Figure S4, available at Carcinogenesis Online). Adnexal tumors were observed in 100% of Lkb1<sup>cko</sup>;Pten<sup>cko</sup>mice examined (<i>N</i> = 12) (Supplementary Figure S4, available at Carcinogenesis Online). Histologic examination of Lkb1<sup>cko</sup>;Pten<sup>cko</sup> tumors showed high-grade papillary serous carcinomas involving the ovarian parenchyma. Some mutant animals
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(N = 3/12) also showed development of ascites (Supplementary Figure S4, available at Carcinogenesis Online), a common occurrence in the later and recurrent stages of human ovarian cancer, with typical cobblestone morphology associated with epithelial cell culture that were cytokeratin (CK)-positive (Supplementary Figure S4, available at Carcinogenesis Online). The Lkb1<sup>fl/fl</sup>;Pten<sup>fl/fl</sup> tumors were CK8-positive, confirming their epithelial origin (Figure 3H and J) and ruling out stromal/granulosa cell tumorigenesis. IHC for AMH shows that the ovarian tumors are devoid of this granulosa cell marker except for its expression in the remaining follicles (Supplementary Figure S3G and H, available at Carcinogenesis Online). In Lkb1<sup>fl/fl</sup>;Pten<sup>fl/fl</sup> controls, CK8 staining was only observed in the OSE (Figure 3G

Fig. 1. Loss of LKB1 in human ovarian serous cancer. (A) Copy number changes analyses show that 200 tumor samples in the TCGA human serous ovarian cancer database have loss of at least one allele of STK11. Venn diagram shows that of the 200 TCGA tumors with LKB1 deletion, 146 were also deleted for TSC1, TSC2 or PTEN. (B) Correlation of CNA with normalized mRNA expression for the indicated genes is shown for 288 TCGA patient samples. A P value (P ≤ 0.05) indicates there is predictive power in CNA with regard to mRNA expression. Pearson’s correlation coefficient (ρ) describes the linearity of the relationship.
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Fig. 2. LKB1 expression in ovarian serous cancer. Representative images (N = 92) showing expression of LKB1 assayed by immunofluorescence revealing strong or scattered expression of LKB1 in 44% of human ovarian serous sections (A) or absence in 56% (B). (C) LKB1 expression in the normal human OSE is shown by immunofluorescence for LKB1 and pS6. Arrows indicate OSE cells that express both. Further examination of human serous ovarian tumors (N = 19) by immunofluorescence showed that loss of LKB1 is coincident with increased pS6 protein expression (D). Nuclei are stained with 4',6-diamidino-2-phenylindole. Bars equal 50 μm. (E) Loss or reduced LKB1 expression compared with control T29 immortalized human OSE cells was observed by western blot in 73% (N = 6/8) of human serous ovarian cancer lines (OVCAR5, OV1063, OV90, SKOV3, EP021, A2780, OAW28, OVCAR4), which was consistent with higher levels of pS6. TSC1 (4/8), TSC2 (8/8) and p53 (8/8) were also reduced in serous cell lines compared with control T29 cells. TOV21G, OVKATE and Kuramochi are non-serous ovarian cancer cell lines shown for comparison.

Discussion

One of the major challenges for the ovarian cancer biology field is the lack of model systems targeting different cell types that faithfully replicate human ovarian disease, especially in the context of serous carcinoma (34). Conditional loss of Brca1 and Tp53 or Brca1, Rb and Tp53 genes—all of which are frequently mutated in human serous patients—in the ovary leads to the development of ovarian leiomyosarcomas (35,36). Individual loss of Tp53, Brca1 or Pten genes is unable to initiate neoplastic changes in the mouse ovary (22,35,36). Recently, Szabova et al. (37) showed that loss of Tp53, Rb, Brca1 or Brca2 in OSE cells using a Cytokeratin 18 promoter-driven Cre causes development of metastatic ovarian serous carcinomas. Those tumors did not express PAX8, which is expressed in 99% of human ovarian serous carcinomas (31,37). Other mouse models targeting the OSE cells have been reported (38–40) but either lack immunohistochemical expression of some of the typical human serous markers (WT1, PAX8, ERx and TP53) or their presence is not described. Genetic modification of mice has resulted in the most promising models to date; although there are several important limitations to modeling human ovarian cancer in mice that could still prove difficult to overcome. For example, unlike human ovaries, murine ovaries have a bursa covering that protects the peritoneal cavity from epithelial cell shedding in the OSE, which could inhibit metastasis. Humans are a mono-ovulatory species but mice are poly-ovulatory; and whereas human menstrual cycling lasts approximately 28 days, mice have an accelerated estrous cycling period of 4–5 days. All of these could have confounding effects on the interpretation of mouse models. Additionally, most ovarian cancers in PeutzJeghers patients have been described as sex cord tumors with only a few reported...
as mucinous cystadenomas (7,41). While still cognizant of these caveats, we have described the development of a mouse model that closely replicates the human disease and could be used to study the mechanisms changed by dysregulated LKB1/mTOR signaling in the etiology of serous ovarian cancer with the goal of identifying new therapeutic targets for the disease.

The mechanisms disrupted by loss of LKB1 that are important for serous histotype differentiation in the mutant ovaries are unknown but are likely to involve changes to the cellular cytoskeleton controlling polarity. For example, conditional deletion of LKB1 in the mammary gland disrupts epithelial cell polarity and architecture in vitro and in vivo, which results in hyperbranching and deterioration of the basement membrane (42). Also, we have shown that conditional deletion of Lkb1 in murine testes leads to severely disrupted spermatogenesis due, at least in part, to defective Sertoli cell tight-junction complexes (43). Several mechanisms for LKB1-mediated control of cellular polarity during tumorigenesis have been proposed that are thought to involve the direct phosphorylation of the microtubule-associated protein (MAP)/microtubule affinity-regulating kinase 2 (MARK2, also known as PAR1) or AMPK (7,44). Now that we have uncovered a

Fig. 3. Ovarian-specific loss of Lkb1 causes OSE cell hyperplasia and contributes to ovarian epithelial cancer development. (A) Control ovaries are covered by single layer of the OSE cells, shown with an arrow in panel D. (B and C) Abnormal papillary growth (arrows in E) and widespread shedding of OSE cells (arrows in F) into the ovarian bursal space of adult Lkb1<sup>fl/fl</sup> mice. (G–I) Normal CK8-positive OSE cells were observed in Lkb1<sup>fl/fl</sup>;Pten<sup>fl/fl</sup> mice. Combined loss of Lkb1 and Pten causes development of CK8-positive invasive, high-grade epithelial cancer in mice usually by 3 months (H and J). Higher magnification images of the boxed areas in panels A–C, G and H are shown in D–F, I and J. (K) Intact 4-week-old Lkb1<sup>cko</sup>;Pten<sup>cko</sup> ovaries do not show signs of OSE shedding (<i>n</i> = 8/8) but shedding is observed in the ovaries of (L) hysterectomized/salpingectomized Lkb1<sup>cko</sup>;Pten<sup>cko</sup> mice at 8 weeks post-surgery (<i>n</i> = 5/6). (M) The shedding OSE cells in the Lkb1<sup>cko</sup>;Pten<sup>cko</sup> mice express PAX8 by IHC. Bars equal 50 µm in A–J and 100 µm in K–M.
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Fig. 4. Lkb1\(^{cko}\);Pten\(^{cko}\) murine ovarian cancer displays histopathological features of the human serous ovarian carcinomas. Hematoxylin and eosin stained sections of control ovaries (A and D), and Lkb1\(^{cko}\);Pten\(^{cko}\) (B and E) and human (C and F) ovarian serous carcinomas. Higher magnification images of the boxed areas in panels A–C are shown in D–F. Human serous ovarian cancer (G–J) and Lkb1\(^{cko}\);Pten\(^{cko}\) murine ovarian tumors (L–O) expressed markers (CK8, WT1, PAX8 and ER\(^{\alpha}\)) by IHC commonly used for the identification of serous ovarian cancer in human patients. Expression of TP53 was observed in the human ovarian serous samples (K) and in Lkb1\(^{cko}\);Pten\(^{cko}\) tumors (P) and sporadically in Lkb1\(^{fl/fl}\) ovaries (inset in P). OSE cells in mouse control ovaries were positive for CK8 and WT1 (Q and R), and were negative for PAX8, ER\(^{\alpha}\) and TP53 staining (S–U). Except for TP53, these markers were expressed in oviductal epithelial cells, which were used as a positive control for the staining of both human and mice tumors (V–Z). Bars equal 50 µm.
link between serous histotype tumor differentiation and LKB1 status, our next efforts will be to determine how loss of LKB1 drives serous histotype-specific ovarian carcinogenesis.

Because of the difficulty in early diagnosis, widely metastatic disease at the time of presentation, and lack of a precursor lesion and preventative screening, the site and cell of origin of serous carcinoma have been unclear. Early serous carcinoma has been detected at a frequency of 0–14% (mean 4%) in prophylactic salpingo-oophorectomy specimens from human patients with BRCA1 or BRCA2 germline mutations (45). Primary tumors in high-risk patients have been found in the ovary and fallopian tube, and both the OSE and FTE have been purported sites of origin for serous carcinoma. The model described here unequivocally demonstrates that serous ovarian cancer can originate in the OSE. However, this does not preclude the oviduct as a source for serous tumorigenesis. For example, a recent report by Kim et al. (46) has shown that conditional deletion of Dicer using the same Amhr2-Cre leads to high-grade cystadenocarcinoma development from the oviducts. They showed that early tumor formation occurs in oviductal stromal cells, which also appear to express epithelial markers, suggesting that mesenchymal to epithelial transition might be a mechanism for tumorigenesis in this setting. However, in early human ovarian serous carcinomas, the fallopian tube lesions to date have only been observed in the epithelium (12), an important caveat recognized by the authors.

In patients with Peutz-Jeghers syndrome, the lifetime risk for developing cancer is >90% (47). On the other hand, lifetime risk in patients with tuberous sclerosis is thought to be much lower but still significantly higher than the general population (48). Our results with deletion of Lkb1, Tsc1 and Tsc2 in the mouse OSE also demonstrate a more pronounced papillary and shedding phenotype with loss of LKB1 compared with loss of TSC1 or TSC2. We speculate that the other functions of LKB1 in the cell, such as maintaining polarity, when disrupted by the loss of LKB1, contribute to the higher risk associated Peutz-Jeghers patients and the more severe phenotype we observed in mice.

Whereas it has been argued that chromosomal abnormalities are a common outcome in cancer cells due to loss of DNA repair mechanisms, and thus a passenger effect that might have little to do with driving progression of the disease, it could also be argued that heterozygosity and dosage in genes involved in the same signaling pathway may have cumulative carcinogenesis-promoting effects. We also showed that one or more of the genes for upstream regulators of mTOR activity are lost in a large majority of human serous ovarian cancers (Figure 1 and Supplementary Figure S1, available at Carcinogenesis Online). A similar trend has been observed for other cancer-causing genes in several different tumor types, which provides a compelling broader, but more difficult to assess, mechanism for tumorigenesis than the conventional two-hit hypothesis associated with hereditary mutations in tumor suppressors such as retinoblastoma and p53 (49).

We and others have shown that PTEN loss can significantly induce cancer progression and severity in the female reproductive tract when combined with activating mutations in oncogenes (22,40) or deletion of tumor suppressor genes (50,51), including Lkb1 in this report and previously (14). The study of these early events in tumorigenesis in this model could lead to the discovery of pathways and markers amenable to development of diagnostic tools for early detection. The challenge now is to determine how combinations of homozygous and heterozygous deletions of the other upstream regulators of mTOR activity can also affect tumorigenesis, histotype differentiation and/or progression.

**Supplementary material**

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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