nature medicine

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

Role of *K-ras* and *Pten* in the development of mouse models of endometriosis and endometrioid ovarian cancer

Daniela M Dinulescu[1,2], Tan A Ince[3,4,5], Bradley J Quade[4,5], Sarah A Shafer[1,2], Denise Crowley[1,2] & Tyler Jacks[1,2]

Epithelial ovarian tumors present a complex clinical, diagnostic and therapeutic challenge because of the difficulty of early detection, lack of known precursor lesions and high mortality rates. Endometrioid ovarian carcinomas are frequently associated with endometriosis, but the mechanism for this association remains unknown. Here we present the first genetic models of peritoneal endometriosis and endometrioid ovarian adenocarcinoma in mice, both based on the activation of an oncogenic *K-ras* allele. In addition, we find that expression of oncogenic *K-ras* or conditional *Pten* deletion within the ovarian surface epithelium gives rise to preneoplastic ovarian lesions with an endometrioid glandular morphology. Furthermore, the combination of the two mutations in the ovary leads to the induction of invasive and widely metastatic endometrioid ovarian adenocarcinomas with complete penetrance and a disease latency of only 7 weeks. The ovarian cancer model described in this study recapitulates the specific tumor histomorphology and metastatic potential of the human disease.

Epithelial ovarian cancer is the deadliest malignancy of the female reproductive tract in Western countries[1]. A better understanding of the pathogenesis and risk factors associated with this disease should contribute not only to improvements in early detection and prevention but also to the development of more effective therapies. Epithelial ovarian cancer includes five major histological (differentiated) subtypes (serous, endometrioid, mucinous, clear cell and transitional adenocarcinomas) that together make up nearly 90% of all adult ovarian cancers. It has become increasingly clear in recent years that different etiological factors are responsible for the development of various ovarian tumor subtypes. The discovery of frequent somatic *PTEN* mutations and loss of heterozygosity at the 10q23 *PTEN* locus in endometrioid ovarian cancer, coupled with absence of such mutations in other histological subtypes, points to a key role for *PTEN* in the etiology of this subtype[2,3]. *K-RAS* is also mutated in endometrioid ovarian cancer, albeit at a lesser frequency (3.7–36.4%), with the majority of studies indicating a mutational frequency of less than 10%[4–7].

The development of accurate mouse models of human ovarian carcinoma is expected to advance our understanding of its pathophysiology. Recently, significant strides have been made toward modeling epithelial ovarian cancer in genetically engineered mice[8–10]. Most of the tumors described in these models were diagnosed as poorly differentiated or undifferentiated carcinomas, with a smaller percentage diagnosed as serous ovarian carcinomas[8–10]. To advance the understanding of ovarian cancer initiation and progression, it is important to develop animal models of ovarian cancer with a well-differentiated histopathology and also identify precursor lesions for ovarian carcinoma. Furthermore, for both therapeutic and early diagnostic purposes it is essential to generate animal models for the other major histological subtypes of epithelial ovarian cancer, such as endometrioid and mucinous ovarian carcinomas.

Endometriosis, a benign disease defined as the presence of functional uterine-like tissue (epithelial glands and stroma) outside the uterus, is frequently associated with endometrioid ovarian adenocarcinoma[11,12]. Endometriosis is very common in the general population, affecting up to 15% of women in the reproductive age group[11]. Notably, women with a history of endometriosis are at an increased risk for developing endometrioid ovarian cancer. Furthermore, 15–40% of endometrioid ovarian carcinoma cases are associated with endometriosis[11]. The possibility that ovarian cancer arises through the malignant transformation of endometriosis has long been suggested[12]; however, a clear genetic link between endometriosis and ovarian cancer has yet to be identified.

RESULTS

Oncogenic *K-ras* mutations induce endometriosis

To achieve specific gene activation within the mouse ovarian surface epithelium (OSE), we delivered a recombinant adenoviral vector expressing Cre recombinase (AdCre) to the bursal cavity that encloses the ovary. We tested two surgical techniques of injecting adenovirus into the bursal cavity. In the first technique, the needle was introduced in the uterine tubal junction, passed through the oviduct and inserted into the ovarian bursa, where the virus was delivered. The second method of injection, in which the needle was inserted into the oviduct near the infundibulum and the adenovirus was delivered through the infundibulum into the ovarian bursa, was described previously[10]. Both methods were found to be equally effective in infecting OSE cells in a *lox*P-Stop-*lox*P LacZ *(Gt(ROSA)26Sor)* reporter strain (**Fig. 1**). Gross anatomical inspection

[1]Center for Cancer Research and [2]Department of Biology, Massachusetts Institute of Technology, 40 Ames Street, Cambridge, Massachusetts 02139, USA. [3]Whitehead Institute, Nine Cambridge Center, Cambridge, Massachusetts 02142, USA. [4]Department of Pathology, Division of Women's and Perinatal Pathology, Brigham and Women's Hospital, Harvard Medical School,75 Francis Street, Boston, Massachusetts 02115, USA.[5]These authors contributed equally to this work. Correspondence should be addressed to T.J. (tjacks@mit.edu).

Published online 26 December 2004; doi:10/nm1173

showed LacZ staining of the ovary (**Fig. 1a**,**b**). In addition, using the first technique, we found traces of staining in utero-tubal cells (**Fig. 1a**), presumably along the needle path. The second technique was somewhat more tissue restrictive, in that it allowed very efficient staining of the ovary in addition to traces of staining detected in the oviduct (**Fig. 1b**). Histological analysis confirmed infection of OSE cells (**Fig. 1c**,**e–g**), with no LacZ staining detected in ovarian stroma; the control (uninjected) ovary is shown in **Fig. 1d**. Some staining is also seen in the bursa (**Fig. 1f**). Featured in **Fig. 1h** is an example of LacZ staining in the tubal epithelium (oviduct).

To induce expression of oncogenic *K-ras* within the OSE, we used the first injection technique to administer AdCre to the bursal cavity of *lox*P-Stop-*lox*P-*K-ras*$^{G12D/+}$ (*LSL-K-ras*$^{G12D/+}$) mice[13,14]. These mice harbor a transcriptionally silenced, oncogenic allele of *K-ras* that can be activated by the expression of Cre recombinase. Oncogenic *K-ras*^{G12D} activation in the OSE resulted in benign epithelial lesions with a simple endometrioid glandular morphology reminiscent of the epithelial component of endometriosis (**Fig. 2a**). These lesions did not seem to be surrounded by endometrial-type stroma; consequently, we classified them as endometriosis-like lesions rather than endometriosis. All AdCre-infected *LSL-K-ras*$^{G12D/+}$ mice analyzed to date ($n = 15$) showed such benign lesions within the OSE but no invasive ovarian tumors were evident up to 10 months following AdCre infection. In contrast, control (uninfected) ovaries of *LSL-K-ras*$^{G12D/+}$ animals ($n = 15$) or ovaries of wild-type control mice injected with AdCre ($n = 18$) did not show any morphological changes in the OSE (see **Supplementary Fig. 1** online). These results suggest that additional mutations or epigenetic alterations, or both, are necessary for the development of ovarian cancer in the mouse.

In addition to developing ovarian lesions, 47% of mice (7 of 15) also developed peritoneal endometriosis (**Supplementary Fig. 2** online). To our knowledge this is the first mouse model of *de novo* endometriosis. Both the benign ovarian lesions and the peritoneal endometriotic lesions were diagnosed on average at approximately 8 months after AdCre injection. The peritoneal lesions were present within the soft tissue surrounding the ovary (**Fig. 2b**), as well as widespread throughout the peritoneum, being associated with the surface of the oviduct, uterus, cervix, liver, intestine, spleen, pancreas and, more rarely, skeletal muscle (**Fig. 2c–g** and **Supplementary Fig. 3** online, and data not shown). The endometriosis shown by AdCre-infected *LSL-K-ras*$^{G12D/+}$ mice recapitulates the histomorphology and biological behavior of human endometriosis. The diagnosis of endometriosis in the peritoneum was made based on the identification of endometrioid glandular epithelium accompanied by endometrial-type stroma (**Fig. 2g**). In some cases, the endometriotic implants were dominated by the epithelial component, with little stroma present. Histologically, the endometriotic lesions consisted of cystically dilated glands with a well-differentiated, single layer of inactive epithelium that displayed a bland cytology, no nuclear atypia and few mitotic figures (**Fig. 2g**). The glands were surrounded by a rim of endometrial-like stroma, consisting of densely packed fusiform cells with scanty cytoplasm (**Fig. 2g**). Both glands and stroma were scattered within dense fibrous tissue; these dense fibrotic adhesions resembled the endometriosis-associated adhesions found in humans. Furthermore, the long-term follow-up of these lesions was benign in all animals; they were never fatal.

Through the use of laser capture microdissection and subsequent PCR of isolated DNA, we confirmed that epithelial cells within the endometriotic glands had undergone Cre-mediated recombination of the oncogenic *K-ras*^{G12D} allele (**Fig. 2h**). To further confirm the diagnosis of endometriosis, we showed that the peritoneal endometriotic lesions shared not only morphological but also immunohistochemical similarities with the uterine endometrium. Thus, both endometriotic and endometrial glands were positive for epithelial markers, such as cytokeratins 7 and 8, but not for cytokeratin 20 (**Fig. 3a**). In addition, they both stained positively for steroid receptors,

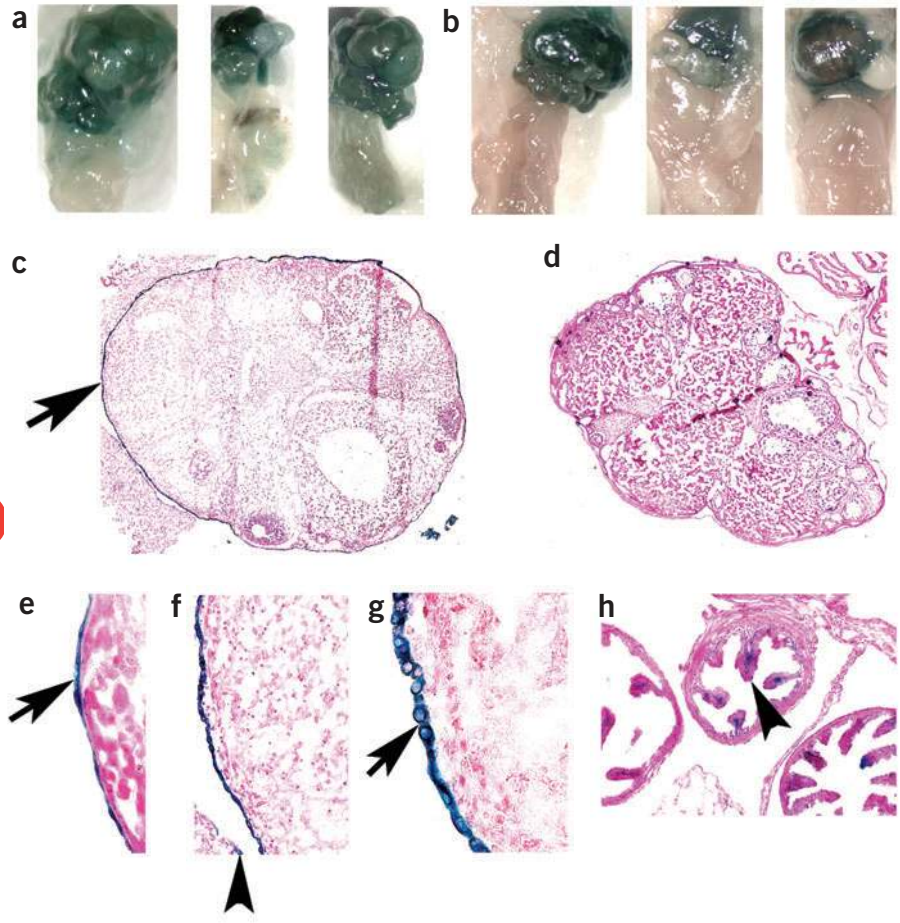

Figure 1 Intrabursal AdCre administration results in efficient infection of the OSE. (**a**,**b**) Whole-mount β-galactosidase staining of ovaries and the reproductive tract in reporter mice, following a single intrabursal injection of AdCre delivered through the uterotubal junction (**a**) or infundibulum (**b**). (**c**,**d**) Histochemical detection of β-galactosidase in the OSE (arrow) of AdCre-injected (**c**) and control ovaries (**d**). (**e–g**) Higher-magnification pictures of LacZ staining within the OSE (arrow) of AdCre-infected mice; AdCre was delivered intrabursally through the uterotubal junction (**e**) or infundibulum (**f**,**g**). Traces of LacZ staining are also detected in the bursa (**f**, arrowhead) and tubal epithelium (oviduct) (**h**, arrowhead).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

estrogen and progesterone receptors (**Fig. 3a**). Furthermore, endometriotic stroma was positive for markers that typically stained endometrial stroma, such as smooth-muscle actin and CD10 (**Fig. 3a**). The presence in the peritoneum of glandular lesions that are cytokeratin 7–, estrogen receptor– and progesterone receptor–positive, surrounded by CD10 and smooth-muscle actin–positive stroma, coupled with the specific histopathological findings and the benign long-term follow-up, suggests a diagnosis of endometriosis.

The development of the first model of *de novo* endometriosis enabled us to gain some insight into the mechanism of endometriosis initiation in mouse models. Thus, we tested the metaplastic theory of endometriosis initiation by delivering AdCre directly into the peritoneum through intraperitoneal injections. Animals were killed approximately 8 months after AdCre injection and the entire peritoneum was carefully analyzed in all cases. Notably, all animals injected intraperitoneally, being either *LSL-K-ras*$^{G12D/+}$ (n = 12) or wild-type controls (n = 13), showed a normal histomorphology and no signs of endometriosis (**Fig. 3b**). These results indicate that at least in this mouse system peritoneal endometriosis does not originate through a process of metaplastic differentiation from the pelvic peritoneum.

In contrast, the 'implantation theory' of endometriosis initiation suggests that the lesions originate from endometrial tissue that is refluxed through the fallopian tubes into the peritoneum. Based on our injection protocol and the subsequent LacZ-staining analysis it is probable that oncogenic *K-ras*^{G12D} is also expressed in some percent of epithelial utero-tubal cells in addition to the OSE. As a result, uterine or tubal cells could acquire a growth advantage and may be able to implant and expand at ectopic peritoneal sites. Alternatively, the endometriotic lesions may arise directly from the OSE through a metaplastic differentiation process induced by oncogenic *K-ras*^{G12D}, and subsequently implant onto peritoneal surfaces. To differentiate between these two possible mechanisms, we used the same method of intrabursal AdCre injection in *LSL-K-ras*$^{G12D/+}$ mice, followed 48 h later by transplantation of the ovary (removed from its bursa and adjoining tissues, uterus, oviduct, etc.) under the ovarian bursa of BALB/c *Rag2*$^{-/-}$ mice. The transplanted mice (n = 4) were killed 5.5 months later. Although all of the mice showed ovarian endometriotic-like lesions (**Fig. 3c**) similar to those described above, none of them had peritoneal endometriosis. Although the number of mice is too small to draw a definitive conclusion from this experiment, the results raise the possibility that the ovarian and peritoneal lesions seen in *LSL-K-ras*$^{G12D/+}$ mice may have distinct origins, with the ovarian lesions arising from the OSE and the peritoneal lesions having a uterine or tubal origin. This possibility is currently under investigation.

K-ras and *Pten* in the OSE causes endometrioid ovarian cancer

As shown above, expression of oncogenic *K-ras*^{G12D} within the OSE results in benign epithelial lesions with a typical endometrioid glandular morphology that do not progress to ovarian carcinoma. Because *PTEN* has been implicated in endometrioid ovarian tumorigenesis in humans[2,3], we tested whether the combined mutation of *K-Ras* and *Pten* in the OSE might cause progression to this tumor type in mouse models. Indeed, all *LSL-K-ras*$^{G12D/+}$ *Pten*$^{loxP/loxP}$ mice developed invasive endometrioid ovarian adenocarcinomas as early as 7 weeks after intrabursal AdCre administration through the infundibulum. In the initial cohort of mice we tested (n = 9), animals showed signs of disease and were killed between 7 and 12 weeks after injection. AdCre-infected ovaries were enlarged (**Fig. 4a**) compared to control uninfected ovaries (**Fig. 4a**). Grossly, the ovarian tumors were solid and cystic, with the cysts containing soft masses and bloody fluid (**Fig. 4a**). Some animals also showed a distended abdomen (**Supplementary Fig. 4** online) and

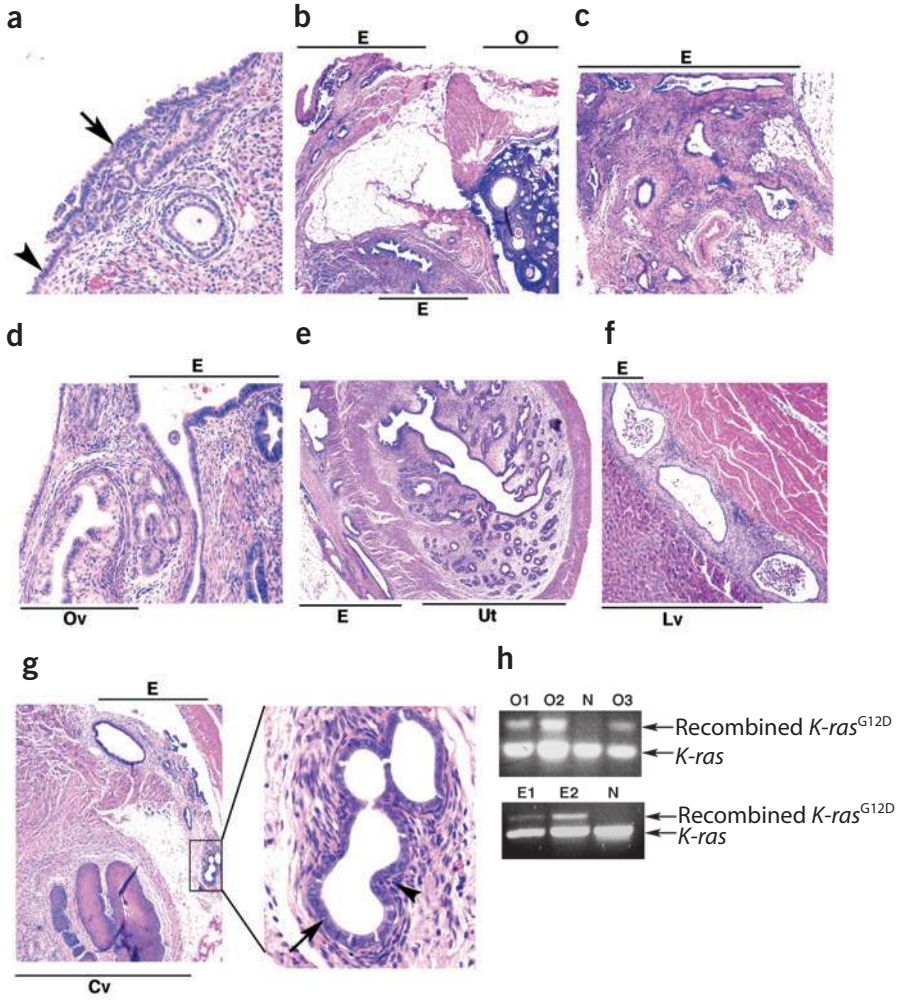

Figure 2 Induction of ovarian endometriotic-like lesions and peritoneal endometriosis by oncogenic *K-ras*^{G12D}. (**a**) Activation of *K-ras*^{G12D} within the OSE results in endometrioid glandular lesions (arrow); normal OSE is seen nearby (arrowhead). (**b–g**) Peritoneal endometriosis (E) in the soft tissue surrounding the ovary (O) (**b**), fat (**c**), on the surface of the oviduct (Ov) (**d**), uterus (Ut) (**e**), liver (Lv) (**f**), and cervix (Cv) (**g**). A higher magnification picture (**g**) shows the presence of both endometriotic epithelial glands (arrow) and stroma (arrowhead). (**h**) Cre-mediated recombination of *K-ras*^{G12D} in ovarian endometriotic-like lesions (O1–O3), peritoneal endometriotic lesions (E1, E2), but not in control OSE (N).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

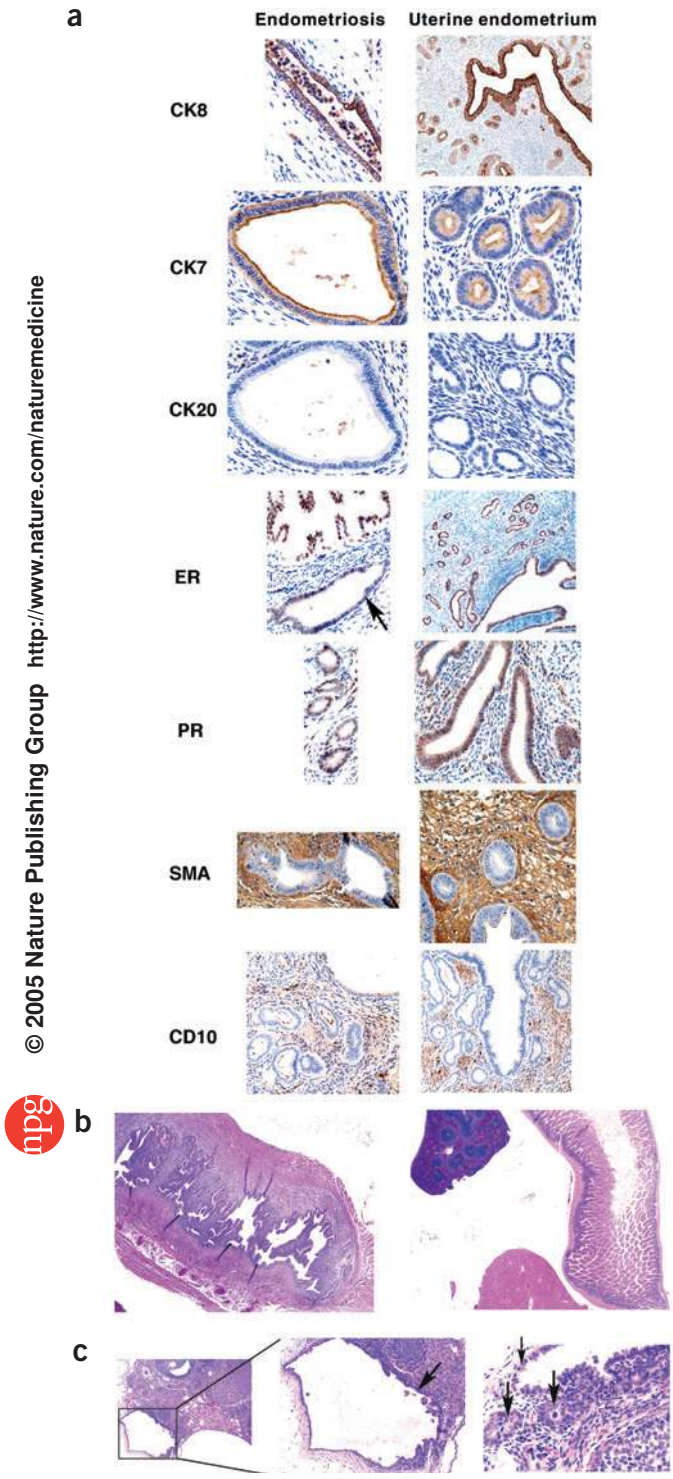

Figure 3 Characterization of endometriotic lesions. (**a**) Peritoneal endometriotic lesions and the uterine endometrium have a similar immunohistochemical profile. Both endometriotic and endometrial glands are positive for cytokeratin 8 (CK8), cytokeratin 7 (CK7), estrogen receptor (ER) and progesterone receptor (PR), but not for cytokeratin 20 (CK20). Endometriotic stroma stains positively with endometrial-type stroma markers, smooth-muscle actin (SMA) and CD10. (**b**) Intraperitoneal injection of AdCre in *LSL-K-ras*$^{G12D/+}$ mice does not result in endometriosis. Organs within the pelvic/peritoneal cavity, such as the uterus, spleen, liver and intestine, show a normal histomorphology. (**c**) Transplanted AdCre-infected *LSL-K-ras*$^{G12D/+}$ ovaries show similar epithelial lesions with endometrioid glandular morphology (arrow).

hemorrhagic ascites upon necropsy (**Fig. 4a**). All tumors were diagnosed as ovarian endometrioid adenocarcinomas. The primary tumor was located in the ovary in all cases; no morphological changes were seen in either the uterus or oviduct. The ovarian tumors appeared to originate from the OSE and extended into and replaced ovarian stroma through a process of destructive infiltrative growth (**Fig. 4b**). Histologically, we identified both confluent glandular tumor patterns and areas of solid growth (**Fig. 4b**). Some hemorrhage was also seen (**Fig. 4b**); in addition, metaplastic squamous differentiation, a diagnostic feature of ovarian endometrioid carcinoma, was observed in some tumors (**Fig. 4b**). Notably, we did not see any histological abnormalities in the ovaries or OSE of AdCre-infected *Pten*$^{loxP/loxP}$ mice ($n = 9$) injected in parallel and examined within the same timeframe (7–12 weeks; **Fig. 4c**). In contrast, invasive peritoneal implants of endometrioid ovarian carcinoma were identified in *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice as early as 7 weeks after AdCre injection. The glandular implants with a cribriform arrangement were widely disseminated throughout the peritoneum (**Fig. 4d**); some tumors also showed villoglandular growth patterns (**Fig. 4e**). In addition, they diffusely infiltrated abdominal organs (*i.e.*, pancreas, liver and intestine; **Fig. 4f**,**g** and data not shown) and showed a desmoplastic response. Furthermore, a high proportion of ovarian tumors (43%) metastasized to the lungs (**Fig. 4h**).

In a separate experiment, we performed a survival study of another cohort of AdCre-infected *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice ($n = 20$) injected using the same method and killed when they were moribund. The majority of animals (18 of 20) survived for 10 and 20 weeks after AdCre administration; the remaining two mice in the study survived for 26 weeks after infection. The tumors analyzed in these animals showed morphological characteristics similar to those described above. In contrast, all AdCre-injected *Pten*$^{loxP/loxP}$ mice ($n = 13$) were alive and showed no signs of disease throughout the study. They were killed and analyzed 26 weeks after AdCre infection, when the last *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice died. Of note, we diagnosed benign epithelial lesions within the OSE in 62% (8 of 13) of animals; these lesions showed a typical endometrioid glandular morphology and were considerably similar to those seen in AdCre-injected *LSL-K-ras*$^{G12D/+}$ OSE (**Fig. 5a**,**b**). Notably, one of these *Pten*$^{loxP/loxP}$ animals developed endometrioid ovarian adenocarcinoma (**Fig. 5c**).

To prove that the ovarian endometrioid tumors seen in Cre-induced *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ originate in the OSE, we transplanted the injected ovaries (removed from the bursa and adjoining tissues, uterus, oviduct, etc.) under the ovarian bursa of BALB/c *Rag2*$^{-/-}$ mice ($n = 8$). The mice were killed 8 weeks later and showed enlarged ovaries on the transplanted side (**Fig. 5d**) compared to the control side (**Fig. 5d**). All animals were diagnosed with endometrioid ovarian adenocarcinomas that had an identical histomorphology (**Fig. 5e**) with the ovarian tumors previously described. This result indicates that the ovarian tumors in our model most probably originate from the OSE.

We further confirmed Cre-mediated recombination of the oncogenic *K-ras*^{G12D} allele and conditional deletion of *Pten* in primary ovarian tumors and peritoneal implants (**Fig. 5f**). Immunohistochemical studies showed that both ovarian tumors and their peritoneal implants stained strongly with epithelial markers (CK8) (**Fig. 5g**). As expected, conditional *Pten* deletion induced strong AKT phosphorylation (p-AKT) in the primary ovarian tumor, peritoneal implants and lung metastases (**Fig. 5h**). Furthermore, the tumor implants showed focal immunostaining for phosphorylated mTOR (p-mTOR), a key downstream effector of p-AKT (**Fig. 5h**), suggesting that activation of the PI3-kinase-AKT-mTOR pathway might be responsible in part for their oncogenic potential. Notably, they also showed increased phosphorylation of the p70 S6 kinase (p-S6K), a downstream target of mTOR (**Fig. 5h**). Thus, this ovarian tumor subtype may be an attractive

target for rapamycin therapy, because low PTEN, high p-AKT and high p-S6K levels are expected to render it sensitive to rapamycin. In addition, the mouse tumors were positive for phosphorylated FKHR (p-FKHR) and phospho-MAPK (p-MAPK), downstream effectors of p-AKT and oncogenic K-RAS, respectively (**Fig. 5h**). Finally, as reported for human endometrioid ovarian carcinomas, the mouse primary ovarian tumors, tumor implants and lung metastases showed high levels of estrogen receptor immunostaining (**Fig. 5h** and data not shown).

DISCUSSION

Here we present the first mouse models of two major gynecological diseases, endometriosis and well-differentiated endometrioid adenocarcinoma of the ovary. In addition to recapitulating the histomorphology of these human diseases, the two models closely mimic their biological behavior. Notably, several studies have analyzed the mutational status of *K-RAS* in human endometriosis and did not find any mutations to date[15–17]. Our results, however, raise the possibility that RAS pathway activation by alternate mechanisms may have a role in human endometriosis. Although the mouse model we developed may not be an exact genocopy of human endometriosis, it is a phenocopy of the human disease from the histomorphological and biological standpoints. As such, we think that it can be a valuable tool for the study of endometriosis, as it will allow a more thorough investigation into the mechanism of endometriosis initiation, propagation, and possibly therapy. Furthermore, although the coexistence of *PTEN* and *K-RAS* mutations in ovarian cancer patients has yet to be investigated, our results obtained in mouse models give further reason to explore this potential in human tumors.

In contrast to the peritoneal endometriotic lesions that only develop in Cre-induced *LSL-K-ras*$^{G12D/+}$ mice, benign appearing (i.e., histologically bland and noninvasive) proliferations of glands on the ovarian surface were observed in both AdCre-injected *LSL-K-ras*$^{G12D/+}$ and *Pten*$^{loxP/loxP}$ animals. These epithelial lesions, although showing a notable endometrioid glandular morphology, lacked a surrounding endometrial-type stroma. Presence of endometrial-type stroma surrounding endometrial glands is pathognomic of endometriosis. Therefore, in contrast to the peritoneal lesions, we have not classified them as endometriosis. However, even in human endometriosis lesions stromal cells can be very sparse or sometimes absent. Consequently, it remains to be determined whether these lesions represent endometriosis or a unique epithelial proliferation. Furthermore, these ovarian endometriotic-like lesions appear to be a precursor for endometrioid ovarian adenocarcinoma, because they were present in the majority of AdCre-induced *Pten*$^{loxP/loxP}$ mice and in one case they probably progressed to endometrioid ovarian cancer. Notably, *PTEN* has been implicated in the progression of ovarian endometriosis to endometrioid ovarian carcinoma in humans[3]. Specifically, three out of five cases of endometrioid ovarian carcinomas associated with endometriosis displayed loss of heterozygosity events at the 10q23.3 *PTEN* locus in both the ovarian carcinoma and endometriotic lesions[3]. In addition, somatic mutations in the *PTEN* gene have been identified in 20% of endometrioid ovarian carcinoma cases as well as 20% of ovarian endometriotic lesions[3].

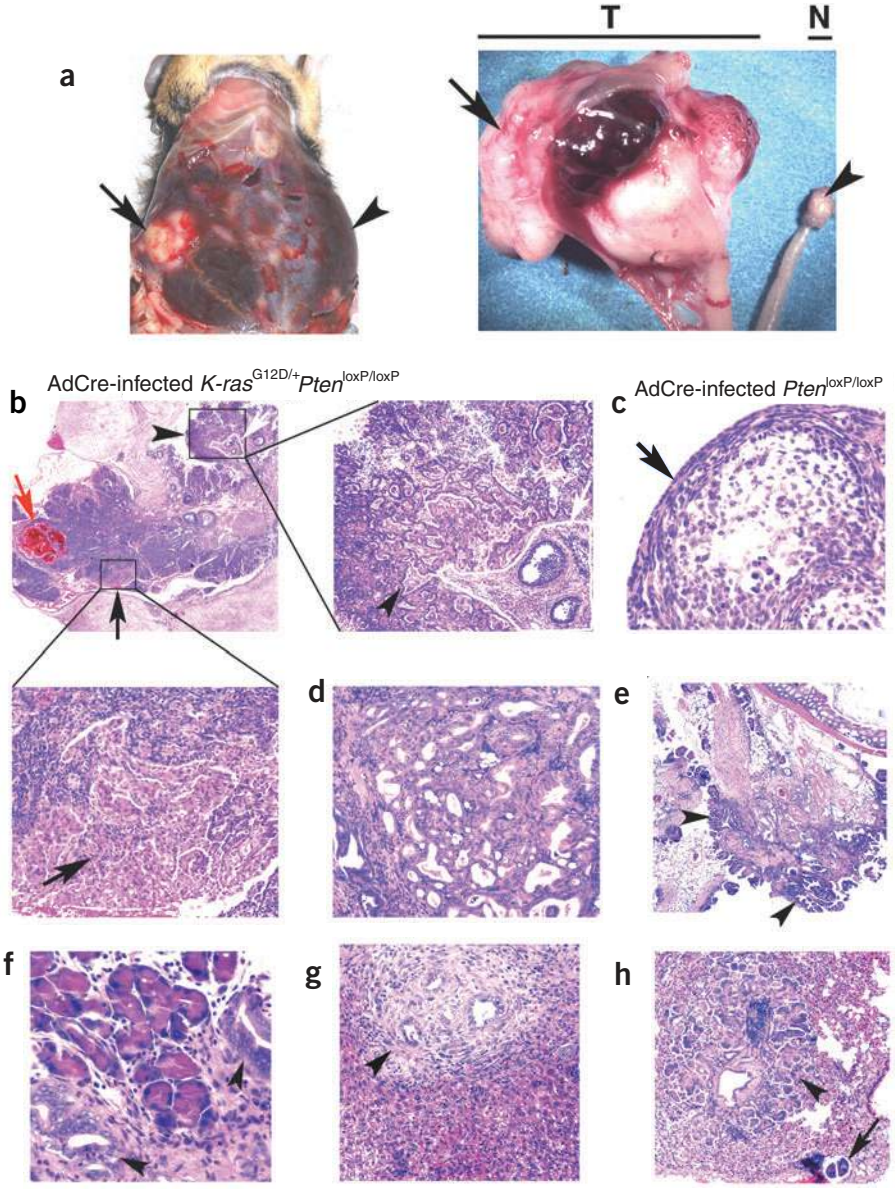

Figure 4 Combined *K-ras*^{G12D} activation and conditional deletion of *Pten* lead to endometrioid ovarian cancer. (**a**) A single intrabursal injection of AdCre in *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice on one side results in hemorrhagic ascites and a large, cystic ovarian tumor (T, arrow); normal (N, arrowhead). (**b**) Histopathology of endometrioid ovarian carcinomas. Areas of solid growth with squamous differentiation (black arrow), confluent glandular pattern areas (black arrowhead) and hemorrhage (red arrow) are shown. The remaining normal ovary is depicted by the white contour and arrow. (**c**) AdCre-infected *Pten*$^{loxP/loxP}$ ovaries show normal OSE (arrow) at 12 weeks after injection. (**d**,**e**) Peritoneal tumor implants with a cribriform glandular architecture (**d**) or a villoglandular growth pattern (**e**, arrowhead). (**f**,**g**) Peritoneal tumor implants (arrowhead) infiltrate the pancreas (**f**) and liver (**g**). (**h**) Tumor cells circulating through a lymphovascular channel (arrow) are seen near the lung metastasis (arrowhead).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

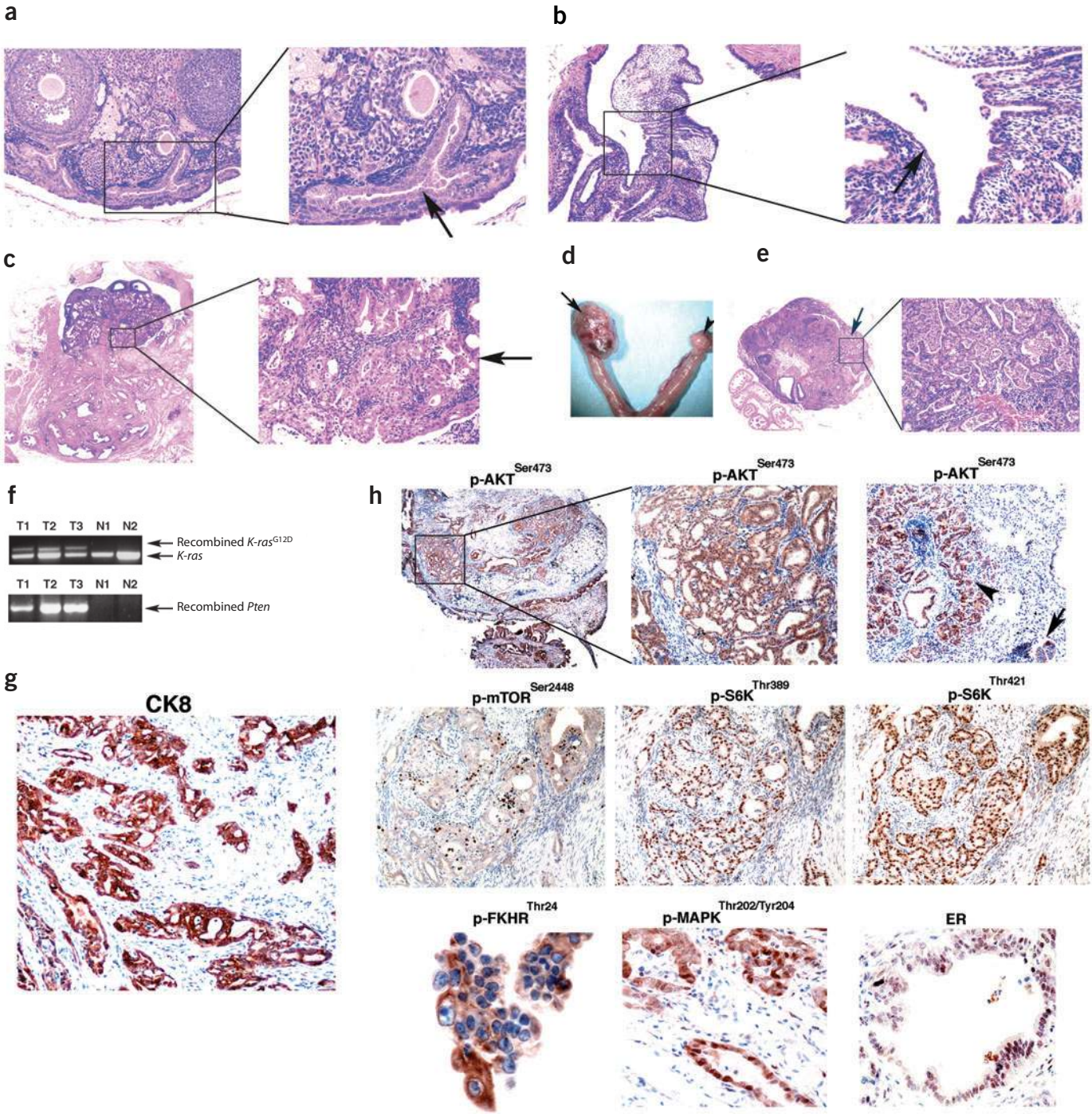

Figure 5 Lesion profiles in AdCre-infected *LSL-K-ras*$^{G12D/+}$ *Pten*$^{LoxP/loxP}$ and *Pten*$^{loxP/loxP}$ mice. (**a**,**b**) Induction of ovarian endometriotic-like lesions (arrow) in AdCre-infected *Pten*$^{loxP/loxP}$ mice at 26 weeks after injection. (**c**) Ovarian endometrioid adenocarcinoma (arrow) detected in 1 of 13 AdCre-infected *Pten*$^{loxP/loxP}$ mice at 26 weeks after injection. (**d**,**e**) Ovarian tumors in BALB/c *Rag2*$^{-/-}$ mice transplanted with AdCre-infected ovaries isolated from *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice. The transplanted ovary is enlarged (**d**, arrow) compared to control (**d**, arrowhead), and shows a similar histopathology (**e**) with the ovarian tumors previously described. (**f**) Cre-mediated recombination of *K-ras*^{G12D} and conditional deletion of *Pten* in tumors (T1–T3) isolated from AdCre-infected *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ animals; controls (N1, N2). (**g**,**h**) Immunohistochemical profile of primary ovarian tumors, peritoneal implants and lung metastases in Cre-induced *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice.

Finally, the studies presented here have potential clinical implications for the therapy of human endometrioid ovarian carcinoma. Our results demonstrate that the mouse tumors show activation of both the PI3K-AKT-mTOR-p70 S6K and MAPK pathways. The Cre-induced *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mouse model of ovarian cancer will be helpful in testing the efficacy of specific inhibitors of these pathways, such as rapamycin and PD 184352 (refs. 18,19), in the treatment of ovarian cancer. In addition, we show that the mouse ovarian tumors exhibit phosphorylation of forkhead transcription factors, which may have a role in ovarian cancer chemoresistance to cisplatin[20]. Furthermore,

because these tumors are estrogen receptor–positive, studies with selective modulators of antiestrogen signaling in combination with conventional chemotherapy are also warranted. The accumulation of these critical preclinical data should lead in turn to the development of more rational clinical trials for human ovarian cancer therapy.

METHODS

Generation of mice. The *Gt(ROSA)26Sor* reporter strain was acquired from the Jackson Lab. The *LSL-K-ras*$^{G12D/+}$ strain was developed in our laboratory, and the *Pten*$^{loxP/loxP}$ mice were provided by H. Wu. *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice were generated by crossing animals heterozygous for the *lox*P-Stop-*lox*P-*K-ras*^{G12D} allele to mice homozygous for the conditional *Pten* allele (*Pten*$^{loxP/loxP}$)[21]. *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/+}$ littermates were then bred to each other to generate *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice. All animals were on a mixed genetic background. Specifically, *LSL-K-ras*$^{G12D/+}$ animals were on a 129 S_4/SvJae background or a 129 S_4/SvJae-C3H/HeJ (F1 mixed background). The original background of the *Pten*$^{loxP/loxP}$ mice received from H. Wu was BALB/c; they were then rederived on a C57BL/6J background and subsequently crossed to *LSL-K-ras*$^{G12D/+}$ mice on a 129 S_4/SvJae background. Animal genotyping was done by PCR analysis of tail DNA according to published methods[13,14,21].

Surgical procedures and administration of recombinant adenovirus. All animal studies and procedures were approved by the Massachusetts Institute of Technology Institutional Animal Care and Use Committee. AdCre was injected into surgically exposed ovaries. The recombinant adenovirus, carrying a cytomegalovirus (CMV) promoter, was provided by the University of Iowa Gene Transfer Vector Core. To synchronize ovulation, animals were injected with 5 U of pregnant mare serum gonadotropin (Sigma) and 48 h later were injected with 5 U of human chorionic gonadotropin (Sigma). Subsequently, animals were given a single intrabursal injection of AdCre (2.5×10^7 plaque-forming units (p.f.u.)) to one ovary only (the contralateral ovary served as control) approximately 1.5 d later. This time point after ovulation was shown to correspond to increased proliferation of epithelial cells on the ovarian surface, as described previously[10]. The adenovirus was delivered using a modified calcium phosphate precipitation protocol[13]. Briefly, we reconstituted Minimum Essential Media powder (Sigma) into 450 ml of sterile water and supplemented it with 2.2 g of sodium bicarbonate. The calcium chloride solution was prepared by dissolving 3 g of calcium chloride in 100 ml of sterile water. We first added 20 μl of AdCre (1×10^7 p.f.u./μl) to 18 μl of MEM, gently mixed the solution, then added 2 μl of calcium chloride for a total of 40 μl final solution (the final phosphate and calcium concentrations were 0.86 mM and 12 mM, respectively). The mixture was incubated for 15–20 min at room temperature and 5 μl of the calcium phosphate precipitate containing AdCre virus was subsequently injected. For the intraperitoneal experiments we administered 100 μl of AdCre (5×10^8 p.f.u.) per animal. In the ovarian transplantation experiments, AdCre-injected ovaries (minus the bursa, uterus and oviduct) were removed from *LSL-K-ras*$^{G12D/+}$ or *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ animals 48 h after injection and transplanted under the ovarian bursa of BALB/c *Rag2*$^{-/-}$ recipients following published methods[22]. The ovary of the recipient mouse was removed prior to transplantation and the procedure was performed on one side only, with the contralateral ovary serving as control.

Histopathology. We monitored *LSL-K-ras*$^{G12D/+}$*Pten*$^{loxP/loxP}$ mice closely for tumor formation or signs of ill health and used *Pten*$^{loxP/loxP}$ animals as controls. Mice that showed obvious tumors or evidence of distress were killed and subjected to full necropsy and gross examination. *LSL-K-ras*$^{G12D/+}$ and wild-type controls did not show any signs of tumor, and they were killed at designated time points between 7 and 10 months after AdCre administration. The histopathological analysis and diagnosis of endometriosis and ovarian carcinomas were performed by T.A.I. and B.J.Q. according to human tumor classifications.

LacZ staining and immunohistochemical studies. The reproductive tract, including the ovaries, was removed from *lox*P-Stop-*lox*P LacZ *(Gt(ROSA)26Sor)* reporter mice 72 h after intrabursal administration of AdCre. We fixed the organs for 30 min in formalin and subjected them to LacZ staining according to published methods[10], froze them in OCT, sectioned them with a cryostat, and then analyzed them. Immunohistochemical analysis was performed on formalin-fixed paraffin sections following antigen retrieval (10 min boiling in 10 mM sodium citrate buffer followed by 20 min antigen unmasking at room temperature). The primary antibodies and dilutions used in these studies are as follows: anti-cytokeratin 8 (1:10, RDI), anti-cytokeratin 7 (NEAT, Abcam), anti-cytokeratin 20 (1:50, RDI), estrogen receptor (1:200, LabVision), progesterone receptor (1:50, LabVision), smooth-muscle actin (1:200, RDI), CD10 (NEAT, Novocastra), phospho-AKT^{Ser473} (1:25, Cell Signaling Technology), phospho-MAPK$^{Thr202/Tyr204}$ (1:50, Cell Signaling Technology), phospho-mTOR^{Ser2448} (NEAT, Cell Signaling Technology), phospho-S6K^{Thr421} (1:50, Cell Signaling Technology), phospho-S6K^{Thr389} (1:100, Cell Signaling Technology), and phospho-FKHR^{Thr24} (1:25, Cell Signaling Technology). Normal estrogen receptor staining in control ovaries is shown in **Supplementary Fig. 5** online. Negative control slides for the immunohistochemistry experiments are shown in **Supplementary Fig. 6** and **7** online.

PCR analysis of Cre-mediated recombination. Laser capture microdissection and DNA isolation were performed as previously described[10]. DNA isolated through this method was then subjected to PCR analysis using specific primers that detected Cre-mediated recombination of the oncogenic *K-ras*^{G12D} allele (absence of the STOP cassette), as reported previously[13,14]. PCR analysis of Cre-mediated deletion of *Pten* was performed on genomic DNA isolated from ovarian tumors using a phenol-chloroform extraction method[21].

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank C. P. Crum and D. H. Castrillon for additional pathological analysis and comments and advice. We are grateful to A.Y. Nikitin, A. Flesken-Nikitin, T. C. Hamilton, and R. Bao for sharing their technical expertise and advice. In addition, we would like to thank E. Jarmon for technical assistance, H. Wu for the gift of *Pten*$^{loxP/loxP}$ mice, and D. Kirsch and D. MacPherson for critical reading of the manuscript. This work was supported by grants from the American Cancer Society and the Shoreline Circle of Hope (D.M.D.), the Anna Fuller Fellowship (D.M.D.), the Mouse Models of Human Cancer Consortium of the National Cancer Institute (T.J.), and a KO8 award (CA 92013) from the National Cancer Institute (T.A.I.). T.J. is an Investigator of the Howard Hughes Medical Institute. This paper is dedicated to the memory of a good friend, I. Triculescu, who fought a courageous battle with ovarian cancer.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 5 October; accepted 24 November 2004
Published online at http://www.nature.com/naturemedicine/

1. Ozols, R.F. *et al.* Focus on epithelial ovarian cancer. *Cancer Cell* **5**, 19–24 (2004).
2. Obata, K. *et al.* Frequent PTEN/MMAC1 mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Res.* **58**, 2095–2097 (1998).
3. Sato, N. *et al.* Loss of heterozygosity on 10q23.3 and mutation of the tumor suppressor gene *PTEN* in benign endometrial cyst of the ovary: possible sequence progression from benign endometrial cyst to endometrioid carcinoma and clear cell carcinoma of the ovary. *Cancer Res.* **60**, 7052–7056 (2000).
4. Cuatrecasas, M. *et al. K-ras* mutations in nonmucinous ovarian epithelial tumors. *Cancer* **82**, 1088–1095 (1998).
5. Gemignani, M.L. *et al.* Role of *KRAS* and *BRAF* gene mutations in mucinous ovarian carcinoma. *Gynecol. Oncol.* **90**, 378–381 (2003).
6. Hogdall, E.V. *et al.* K-ras alterations in Danish ovarian tumor patients. From the Danish "Malova" Ovarian cancer study. *Gynecol. Oncol.* **89**, 31–36 (2003).
7. Okuda, T. & Okai, T. p53 mutations and overexpression affect prognosis of ovarian endometrioid cancer but not clear cell cancer. *Gynecol. Oncol.* **88**, 318–325 (2003).
8. Orsulic, S. *et al.* Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell* **1**, 53–62 (2002).
9. Connolly, D.C. *et al.* Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. *Cancer Res.* **63**, 1389–1397 (2003).
10. Flesken-Nikitin, A., Choi, K.C., Eng, J.P., Shmidt, E.N. & Nikitin, A.Y. Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res.* **63**, 3459–3463 (2003).
11. Clement, P.B., Seidman, J.D., Russell, P. & Kurman, R.J. in *Blaustein's Pathology of the Female Genital Tract* 5th edn (ed. Kurman, R.J.) 746–768, 791–905 (Springer, New York, 2002).
12. Swiersz, L.M. Role of endometriosis in cancer and tumor development. *Ann. NY Acad. Sci.* **955**, 281–292 (2002).
13. Jackson, E. L. et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-RAS. *Genes Dev.* **15**, 3243–3248 (2001).
14. Tuveson, D.A. *et al.* Endogenous oncogenic *K-ras*^{G12D} stimulates proliferation and

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

widespread neoplastic and developmental defects. *Cancer Cell* **5**, 375–387 (2003).
15. Vercellini, P. *et al.* Analysis of p53 and ras gene mutations in endometriosis. *Gynecol. Obstet. Invest.* **38**, 70–71 (1994).
16. Amemiya, S. *et al.* Malignant transformation of endometriosis and genetic alterations of K-ras and microsatellite instability. *Int. J. Gynecol. Obstet.* **86**, 371–376 (2004).
17. Otsuka, J. *et al.* K-ras mutation may promote carcinogenesis of endometriosis leading to ovarian clear cell carcinoma. *Med. Electron Microsc.* **37**, 188–192 (2004).
18. Grunwald, V. *et al.* Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. *Cancer Res.* **62**, 6141–6145 (2002).
19. Sebolt-Leopold, J.S. *et al.* Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nat. Med.* **5**, 810–816 (1999).
20. Arimoto-Ishida, E. *et al.* Inhibition of phosphorylation of a forkhead transcription factor sensitizes human ovarian cancer cells to cisplatin. *Endocrinology* **145**, 2014–2022 (2004).
21. Lesche, R. *et al.* Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene. *Genesis* **32**, 148–149 (2002).
22. Hogan, B., Beddington, R., Constantini, F. & Lacy, E. in *Manipulating the Mouse Embryo* 2nd edn 185–186 (Cold Spring Harbor Laboratory Press, Plainview, NY, 1994).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine