Disease Models & Mechanisms 3, 181-193 (2010) doi:10.1242/dmm.004440

RESEARCH ARTICLE

Lkb1 inactivation is sufficient to drive endometrial cancers that are aggressive yet highly responsive to mTOR inhibitor monotherapy

Cristina M. Contreras[1,2], Esra A. Akbay[1,2], Teresa D. Gallardo[1,2], J. Marshall Haynie[1,2], Sreenath Sharma[3], Osamu Tagao[4], Nabeel Bardeesy[3], Masaya Takahashi[4], Jeff Settleman[3], Kwok-Kin Wong[5] and Diego H. Castrillon[1,2,*]

SUMMARY

Endometrial cancer – the most common malignancy of the female reproductive tract – arises from the specialized epithelial cells that line the inner surface of the uterus. Although significant advances have been made in our understanding of this disease in recent years, one significant limitation has been the lack of a diverse genetic toolkit for the generation of mouse models. We identified a novel endometrial-specific gene, *Sprr2f*, and developed a *Sprr2f-Cre* transgene for conditional gene targeting within endometrial epithelium. We then used this tool to generate a completely penetrant *Lkb1* (also known as *Stk11*)-based mouse model of invasive endometrial cancer. Strikingly, female mice with homozygous endometrial *Lkb1* inactivation did not harbor discrete endometrial neoplasms, but instead underwent diffuse malignant transformation of their entire endometrium with rapid extrauterine spread and death, suggesting that *Lkb1* inactivation was sufficient to promote the development of invasive endometrial cancer. Mice with heterozygous endometrial *Lkb1* inactivation only rarely developed tumors, which were focal and arose with much longer latency, arguing against the idea – suggested by some prior studies – that *Lkb1* is a haploinsufficient tumor suppressor. Lastly, the finding that endometrial cancer cell lines were especially sensitive to the mTOR (mammalian target of rapamycin) inhibitor rapamycin prompted us to test its efficacy against *Lkb1*-driven endometrial cancers. Rapamycin monotherapy not only greatly slowed disease progression, but also led to striking regression of pre-existing tumors. These studies demonstrate that *Lkb1* is a uniquely potent endometrial tumor suppressor, but also suggest that the clinical responses of some types of invasive cancers to mTOR inhibitors may be linked to Lkb1 status.

INTRODUCTION

Endometrial cancer represents 6% of all cancer and is the most common cancer of the female reproductive tract, with 40,880 new cases and 7310 cancer-related deaths in 2005 in the USA alone (Jemal et al., 2007). Although surgical therapy (hysterectomy and salpingo-oophorectomy) is effective against early disease, the median survival of women with advanced endometrial cancer is less than one year, and in the last 30 years there has not been a significant improvement in survival of advanced disease. Neither cytotoxic agent nor radiation therapies significantly increase long-term survival (Ellenson and Wu, 2004). Ovarian cancer has received more attention than endometrial cancer, although it is frequently overlooked that nearly 30% of ovarian cancers are endometrioid adenocarcinomas arising in foci of endometriosis (implants of endometrium outside the uterus) and are thus believed to be of endometrial origin.

The endometrium – or inner lining of the uterus – is comprised of endometrial epithelial glands set in a specialized stroma. Endometrial adenocarcinomas, which account for 95% of uterine body malignancies, arise from the epithelial cells. Inactivating mutations of the tumor suppressor *PTEN* represent the most common known genetic abnormality in endometrial adenocarcinomas. *PTEN* mutations have been identified in approximately 50% of tumors (Tashiro et al., 1997), and are also frequently present in premalignant hyperplasias, demonstrating that *PTEN* mutations occur relatively early in the pathogenesis of the disease. Some studies have suggested that *PTEN* mutations precede the development of histologically recognizable hyperplastic lesions, and thus may represent true initiating events. Other endometrial cancer genes include *KRAS*, mutated in 10-30% of cases, and *CTNNB1* (β-catenin), which has a mutation rate of ~10%, although β-catenin accumulates in 40% of cases (Ellenson and Wu, 2004). These alterations are also frequently observed in hyperplasias, arguing that they too represent relatively early events in endometrial tumor progression. The high mutation rate of both *PTEN* and *KRAS* have implicated phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) signaling as a crucial endometrial cancer network, a notion recently corroborated by the discovery that *PIK3CA* mutations are common (36%) in endometrial cancers (Oda et al., 2005). Mutations in *TP53* are found in approximately 10-20% of all endometrioid carcinomas, typically in high-grade tumors, and may synergize with accelerated telomere shortening (Akbay et al., 2008). Thus, although some signature molecular lesions underlying endometrial cancer have been identified, most, with the possible exception of *TP53*, are early events that drive the formation of hyperplasias. The molecular underpinnings of the transition of hyperplasias to invasive, lethal cancers remain poorly understood.

Genetically engineered mouse models (GEMMs) of endometrial cancer are poised to accelerate our understanding of the impact of

[1]Department of Pathology, [2]Simmons Comprehensive Cancer Center and [4]UT Southwestern Medical Center, UT Southwestern Medical Center, Dallas, TX 75390, USA
[3]Massachusetts General Hospital Cancer Center and Harvard Medical School, Boston, MA 02114, USA
[5]Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115, USA
*Author for correspondence (diego.castrillon@utsouthwestern.edu)

individual genetic lesions on tumor initiation and progression, and facilitate preclinical testing of novel therapeutic targets. One significant limitation in the development of such models has been the lack of an endometrial epithelium-specific gene or promoter that could be exploited to direct the expression of transgenes (e.g. Cre recombinase) specifically within endometrial epithelium. Some Cre lines with endometrial activity, such as the progesterone receptor Cre knock-in *PR-Cre*, have been described and used to explore the role of putative endometrial cancer genes such as *Pten* and *Ctnnb1* in endometrial cancer (Daikoku et al., 2008; Jeong et al., 2009). However, the utility of *PR-Cre* may be limited in some experimental contexts by the expression of the progesterone receptor (and hence *PR-Cre*) throughout the uterus, including endometrial stroma, and in progesterone-responsive tissues such as the ovary, mammary gland and brain.

Inherited mutations in the *LKB1* locus result in Peutz-Jeghers syndrome (PJS), an autosomal dominant condition characterized by hamartomatous polyps of the gastrointestinal (GI) tract (Alessi et al., 2006; Shackelford and Shaw, 2009) and a significantly elevated (>15×) general risk of cancer including clinically and histopathologically distinctive neoplasms of the female reproductive tract. *LKB1* is a significant tumor suppressor in the lung and was the first gene that was found to be mutated in a significant proportion (>20%) of cervical cancers. Interestingly, the majority of cervical cancer cell lines, including HeLa, harbor homozygous *LKB1* deletions (Wingo et al., 2009). An important unresolved controversy is whether bialleleic inactivation is required for tumorigenesis, or if *LKB1* is a haploinsufficient tumor suppressor. Intriguingly, mouse models suggest that GI polyposis is due to *Lkb1* haploinsufficiency of the stroma and not the epithelium, and thus may not be a cell-autonomous process. In human cancers, the frequency of single-copy allelic loss at chromosome 19p spanning the *LKB1* locus (without inactivation of the second allele) has been interpreted as possible evidence for a haploinsufficient tumor suppressor function (Hezel and Bardeesy, 2008; Ji et al., 2007).

In mouse models, conditional *Lkb1* inactivation consistently promotes aggressive invasive and metastatic growth. In a *Kras*-driven mouse model of lung cancer, *Lkb1* inactivation provided the strongest cooperation in terms of tumor latency and frequency of metastasis [when compared with classic tumor suppressors such as *Tp53* (also known as *Trp53*) and *Ink4a/Arf* (also known as *Cdkn2a*)] (Ji et al., 2007). Carcinogen-treated mice with an engineered knockout of *Lkb1* in epidermis were prone to highly invasive squamous cell carcinomas of the skin that arose apparently de novo without progressing through an in situ (papilloma) stage (Gurumurthy et al., 2008). Finally, inactivation of *Lkb1* in mouse prostatic epithelium led to prostatic neoplasia and reduced longevity (Pearson et al., 2008). Consistent with the idea that *LKB1* inactivation promotes invasion and metastasis, as first suggested by these mouse models, *LKB1* mutations in primary cervical tumors were strongly associated with accelerated disease progression and death (Wingo et al., 2009), and decreased LKB1 protein expression in endometrial cancers correlates with a higher grade and stage (Contreras et al., 2008a). These findings suggest that assays based on *LKB1* may prove useful for prognostication in cancers characterized by *LKB1* inactivation.

In addition to intestinal polyposis, $Lkb^{+/-}$ female mice develop endometrial cancers, but with long latency and incomplete penetrance. We previously explored the role of *Lkb1* as an endometrial tumor suppressor through the instillation of Cre-expressing adenovirus (Ad-Cre) into the uterine lumen of adult females homozygous for the floxed allele ($Lkb1^{L/L}$) (Contreras et al., 2008a). As in $Lkb1^{+/-}$ mice, tumors were observed, but with long latency and incomplete penetrance (65% incidence by 9 months of age). These studies established *Lkb1* as a bona fide endometrial tumor suppressor that functions in a cell-autonomous manner and plays a special role in promoting invasion (Contreras et al., 2008a). However, a significant limitation of this Ad-Cre approach was its extremely low efficiency. Semi-quantitative PCR analysis indicated that the efficiency of recombination was <1% in endometrial epithelial cells, with no recombination detectable in ~50% of animals (unpublished data). Thus, it was unclear whether the relatively low observed cancer rate reflected the inefficiency of Ad-Cre infection, or the need for additional cooperating oncogenic mutations that characterize most murine cancer models. Furthermore, incomplete penetrance and long latency would have limited the utility of the model for preclinical drug testing.

LKB1 encodes a protein kinase that regulates the AMP-dependent kinase (AMPK)-mTOR signaling pathway. LKB1 directly phosphorylates, and thereby activates, AMPK, a sensor of intracellular ATP levels. AMPK controls protein synthesis by regulating mTOR through phosphorylation of Tsc2 (tuberin) (Shackelford and Shaw, 2009). Through this pathway, loss of LKB1 activity in tumors de-represses protein synthesis and promotes cell growth, proliferation and tumorigenesis. Intriguingly, Lkb1 is involved in the regulation of cell polarity, a role mediated at least in part by AMPK, which, in addition to its roles in the mTOR pathway, also regulates the assembly of epithelial tight junctions and thus directly contributes to the maintenance of epithelial cell polarity. Although these findings suggest that AMPK is of central importance as a downstream effector of LKB1 inactivation, LKB1 is also the master upstream kinase of at least 13 AMPK-related kinases, raising the possibility that LKB1 contributes to tumorigenesis through other mechanisms. Thus, misregulation of the AMPK-mTOR pathway probably contributes to LKB1's role as a tumor suppressor, but probably does not entirely account for its role in mediating invasion (Hezel and Bardeesy, 2008). Nonetheless, misregulation of mTOR in LKB1-deficient tumors represents an opportunity for the development of individualized targeted therapies.

There has been substantial interest in the use of mTOR inhibitors (rapamycin and its derivatives) in the treatment of cancer. For example, temsirolimus (CCI-779) is approved for the treatment of advanced renal cell cancer and also leads to objective responses in women with endometrial cancer (Meric-Bernstam and Gonzalez-Angulo, 2009). However, for both types of cancer, only a fraction of patients respond, and it has not been possible to predict responses on the basis of clinical features or biomarkers of PI3K/Akt/Pten status. In this study, we present a novel mouse model of endometrial cancer where Lkb1 deficiency is sufficient to drive invasive endometrial cancers with 100% penetrance. We then used this in vivo model to explore the hypothesis that mTOR inhibition and Lkb1 deficiency constitute a synthetic lethal interaction and, thus, that the state of Lkb1 deficiency in a tumor confers unique sensitivity to mTOR inhibition. Our data lend support to this hypothesis, suggesting that Lkb1 deficiency in

human tumors may be predictive not only of aggressive behavior but also of clinical responses to mTOR inhibitors.

RESULTS

Discovery of *Sprr2f* as a gene expressed only in endometrial epithelium

We previously developed a microarray-based method for the detection of genes expressed in specific tissues or cellular compartments. Having successfully employed this method for the systematic discovery of ovary-specific fertility factors (Gallardo et al., 2007b), we then applied the method to the mouse uterus, leading to the identification of five candidate genes. Subsequent analyses demonstrated that one of these genes, *Sprr2f*, was highly expressed in the uterus, but not in other tissues (Fig. 1). Northern analysis of total RNA using an *Sprr2f* 3′-untranslated region (UTR) probe confirmed our digital northern analysis (Fig. 1A), showing that *Sprr2f* was highly expressed only in the uterus (Fig. 1B). Because genes that are highly expressed in the uterus may be estrogen dependent, we analyzed the expression of *Sprr2f* throughout the 5-day murine estrus cycle [day (D)1 to D5]. *Sprr2f* expression in the uterus was induced in days of the estrus cycle associated with high circulating estrogen levels (D3 and D4), strongly suggesting that *Sprr2f* expression is estrogen dependent. This was confirmed by treatment of 3-week-old (prepubertal) mice with estrogen, which resulted in potent induction of *Sprr2f* expression. However, *Sprr2f* was not expressed in other sex steroid hormone-dependent organs such as the ovary or mammary gland (Fig. 1C).

RNA in situ hybridization was then employed to identify the specific uterine cell type(s) expressing *Sprr2f*. An antisense probe showed strong expression in endometrial epithelium including both the luminal (surface) and glandular (deep) compartments, but no expression in endometrial stroma or myometrium. Expression was temporally regulated, being detectable at D4 but not D1, as expected (supplementary material Fig. S1A-C). Immunohistochemistry using an antibody that recognizes all members of the Sprr2 family confirmed that, within the uterus, the expression of Sprr2 proteins is confined to the endometrial epithelium (supplementary material Fig. S1). In summary, several properties of *Sprr2f* expression suggested that its promoter could serve as the basis for useful tools for the genetic analysis of endometrial epithelium: (1) its high expression in uterine epithelium, (2) its lack of expression in other uterine compartments such as endometrial stroma, (3) its absence of expression in other tissues and (4) its expression not only in luminal endometrium but also in the deep glandular epithelium where presumptive endometrial stem cells may reside (Gargett et al., 2007).

Sprr2f is part of the *Sprr2* tandem gene cluster on chromosome 3. The 11 members of this cluster (*a-k*) encode small proline-rich repeat proteins that provide structural integrity and serve barrier functions in epithelial cells. *Sprr2* family members are variably expressed in different organs such as the skin and esophagus, where their expression is confined to the squamous or columnar epithelial cells (Patel et al., 2003; Song et al., 1999). *Sprr2* gene expression in the female reproductive tract had been documented previously, but the uterine-specific expression of *Sprr2f* was not known prior to this study (Hong et al., 2004; Tan et al., 2003; Tan et al., 2006). To understand the basis of the observed estrogen dependence of *Sprr2f* expression, the 4 kbs of genomic sequence upstream of each transcriptional start site were scanned with the Dragon ERE (estrogen response element) finder (Bajic et al., 2003). Using stringent search criteria, only *Sprr2f* among the 11 *Sprr2* family members was associated with a high scoring ERE (supplementary material Fig. S2A,B), suggesting that this ERE is physiologically significant and contributes to the estrogen-dependent expression of *Sprr2f*. Consistent with this hypothesis, gel retardation assays with an oligonucleotide probe spanning this ERE showed specific binding to the estrogen receptor α (ERα) protein that was abrogated following deletion or mutation of both palindromic half-sites (supplementary material Fig. S2C).

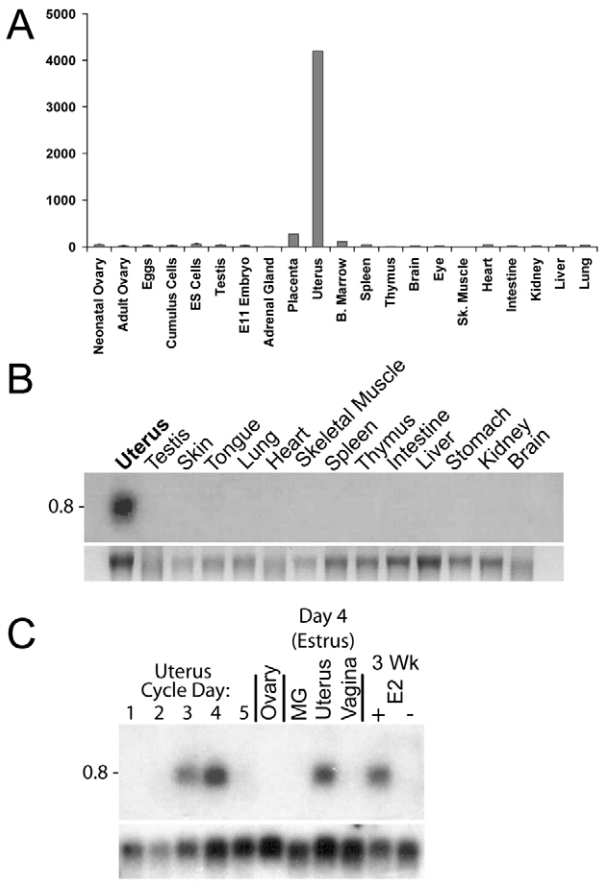

Fig. 1. Identification of *Sprr2f* as an estrogen-dependent gene that is specifically expressed in the uterus. (A) Digital northern representation, per Affymetrix 430 2.0 array data, of *Sprr2f* expression in total RNA from various tissues and developmental stages. The Y-axis corresponds to the relative expression levels of the *Sprr2f* probe set (1449833_at) across the samples shown. Tissues were obtained from adult mice unless specified otherwise. (B) Northern analysis of total RNA using an *Sprr2f* 3′-UTR probe demonstrates that *Sprr2f* (0.8 kb transcript) is expressed only in the uterus and in no other tissues obtained from adult female mice. A probe corresponding to the 3′-UTR was selected to avoid cross-hybridization with other *Sprr2* members, which are highly conserved. Bottom panel=loading control. (C) Northern analysis of total RNA using 3′-UTR probe, showing that *Sprr2f* expression is cyclical in relation to estrus and is induced by estrogen; MG=mammary gland. Uterus cycle days D1-D5 correspond to diestrus, proestrus, early estrus, estrus and postestrus. The last two lanes represent uteri from 3-week-old mice injected with estrogen or vehicle. Bottom panel=loading control.

Cloning of an *Sprr2f* promoter fragment containing the ERE and generation of *Sprr2f-Cre* transgenic mice

Next, we sought to (1) determine whether an *Sprr2f* promoter fragment could be used to direct the expression of transgenes specifically within endometrial epithelium and (2) simultaneously develop a novel Cre transgenic mouse line that would be useful for the generation of genetically engineered endometrial cancer models. First, a 5845-bp *Sprr2f* promoter fragment was cloned by PCR. This genomic DNA fragment contains the putative TATA box at –29, the putative ERE, a ~300-bp dinucleotide (TC_n) repeat sequence, and the transcriptional start site including 32 bases of the first (non-coding) exon (supplementary material Fig. S2B). This *Sprr2f* promoter fragment was ligated to a cassette of the Cre recombinase open-reading frame (ORF) with an engineered nuclear localization sequence (CreN) (Gu et al., 1993). Following linearization and oocyte microinjection of this construct, over 20 transgenic founder lines with transgene integration confirmed by Southern analysis were identified (not shown). Subsequent screening of these transgenic founder lines was performed by genetic crosses to the β-galactosidase reporter *R26R* (Soriano, 1999), followed by X-gal staining of tissues of the *Sprr2f-Cre*; *R26R* progeny. One line with the desired endometrial-specific Cre-mediated recombination was selected (Fig. 2).

Efficient Cre-mediated recombination was observed in both the luminal and glandular endometrial epithelium of *R26R* mice harboring *Sprr2f-Cre* (Fig. 2A-C, supplementary material Fig. S3A). Recombination occurred in >50% of endometrial epithelial cells by 6 weeks of age, with no evidence of recombination in non-epithelial cells of the uterus, including endometrial stroma, myometrium, blood vessels, etc. (Fig. 2D). Cre-mediated recombination extended into the squamocolumnar junction of the cervix, with recombination clearly evident in both the superficial

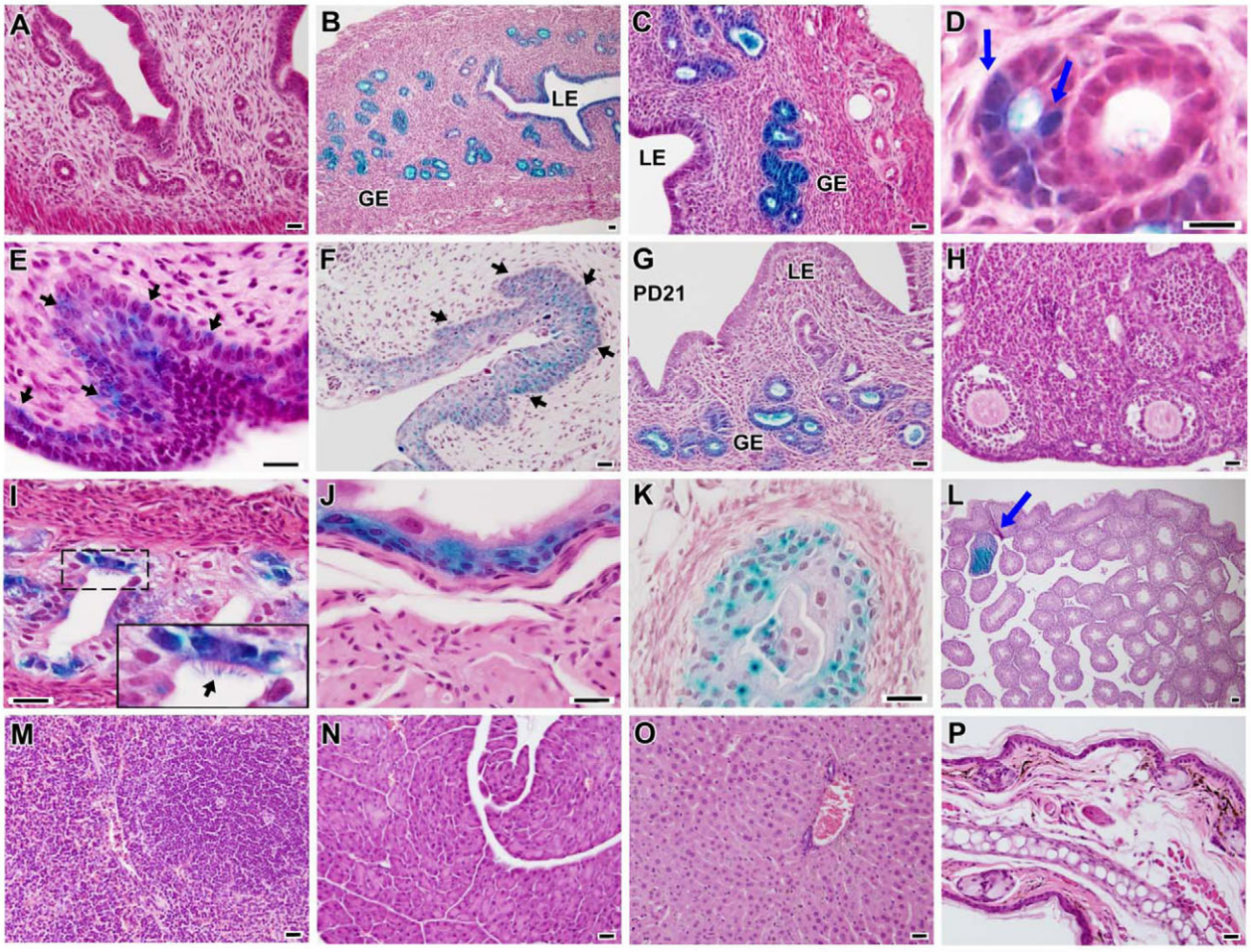

Fig. 2. Cre activity in gonads and other tissues in *Sprr2f-Cre*; *R26R* reporter mice. Tissues were fixed in formalin, stained with X-gal, and sectioned. All tissues are from adult mice (6-week old), except where noted. (A) Uterus; the uterus of a *R26R* non-transgenic sibling (negative control) shows an absence of Cre activity. (B,C) Uterus (higher magnification); the uterus shows efficient and specific Cre-mediated recombination within endometrial epithelium. LE=luminal epithelium, GE=glandular epithelium. (D) Uterus; a close-up of shows a mosaic pattern of recombination where only some cells have undergone Cre-mediated recombination (arrows). (E) Cervix; the squamocolumnnar junction (arrows show efficient recombination in the superficial and reserve layer). (F) Vaginal fornix. (G) Uterus from a 3-week-old mouse (postnatal day 21). Recombination was already efficient in the glandular epithelium in these prepubertal animals (it ranged from ~30 to 50%); the field shown represents a region of efficient recombination. (H) Ovary. (I) Oviduct; the inset shows specific recombination in ciliated epithelial cells (arrows). (J) Bladder. (K) Ureter. (L) Testis; the arrow shows a rare tubule with focal Cre activity. (M) Spleen. (N) Pancreas. (O) Liver. (P) Ear with skin, cartilage and connective tissue. Bars, 20 μm (A-C,E-J,L-P); 40 μm (D,K).

and reserve epithelial layers (Fig. 2E). There was also recombination in the vaginal fornices (Fig. 2F, supplementary material Fig. S3B). Efficient Cre-mediated recombination was detectable in prepubertal animals by postnatal day 21, suggesting that gestational or postnatal estrogen levels were sufficient to induce *Sprr2f-Cre* expression in the endometrium. At this age, most recombination occurred within the glandular epithelium (Fig. 2G). No Cre activity was evident in the ovary (Fig. 2H), but recombination was efficient in the ciliated cells of the oviduct (Fig. 2I, supplementary material Fig. S3C). In summary, *Sprr2f-Cre* was expressed in most Müllerian epithelial cells of the uterus, oviduct and cervix. Cre-mediated activity was also evident in the urothelium of the bladder and ureters (Fig. 2J,K), and in the testis where, in rare animals, very focal recombination involving at most one tubule was observed (Fig. 2L). Ectopic activity was observed in the cerebellum (not shown) but not in other regions of the brain or in other organs (Fig. 2M-P).

Generation of a completely penetrant mouse model of Lkb1-deficient invasive endometrial cancer

To address the questions raised by our previous Ad-Cre-based model of endometrial cancer, we bred *Sprr2f-Cre* mice together with $Lkb1^{L}$ mice. *Sprr2f-Cre*; $Lkb1^{L/L}$ mice were born at expected mendelian ratios, and were externally normal and initially in good health. However, *Sprr2f-Cre*; $Lkb1^{L/L}$ females exhibited a striking increase in mortality. They began to die as early as 120 days (17 weeks) of age and all were dead in a remarkably short window of time, by 212 days (30 weeks) (n=25, P<0.0001 log-rank test) (Fig. 3A). As shown below, this mortality was because of invasive endometrial cancers that arose and progressed in a highly stereotypical manner. *Sprr2f-Cre*; $Lkb1^{L/L}$ uteri developed normally and were of normal weight through the onset of sexual maturity. However, by 16 weeks of age there was significant uterine enlargement and, in females that survived to 28 weeks, the average uterine weights were increased nearly tenfold relative to controls owing to extensive involvement by invasive endometrial cancer (Fig. 3B, Fig. 4A).

To study the contribution of *Lkb1* haploinsufficiency to tumor progression, we studied *Sprr2f-Cre*; $Lkb1^{L/+}$ (heterozygous) females. In sharp contrast to females with homozygous *Lkb1* inactivation, there was no difference in the overall survival relative to control females of the same genetic background (P<0.0001, log-rank test) (Fig. 3A). The single *Sprr2f-Cre*; $Lkb1^{L/+}$ death was of unrelated causes (the uterus was normal). At approximately 600 days of age, all *Sprr2f-Cre*; $Lkb1^{L/+}$ females were euthanized and their uteri inspected carefully. Uteri were not enlarged and only one harbored a uterine neoplasm, which, unlike the diffuse tumors seen in all *Sprr2f-Cre*; $Lkb1^{L/L}$ animals (see below), was discrete (focal). These findings suggest – contrary to some previous studies – that *Lkb1* is not a haploinsufficient tumor suppressor and that inactivation of both alleles is required, at least in the context of endometrial cancer.

Gross and microscopic examinations confirmed that tumor progression occurred in a highly stereotypical manner. At 6 weeks of age, *Sprr2f-Cre*; $Lkb1^{L/L}$ uteri were indistinguishable from sibling controls with no weight increase or microscopic evidence of neoplasia or invasion. However, by 12 weeks of age, diffuse infiltration into the myometrium was observed in most animals. This infiltration was progressive throughout the uterus, leading to diffuse uterine enlargement with increasing age owing to the growth of the tumor and associated stroma (Fig. 4A,B). At later time points, invasive endometrial carcinoma spread to adjacent organs, particularly the ovary, cervix and bladder. The cause of death in most animals was infiltration into the urinary bladder (which lies directly on the anterior aspect of the uterus) with ensuing urinary tract obstruction and hydronephrosis (Fig. 4). Invasion through the uterine wall also led to acute peritonitis and sepsis, contributing to morbidity. Distant metastases were observed only occasionally (i.e. one mouse harbored subcutaneous and pulmonary nodules that were histologically consistent with metastases from the uterine primary tumor). In this respect, this model closely resembles human endometrial cancer, which results in morbidity owing to local infiltration and spread, but rarely metastasizes to distant sites (Barakat et al., 2000).

Systematic drug screens reveal sensitivity of endometrial cell lines to rapamycin

Lkb1 loss and rapalog therapy should, in principle, represent a synthetic lethal interaction. To determine whether rapamycin might be an effective agent against endometrial cancers in general, we analyzed data from a previously described, high-throughput cancer cell line drug sensitivity study (McDermott et al., 2007). Of 690 cancer cell lines from tumors of diverse anatomic origin, ten were derived from primary endometrial adenocarcinomas.

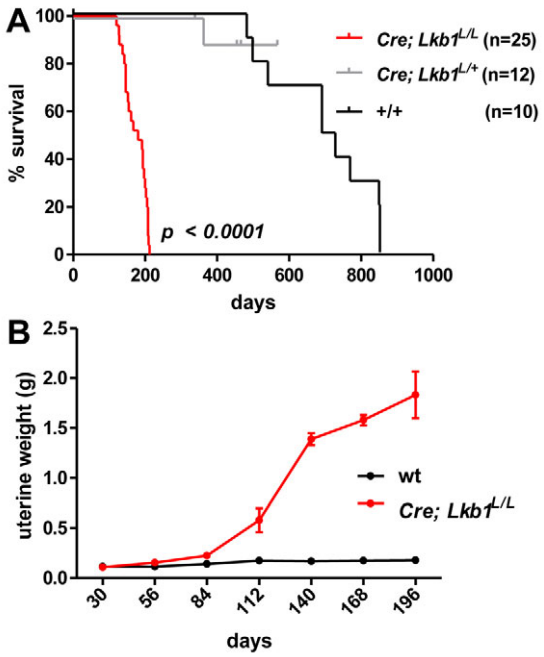

Fig. 3. Homozygous but not heterozygous inactivation of *Lkb1* results in accelerated mortality owing to endometrial cancer. (A) Survival curve; P<0.0001 for both *Sprr2f-Cre*; $Lkb1^{L/L}$ versus wild-type controls and *Sprr2f-Cre*; $Lkb1^{L/L}$ versus *Sprr2f-Cre*; $Lkb1^{L/+}$ sibling heterozygotes. The +/+ curve represents non-sibling wild-type animals of the same genetic background. Statistical significance was calculated with the log-rank test. (B) Uterine weights of *Sprr2f-Cre*; $Lkb1^{L/L}$ and wild-type animals, n=1-5 uteri per time point. Error bars=standard error of the mean (S.E.M.).

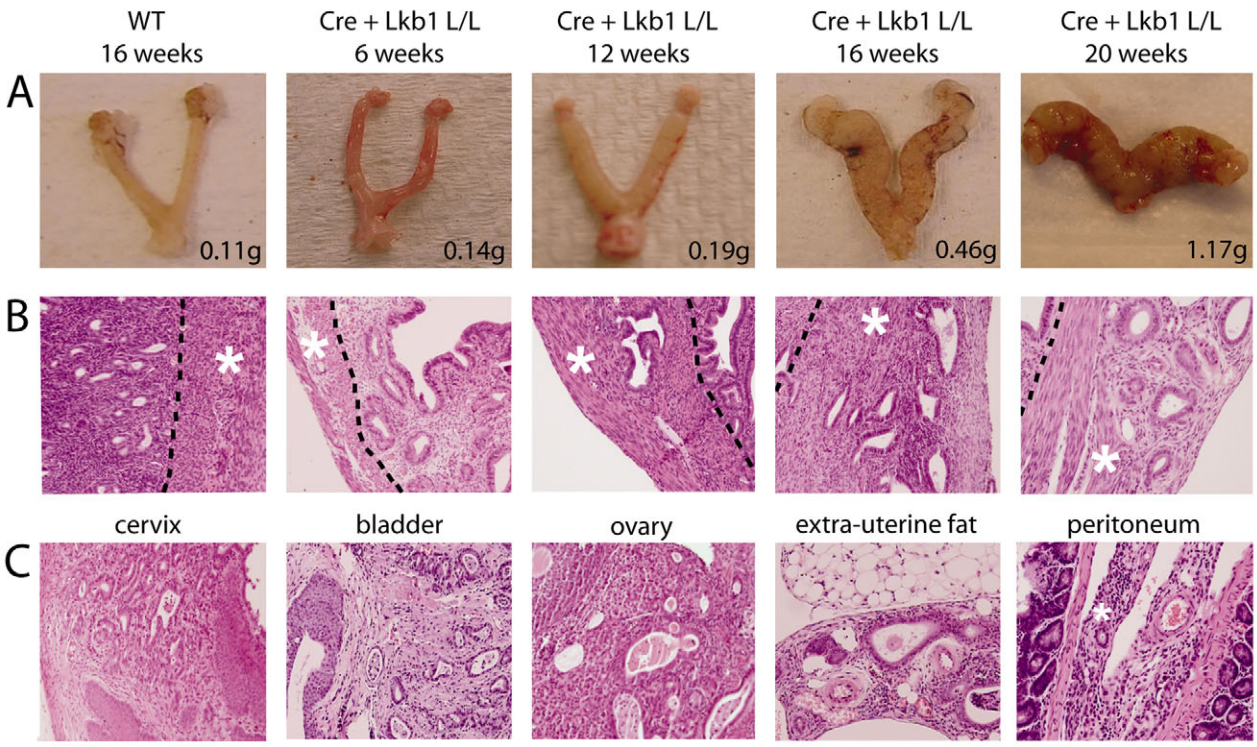

Fig. 4. Stereotypical endometrial cancer initiation and progression in *Sprr2f-Cre; Lkb1$^{L/L}$* mice. (A) Gross pictures of intact uteri at 6 to 20 weeks of age; a control (wild-type) uterus at 16 weeks is shown on the left. The weight for each uterus is shown in the lower right corner. (B) Microscopic analysis shows a lack of invasion at 6 weeks and progressive infiltration after 12 weeks. The endometrial-myometrial interface is shown by a dashed line, with the myometrial layer marked by an asterisk. Note: distinctive features of *Lkb1*-deficient endometrial tumors include their extremely well-differentiated histology and the absence of a morphologically distinctive in situ precursor. Infiltration of glands into the myometrium (which does not normally harbor endometrial glands) serves as definitive histological evidence of invasion (Contreras et al., 2008a). (C) Growth of invasive endometrial adenocarcinoma in characteristic anatomic locations. At each site, the tumor retains a well-differentiated appearance, consisting of well-defined interspersed glands associated with abundant stroma.

Strikingly, when the 690 cell lines were ranked in order of sensitivity to 10 μM rapamycin (a concentration that lies within the effective therapeutic range), nine of the ten cell lines were ranked in the top quartile (Fig. 5A,B). The overall rank order was highly statistically significant (P=3×10^{-5}, see Methods). Highly significant rank orders (P<3×10^{-3}) were also observed with lower concentrations of rapamycin (1 and 0.1 μM). Notably, among the 20 distinct tumor types represented in the study, endometrial cancers as a group exhibited greater sensitivity to rapamycin than any other cancer type (Fig. 5C). The biological basis of this sensitivity and its relationship to LKB1 status in these cell lines is currently under investigation.

Lkb1-deficient endometrial tumors are highly sensitive to rapamycin monotherapy

The general sensitivity of human endometrial cell lines to rapamycin combined with theoretical considerations that LKB1 deficiency should sensitize cells to rapamycin prompted us to explore this agent in our *Sprr2f-Cre*; *Lkb1$^{L/L}$* model of invasive endometrial cancer. Twelve-week-old *Sprr2f-Cre*; *Lkb1$^{L/L}$* females (which already harbor early invasive cancers) were randomly assigned to treatment and control groups. Treatment consisted of daily intraperitoneal (IP) injections using a standard dosing regimen (2 mg/kg/day) (Wei et al., 2008). Control group animals received daily injections of vehicle. The treatment was well tolerated by animals with no side effects or overall weight loss, and all animals were euthanized after 4 weeks of treatment. Control untreated *Sprr2f-Cre*; *Lkb1$^{L/L}$* females had (as expected) significantly enlarged uteri owing to progression of the animals' pre-existing invasive endometrial adenocarcinomas. However, rapamycin dramatically slowed disease progression in every animal, resulting in a 2.4-fold decrease in mean uterine weights (0.25 vs 0.60 grams; P<0.0001, unpaired t-test). Following the rapamycin regimen, the mean *Sprr2f-Cre*; *Lkb1$^{L/L}$* uterine weights were only slightly increased relative to wild-type uteri. Rapamycin did not have a measurable effect on wild-type uteri (Fig. 6A).

An assessment of histopathology and biomarkers suggested that rapamycin exerted combined cytostatic and cytocidal effects on Lkb1-deficient tumors. At the end of the treatment protocol, the tumor burden was greatly reduced, but the scattered, residual invasive tumor glands remaining in myometrium were histologically similar to those in untreated animals (Fig. 6B). Lkb1 inactivation resulted in increased proliferation of endometrial epithelial cells, but this effect was reversed by rapamycin (P=0.004, unpaired t-test). Concordantly, although Lkb1 deficiency led to a decrease in the spontaneous apoptosis of endometrial epithelial cells [per TdT-mediated dUTP nick-end labeling (TUNEL) assay], rapamycin treatment led to a significant increase in cell death

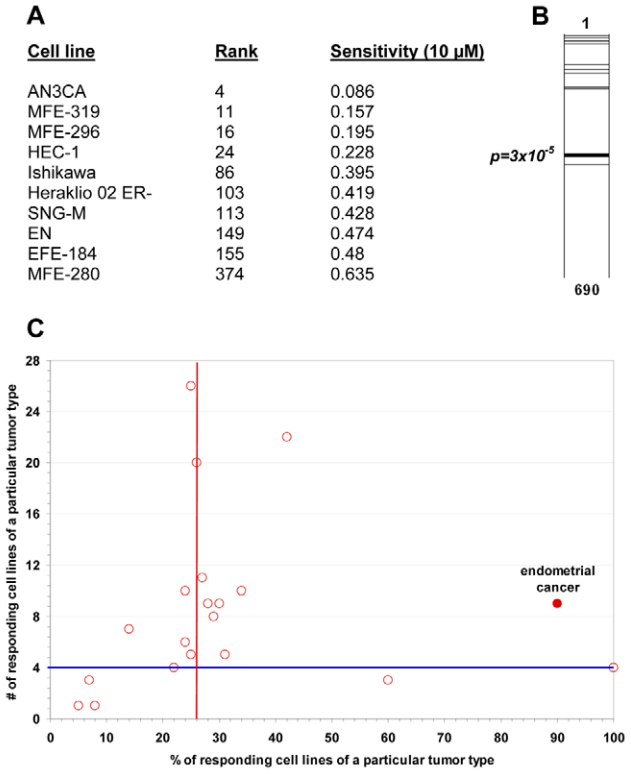

Fig. 5. Endometrial cancer cell lines are especially sensitive to rapamycin. (A) Rank order of each endometrial cancer cell line (*n*=10) among a total of 690 cancer cell lines in the study. Sensitivity was calculated as the fraction of viable cells relative to untreated controls following treatment with 10 μM rapamycin. The *P* value was calculated as described in the methods section. (B) Graphical representation of ranks. Each horizontal bar represents the rank order position of a single endometrial cancer cell line among the 690 cell lines, except for the thick bar, which represents the median rank. Note that nine of the ten cell lines are in the top half. (C) Tumor type rapamycin sensitivity chart. Each circle corresponds to a tumor type (e.g. pancreatic, lung, breast). The vertical red line indicates the overall sensitivity of all cell lines (*n*=690) to the compound; i.e. 26% of human tumor-derived cell lines were sensitive to rapamycin (the criteria for sensitivity was >50% killing at a high concentration of the drug). The horizontal blue line at 4 on the *y*-axis is an empirically-derived gate indicating that, for any sensitivity to be considered meaningful, it has to occur in >4 cell lines of a particular tumor type. Tumor types in the top right-hand quadrant are thus more sensitive to the drug. The endometrial adenocarcinoma group at 90% (highlighted in red) includes all of the endometrial adenocarcinoma cell lines (*n*=10).

(*P*=0.013, unpaired *t*-test) (Fig. 6C). We then analyzed the ribosomal protein p70S6 kinase (S6K), a direct phosphorylation target of the mTOR kinase. Phosphorylated S6K (p-S6K) serves as a reliable in vivo biomarker of mTOR pathway activity. In normal endometrial cells, p-S6K was detectable in a minority of cells in a variegated pattern. p-S6K was significantly increased in Lkb1-deficient cells; however, rapamycin led to a dramatic suppression of p-S6K (*P*<0.001, unpaired *t*-test) to below the baseline in untreated uteri (Fig. 6C,D). These results suggest that the potent effects of rapamycin against Lkb1-driven endometrial cancers can be explained by its effects in counteracting the mTOR hyperactivity that follows Lkb1 inactivation, thereby promoting a shift towards decreased proliferation and cell survival.

We then studied the clinical impact of rapamycin treatment in mice with more advanced disease. Three *Sprr2f-Cre*; *Lkb1*$^{L/L}$ females with very advanced disease were selected for treatment with the same rapamycin dosing regimen, but combined with serial monitoring of their tumor burden by magnetic resonance imaging (MRI). The mice were all 207 days of age (30 weeks) when treatment was initiated, had large advanced tumors (Fig. 7A), and were obviously ill. Each mouse exhibited difficulty urinating with absent micturition reflexes, poor grooming and posture, decreased mobility, and diminished feeding, such that each animal would have imminently required euthanasia per compassionate animal use guidelines. Within one to two weeks of treatment, all animals showed significant improvements in overall health, with complete restoration of micturition reflexes, and normal mobility and feeding. Despite the relentless tumor growth that was consistently observed in untreated *Sprr2f-Cre*; *Lkb1*$^{L/L}$ animals (Figs 3, 4), tumor volume measurements by MRI showed dramatic therapeutic responses with decreased tumor volumes at 2 and 4 weeks after the initiation of therapy in all three animals, with an average reduction in tumor volume of 52% after 4 weeks of treatment (Fig. 7B). There was no further objective decrease in tumor burden at 6 weeks despite continued treatment. At this time, the animals were vigorous and in excellent health. We note that, by this point in the experimental protocol, the three animals were already each 249 days (36 weeks) of age, far beyond the maximal age of 212 days for any untreated animals (Fig. 3A). To determine whether rapamycin therapy continued to have a biological effect despite the lack of further decline in tumor volumes, therapy was ceased at 6 weeks. In all three animals, the tumors grew back with extreme rapidity. The animals became ill and moribund, requiring euthanasia at approximately 2 or 4 weeks following therapy cessation, or at 275, 283 and 283 days of age (Fig. 7A). Thus, rapamycin monotherapy not only halted the progression of Lkb1-deficient tumors but also led to significant and sustained reductions in tumor burden even in animals with very advanced disease, leading to a significant life span extension (*P*=0.010, log rank test) and an improved quality of life.

DISCUSSION

We identified *Sprr2f* as a gene that is specifically expressed in the endometrial epithelium of the mouse and demonstrated that an *Sprr2f*-derived promoter fragment can drive the expression of transgenes specifically within endometrial epithelium. The *Sprr2f-Cre* transgene developed and validated as a part of this study permitted the generation of a highly penetrant mouse model of invasive endometrial cancer with novel properties. Although well suited for the generation of GEMMs, the mosaic pattern of *Sprr2f-Cre*-induced recombination (Fig. 2) may limit its utility for studies of endometrial physiology or implantation. The relatively modest ectopic recombination observed with *Sprr2f-Cre* was not problematic in this *Lkb1* model, as it did not result in disease outside the uterus. However, this ectopic activity may limit the utility of this *Sprr2f-Cre* transgene in other experimental contexts.

In contrast to our earlier mouse model of *Lkb1*-deficient endometrial cancer, which was based on postnatal gene inactivation through intrauterine injection of Ad-Cre (Contreras et al., 2008a), *Sprr2f-Cre*; *Lkb1*$^{L/L}$ females developed highly invasive adenocarcinomas by only 12 weeks of age. These invasive cancers

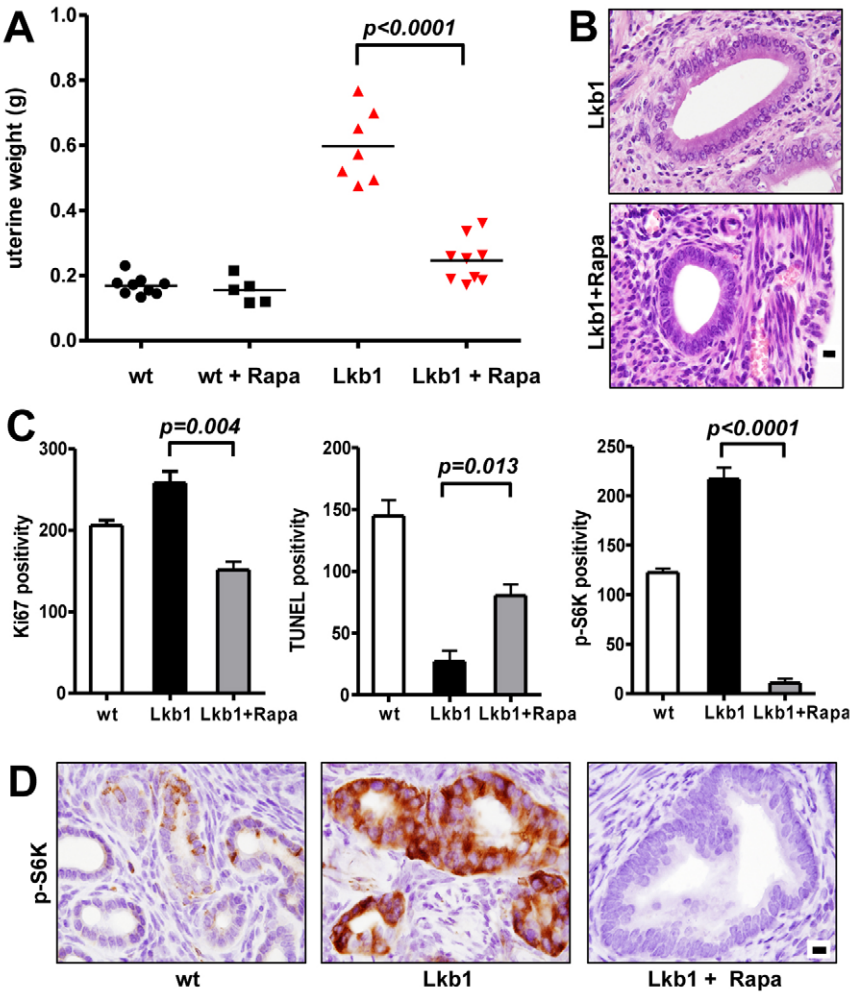

Fig. 6. Rapamycin monotherapy slows the progression of Lkb1-deficient endometrial adenocarcinomas. (A) Uterine weights following 4 weeks of rapamycin monotherapy. Samples are wild-type mice injected with vehicle only (wt; *n*=9), wild-type mice treated with rapamycin (wt+Rapa; *n*=5), *Sprr2f-Cre*; *Lkb1*$^{L/L}$ mice injected with vehicle only (Lkb1; *n*=7), and *Sprr2f-Cre*; *Lkb1*$^{L/L}$ mice treated with rapamycin (Lkb1+Rapa; *n*=9). (B) The tumor burden is reduced greatly by rapamycin therapy but, histologically, the few residual tumor glands closely resemble the tumor glands in untreated animals. (C) Ki67, TUNEL and p-S6K positivity in endometrial epithelium. Bars represent relative counts in tissue sections. (D) Representative regions of tissue sections that were immunohistochemically stained for p-S6K and counterstained with hematoxylin. Bars, 100 μm (B,D).

progressed in a highly stereotypical manner, leading to diffuse uterine enlargement and involvement by invasive adenocarcinoma, with eventual spread to adjacent organs and death of all animals by 30 weeks of age. With the Ad-Cre system, endometrial tumors were, by contrast, always discrete (focal) and arose in only 50% of the animals, and with much longer latency, with no deaths even by ~40 weeks of age (Contreras et al., 2008a). We conclude that these differences reflect the earlier (starting at 3 weeks of age) and much greater efficiency of Cre-mediated recombination and *Lkb1* inactivation with *Sprr2f-Cre*. In other words, the sporadic and focal nature of the tumors that were observed following Ad-Cre administration was due to the inefficiency of Cre-mediated genetic inactivation. In retrospect, that tumors arose at all with Ad-Cre given its inefficiency in inducing recombination is consistent with our other data that Lkb1 is a remarkably potent endometrial tumor suppressor.

This refined *Sprr2f-Cre*-based model demonstrates that Lkb1 serves unique biological roles and is an extremely significant tumor suppressor gene in the endometrium. The short latency, complete penetrance, diffuse growth pattern and absence of a definable morphologic precursor are features that, together, strongly argue that Lkb1 inactivation is sufficient for the malignant transformation of endometrial epithelium into invasive adenocarcinoma, without the requirement for cooperating oncogenic mutations. Consistent with this interpretation, the uterine tumors were always extremely well differentiated with minimal (if any) nuclear atypia or abnormal mitotic figures, suggesting that widespread genomic instability was not a feature of these tumors (Susini et al., 2007). Although we recognize that it is impracticable to prove that no cooperating mutations were present, the early, rapid, stereotypical and diffuse growth of the tumors supports the conclusion that Lkb1 inactivation in endometrial epithelium was sufficient to drive invasive growth. In these respects, this model – in which only one tumor suppressor was inactivated – appears to be unprecedented in GEMMs of cancer, where tumor kinetics and growth patterns have been uniformly consistent with the requirement for cooperating genetic mutations, e.g. in models where only one genetic lesion was engineered (Frese and Tuveson, 2007). It is also notable that, as a tumor suppressor, Lkb1 has remarkably tissue-specific attributes; for example, Lkb1 inactivation is not sufficient to drive tumorigenesis in most other cell types. In *Kras*-driven mouse models of lung cancer, Lkb1 inactivation represented a potent cooperating oncogenic event, but homozygous Lkb1 inactivation by itself did not promote tumor formation (Ji et al., 2007). Lkb1 inactivation in the pancreas led to diffuse acinar polarity defects and the development of neoplasms in 100% of

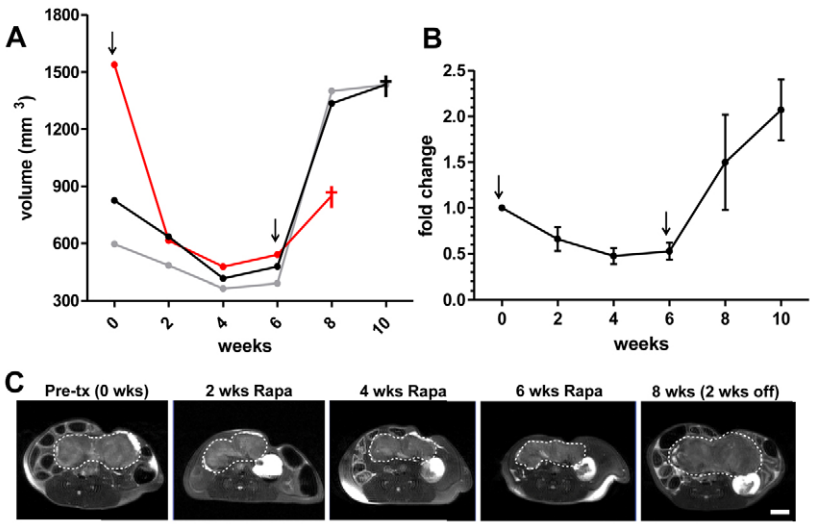

Fig. 7. Rapamycin monotherapy leads to dramatic responses even in animals with very advanced tumors. (A) Uterine volumes (in cubic millimeters), as determined by serial MRI performed every two weeks. Each line represents a single animal. Death, or severe morbidity requiring euthanasia prior to the next scheduled MRI scan, is indicated by a dagger (†) symbol. (B) Relative fold-change of uterine volume, expressed as an average for all three animals (the same animals as shown in panel A). Error bars=S.E.M. For panels A and B, the first arrow marks the initiation of therapy, and the second arrow marks cessation of therapy. (C) Representative MRI images of axial sections showing the effect of rapamycin therapy. Top=ventral. In each image, a dashed white line indicates the uterine margin. The animal shown corresponds to the animal represented by a black line in panel A. Bar, 5 mm.

animals, but these appeared to be benign in nature (serous cystadenomas) (Hezel et al., 2008).

Another significant aspect of this study is our finding that *Lkb1* heterozygosity makes a minimal contribution to endometrial tumor progression, if any. Overall survival was unaltered in *Sprr2f-Cre*; *Lkb1*$^{L/+}$ females, and the only L/+ animal that developed an endometrial cancer harbored a tumor that was more focal than those observed in L/L females. These results are consistent with the notion that loss of both *Lkb1* alleles is required in the epithelium in a cell-autonomous manner for carcinoma formation in the female reproductive tract. Thus, although loss-of-heterozygosity (LOH) studies have led to conflicting results (perhaps owing to the highly stromal nature of Lkb1-deficient polyps and tumors), this rigorous genetic analysis demonstrates that bialleleic *Lkb1* loss in the epithelium is needed for tumor formation, at least in the endometrium. These results are also consistent with studies of human cervical cancer cell lines, the majority of which harbored *LKB1* mutations (intragenic deletions or point mutations) that were demonstrably biallelic in each case (Wingo et al., 2009).

LKB1 mutations have been documented in at least 30% of human lung cancers, although recent studies suggest that the mutation rate may be as high as 45% (Strazisar et al., 2009). Given that lung cancer is one of the more common malignancies in the USA, with over 200,000 cases each year (Jemal et al., 2007), we estimate that there are more than 60,000 new cancer patients in the USA each year whose tumors harbor *LKB1* mutations. Furthermore, our recent discovery that over 20% of cervical primary tumors harbor *LKB1* mutations (Wingo et al., 2009) suggests that other types of cancers characterized by *LKB1* mutations remain to be discovered and, thus, that the number of *LKB1*-deficient tumor cases may be even higher than this estimate. These findings underscore the urgent need for the development of targeted individualized therapies that are effective against *LKB1*-deficient tumors, particularly as the state of *LKB1* deficiency in tumors has been linked to a poor prognosis (Ji et al., 2007; Wingo et al., 2009).

Loss or decreased expression of the LKB1 protein appears to be an important event driving endometrial cancer progression in women. In one study, western blotting showed a loss of LKB1 expression in 20% of primary tumors, which correlated with activation of the mTOR pathway (Lu et al., 2008). Similarly, we found that 20% of primary tumors showed immunohistochemical loss of LKB1 expression and, furthermore, that loss of the LKB1 protein significantly correlated with tumor stage, and hence, prognosis. However, a correspondingly high incidence of *LKB1* mutations has not been reported in endometrial cancer (Contreras et al., 2008b). Thus, although data from multiple laboratories have implicated LKB1 loss or downregulation in endometrial cancer progression, and suggest that this loss is clinically significant, the precise mechanisms remain to be elucidated.

Because mTOR lies at the nexus of LKB1-AMPK and PI3K-AKT signaling, and because mTOR hyperactivation defines most tumors, much effort has been devoted to the study of mTOR inhibitors, including rapamycin and its analogs, in the treatment of cancer, particularly as the side effects associated with these agents have been relatively mild. Overall, clinical results have been somewhat disappointing, with objective responses observed in only a minority of studies and only against some tumor types. Rapalogs appear to be cytostatic and improve survival primarily by stabilizing disease. Unfortunately, rapalogs have failed to show comparable single agent activity against many human tumor subtypes characterized by PTEN loss and PI3K activation, such as melanoma and glioblastoma, and PTEN-PI3K pathway status has not been a reliable predictor of response in any clinical study (Meric-Bernstam and Gonzalez-Angulo, 2009). In mouse models of PI3K-driven neoplasia (e.g. endometrial hyperplasias driven by *Pten* heterozygosity), rapamycin does slow the rate of disease progression, although most studies have not documented shrinkage of tumors of invasive cancers (Milam et al., 2007; Neshat et al., 2001). Interestingly, a recent phase II study of temsirolimus (an ester derivative of rapamycin) in endometrial cancer demonstrated a 26% objective response rate, similar to the 20% rate of endometrial tumors that express low to undetectable levels of the LKB1 protein (Contreras et al., 2008a; Lu et al., 2008). This leads us to speculate that LKB1-AMPK pathway status may be a better predictor of responses to rapalogs than the PI3K-AKT axis, particularly as PI3K

pathway status did not correlate with endometrial cancer responses in the temsirolimus trial (Oza et al., 2006).

Consistent with this possibility, rapamycin monotherapy in *Lkb1*$^{+/-}$ mice with established polyposis led to decreased polyp burden and size (Shackelford et al., 2009; Wei et al., 2008). Rapamycin treatment initiated prior to the onset of polyposis also led to a dramatic reduction of polyp size and overall burden (Wei et al., 2009), demonstrating that rapamycin is effective in both treatment and prophylaxis in a faithful animal model of PJS polyposis. Clinical trials with RAD001 (everolimus) are currently in progress to determine whether mTOR inhibition can decrease the size and/or number of intestinal polyps in patients with PJS.

Although the above results obtained with a PJS polyposis model are consistent with the notion that mTOR inhibitors are effective against tumors that are defined by misregulation of Lkb1, it should be noted that polyps reportedly do not result from LOH in the epithelium but rather from *Lkb1* heterozygosity in the stroma (i.e. they are a cell-non-autonomous phenomenon). By contrast, our study clearly demonstrates that *Lkb1*-deficient endometrial cancer requires biallelic inactivation in a cell-autonomous manner. Thus, this study is significant in that it represents the first demonstration that rapamycin is effective in the prevention and treatment of Lkb1-deficient *invasive* cancer in vivo, and furthermore, that rapamycin is not merely cytostatic, but leads to significant clinical remissions. Our findings further suggest that clinical investigation of mTOR inhibitors against LKB1-deficient human cancers is warranted, particularly in individuals with lung or uterine cancers where *LKB1* mutations can be prospectively identified, i.e. by sequencing or multiplex-ligation probe amplification (MLPA) (Contreras et al., 2008a; Ji et al., 2007; Wingo et al., 2009). Our studies suggest that if rapalog treatment were indeed found to be efficacious in Lkb1-deficient human tumors, treatment would probably need to be permanent, as appears to be the case in patients with tuberous sclerosis (Bissler et al., 2008). It will be of interest to make further use of this mouse model of endometrial cancer to study the consequences of prolonged rapamycin administration on tumor growth, and should relapses occur, to identify and study mechanisms of resistance during Lkb1-deficient tumor progression.

METHODS

Identification of *Sprr2f* and analysis of gene expression

To identify uterine-specific genes in the mouse, mouse uteri harvested on the day of estrus, and a panel of 13 other tissues, were profiled using Affymetrix 430 2.0 arrays exactly as described (Gallardo et al., 2007b). All data sets were normalized by global scaling, and each probe set in the array was ranked by the signal ratio of the uterine sample relative to the average of the 13 somatic tissues. The top five genes for which the corresponding Affymetrix probe set was consistent with uterine-specific gene expression (as in Fig. 1A) were selected for further validation. Full-length cDNAs were obtained for the five genes, including *Sprr2f*, and used as probes in northern analysis. Of the five genes, only *Sprr2f* was expressed highly in the uterus but not in other tissues. The Affymetrix *Sprr2f* probe set identifier on this array is 1449833_at.

For northern analysis, RNA isolation was performed using Tripure reagent (Roche Applied Science) and resuspended in RNAse-free water. Five micrograms of total RNA was loaded onto 2.2 M formaldehyde gels. Transfer was completed overnight, in 20× SSC, to Hybond-N+ membranes (Amersham, Piscataway, NJ). The radioactive probe corresponding to a 3′-UTR fragment (254 bp) of the *Sprr2f* cDNA (GenBank reference sequence NM_011472.2) was prepared by reverse transcription PCR (RT-PCR) of endometrial total RNA (primer sequences available upon request) with the Radprime DNA labeling system (Invitrogen). Overnight hybridizations were performed in Church-Gilbert hybridization buffer (0.5 M sodium phosphate, pH 7.0, 1% BSA, 7% SDS and 1 mM EDTA), with 10^6 counts/ml at 65°C, and exposed to BioMax MS film (Kodak, Rochester, NY). The 18s ribosomal RNA subunit, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), served as loading controls (Fig. 1B and 1C, respectively).

Generation of *Sprr2f-Cre* transgenic mice and animal care

Mice were housed in a pathogen-free animal facility in microisolator cages and fed ad libitum on standard chow under standard lighting conditions; experiments were conducted with the approval of the UTSW Animal Care and Use Committee.

To generate the *Sprr2f-Cre* construct, a 5.5-kb *Sprr2f* promoter fragment was cloned by PCR from strain FVB. DNA was amplified with the primers: oSprr2f-1, 5′-ATGGCGCGCCCTCA-ATAAACTACCTCTTCTCTGTCTCCCTCTG-3′ and oSprr2f-2, 5′-GCTTAATTAAAAAGTATTCCAGGACGTGTGTACCAGG-AGTC-3′. *Asc*I and *Pac*I restriction sites (underlined) were engineered into the 5′-ends of these primers. PCR reactions were performed using the LA PCR kit 2.1 (Takara) in 50 µl volumes with genomic DNA (0.50 µl), H_2O (13 µl), 2×GC buffer 1 (25 µl) (Takara), 25 mM $MgCl_2$ (1.5 µl), 2.5 mM dNTP (8 µl), 10 µm forward and reverse primers (1 µl each), and LA Taq polymerase 5U/µl (0.5 µl) (Takara). The PCR conditions were: 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 68°C for 10 minutes; and then 72°C for 10 minutes. Following amplification, the PCR product was digested with *Asc*I and *Pac*I, and cloned into a *Asc*I- and *Pac*I-digested pCreN vector (Gallardo et al., 2007a). Following linearization with *Sal*I, a 7.5-kb fragment was gel purified, cleaned on an Elutip-D column (Schleicher & Schuell), and microinjected into FVB oocytes by standard protocols (Nagy et al., 2003) at the UT Southwestern Transgenic Core Facility. Genotyping for *Sprr2f-Cre* was performed as follows: DNA was prepared by digesting overnight at 55°C in PCR buffer with nonionic detergents (PBND buffer) (50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% v/v NP40 and 0.45% v/v Tween 20) with 1.5 µl of proteinase K 10 mg/ml (Invitrogen). PCR reactions were performed in 25 µl volumes with tail DNA (0.50 µl), H_2O (18 µl), 10× buffer (2.5 µl), 25 mM $MgCl_2$ (1.5 µl), 25 mM dNTP (0.2 µl), 10 µm forward and reverse primers (1 µl each), and 5 U/µl Hotstart Taq polymerase (0.25 µl). The primer sequences are (Forward) 5′-GGTACACACGTCCTGGAATAC-3′ and (Reverse) 5′-TTCC-CATTCTAAACAACACCCTGAA-3′. The PCR conditions were: 95°C for 10 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and then 72°C for 7 minutes. Note: the forward primer corresponds to the *Sprr2f* promoter, and the reverse primer corresponds to the globin intron of the expression construct. *R26R* mice were purchased from Jackson Laboratories.

Histologic analysis and X-gal staining

Whole-mount X-gal staining was performed by fixing tissues in 4% paraformaldehyde/PBS (Electron Microscopy Sciences) for 1 hour at 4°C, then rinsing twice for 20 minutes each in buffer I (100 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP40) at room temperature. Tissues were then incubated at 50°C in buffer II (100 mM HEPES, 5 mM DTT, 1 mM $MgSO_4$, 2% Triton X-100) for 1 hour. Tissues were again rinsed in buffer I and then incubated in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal and brought up to volume in buffer I) for a period of between 1.5 hours and overnight, and then refixed in formalin overnight. Tissues were embedded in paraffin, cut into 5-μm sections, and counterstained with hematoxylin and eosin.

ERE scans and gel retardation assays

For the digital ERE analyses, 4-kb regions upstream of each start site were scanned with the Dragon ERE finder using the most stringent search criteria (0.75 sensitivity), with the analysis performed in both orientations (Bajic et al., 2003). For the gel retardation assays, sense and antisense oligonucleotides for wild-type and mutant versions of the *Sprr2f* ERE were annealed at 10 μM in 2×SSC buffer. The binding reaction with recombinant human ERα (Invitrogen; cat. no. P2187) was carried out in binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mg/ml BSA), in the presence of 10 nM estradiol at room temperature for 30 minutes, as described previously (Lu et al., 2006). Samples were resolved on 6% native polyacrylamide gel electrophoresis (PAGE) and stained with ethidium bromide (EtBr).

Cell line chemosensitivity studies

The cell lines and methods for the high-throughput drug sensitivity screen have been described previously (McDermott et al., 2007; McDermott et al., 2008). A simulation study was employed to assess the *P* value of the observed ranks, with rapamycin as the index agent. The null hypothesis was that there was no association between the cell lines and drug sensitivity. *P* values were assessed by computer-simulated observations (ten random ranks) generated under this null hypothesis.

Tissue processing and immunohistochemistry

For immunohistochemistry, tissues were fixed in formalin for 48 hours, then processed and embedded in paraffin. Five-micrometer sections were deparaffinized in xylene, and hydrated in an ethanol series. Slides were subjected to antigen retrieval by boiling in 10 mM of sodium citrate and then cooling at room temperature for 20 minutes. The antibodies used were: Ki67 (1:250; Lab Vision, cat. no. RM9106) and p-S6K (1:100; Cell Signaling Technologies, cat. no. 9234). The detection system was Immpress (Vector, Burlingame, CA). TUNEL was performed with the Apoptag kit (Millipore; cat. no. S701) per the manufacturer's instructions.

Rapamycin treatment of mice

Rapamycin (LC Laboratories; cat. no. R-5000) was diluted in DMSO to a stock concentration of 10 mg/ml and stored at –20°C. Fresh dilutions were made daily using vehicle (5% Peg400, 5% Tween-20). Mice were administered with 2 mg/kg rapamycin or vehicle only IP every day.

Magnetic resonance imaging and data analysis

MRI was conducted with a 7-T small animal MRI system (Varian Inc., Palo Alto, CA) with a 40-mm (I.D.) radio frequency (RF) coil. Animals were anesthetized with 1-2% isoflurane (AErrane, Baxter Healthcare Corporation, IL) mixed in 100% oxygen and placed supine with the respiratory sensor, and head first with the abdomen centered with respect to the center of the RF coil. Low-resolution multi-slice gradient echo imaging, serving as the localizer, was first performed on the abdominal region to confirm the location and orientation of the uterus. For the volume measurements of tumerous uteri, axial and sagittal T2-weighted multi-slice images encompassing an entire uterus were obtained with a fat suppression

TRANSLATIONAL IMPACT

Clinical issue

The endometrium, the specialized lining of the uterus, is the most common target tissue for cancers of the female reproductive tract. Early-stage endometrial cancers can be cured, but patients diagnosed with late-stage disease have a median survival of less than a year, and the effectiveness of treatment has not improved in the last 30 years. Animal models of endometrial cancer have been developed but, to date, none has solely endometrial-specific expression, a prerequisite for the precisely targeted genetic manipulation that is needed to study the molecular biology of the disease. The tumor suppressor gene *LKB1* is frequently mutated in human cancers, and decreased *LKB1* expression in endometrial cancer correlates with a higher grade and stage. *LKB1* encodes a protein kinase that normally restrains the activation of the AMP-dependent kinase (AMPK)-mammalian target of rapamycin (mTOR) signaling pathway, whose deregulation is important for the initiation and progression of many cancers. Inhibitors of the mTOR protein itself, such as temsirolimus, a rapamycin derivative, have been used to treat renal cell and endometrial cancers, although only a minority of patients respond.

Results

This paper addresses the dual need for the development of a new model system for endometrial cancer and the study of *LKB1*-driven neoplasia. The authors identify an endometrial-specific promoter and use it to drive expression of Cre recombinase in transgenic mice, meaning that any gene of interest can be manipulated in the endometrial epithelium. They use these mice to explore the consequences of deletion of the mouse *Lkb1* gene in endometrial cancer. Aggressive, invasive endometrial cancers with an early onset and 100% incidence developed, resulting in extrauterine tumor spread and death. The effects of loss of *Lkb1* were partially reversed by blocking the resulting hyperactivation of the AMPK-mTOR pathway using the mTOR inhibitor rapamycin. Prolonged rapamycin administration not only slowed the growth of early tumors, but also resulted in shrinkage of advanced tumors, and significant clinical remissions.

Implications and future directions

Lkb1 is a remarkably potent endometrial tumor suppressor, as its inactivation is sufficient to drive the rapid formation of invasive and lethal endometrial cancers. In addition to developing a new model for endometrial cancer, this is the first study demonstrating that rapamycin is an effective agent against invasive cancers that are driven by Lkb1 deficiency. The model described here can be used to further dissect the initiation and progression of endometrial cancer, and the data also provide a rationale for the setup of clinical trials to test the idea that LKB1 deficiency is a sensitive indicator of responsiveness to anticancer therapies using rapamycin analogs.

doi:10.1242/dmm.005298

fast spin-echo sequence (2500 msecs repetition time, 42 msecs effective echo time, 35×35 mm field of view, 128×128 matrix, 1-mm slice thickness, 25 slices, gapless, eight excitations, fat suppression, scan time of 5 minutes 20 seconds). Volumes were calculated with imaging processing software (ImageJ, version 1.40g; National Institutes of Health, Bethesda, MD), as described previously (Li et al., 2008).

ACKNOWLEDGEMENTS

We thank Lane Shirley and Dr Preeti Kshirsagar for technical assistance, and Dr Song Zhang and Dr Chul Ahn of the Department of Clinical Sciences and the Simmons Comprehensive Cancer Center for help with statistical analysis. This work was supported by grants from the National Cancer Institute (1R01CA13718 to D.H.C. and 1U01CA14157 to D.H.C., K.-K.W. and N.B.), Sidney Kimmel Translational Science Awards (to D.H.C., K.-K.W. and N.B.), the American Cancer Society (RSG0907901MGO to D.H.C.), the Cancer Prevention and Research Institute of Texas (RP100550 to D.H.C.) and the Simmons Comprehensive Cancer Center. C.M.C. was funded through an NIH NRSA F31 fellowship. Deposited in PMC for release after 12 months. This article is freely accessible online from the date of publication.

COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

C.M.C., S.S., J.S., K.-K.W. and D.H.C. conceived and designed the experiments. E.A.A., T.D.G., J.M.H. and O.T. performed the experiments. C.M.C., S.S., J.S., K.-K.W., M.T., N.B. and D.H.C. analyzed the data. C.M.C. and D.H.C. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.004440/-/DC1

Received 8 September 2009; Accepted 29 October 2009.

REFERENCES

Akbay, E. A., Contreras, C. M., Perera, S. A., Sullivan, J. P., Broaddus, R. R., Schorge, J. O., Ashfaq, R., Saboorian, H., Wong, K. K. and Castrillon, D. H. (2008). Differential roles of telomere attrition in type I and II endometrial carcinogenesis. *Am. J. Pathol.* **173**, 536-544.

Alessi, D. R., Sakamoto, K. and Bayascas, J. R. (2006). Lkb1-dependent signaling pathways. *Annu. Rev. Biochem.* **75**, 137-163.

Bajic, V. B., Tan, S. L., Chong, A., Tang, S., Strom, A., Gustafsson, J. A., Lin, C. Y. and Liu, E. T. (2003). Dragon ERE Finder version **2**, A tool for accurate detection and analysis of estrogen response elements in vertebrate genomes. *Nucleic Acids Res.* **31**, 3605-3607.

Barakat, R. R., Grigsby, P. W., Sabbatini, P. and Zaino, R. J. (2000). Corpus: Epithelial Tumors (in Principles and Practice of Gynecologic Oncology). Philadelphia: Lippincott Williams and Wilkins.

Bissler, J. J., McCormack, F. X., Young, L. R., Elwing, J. M., Chuck, G., Leonard, J. M., Schmithorst, V. J., Laor, T., Brody, A. S., Bean, J. et al. (2008). Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *N. Engl. J. Med.* **358**, 140-151.

Contreras, C. M., Gurumurthy, S., Haynie, J. M., Shirley, L., Akbay, E. A., Wingo, S. N., Schorge, J. O., Broaddus, R. R., Wong, K. K., Bardeesy, N. et al. (2008a). Loss of Lkb1 provokes highly invasive endometrial adenocarcinomas. *Cancer Res.* **68**, 759-766.

Contreras, C. M., Gurumurthy, S., Haynie, J. M., Shirley, L. J., Akbay, E. A., Wingo, S. N., Schorge, J. O., Broaddus, R. R., Wong, K. K., Bardeesy, N. et al. (2008b). Loss of Lkb1 provokes highly invasive endometrial adenocarcinomas. *Cancer Res.* **68**, 759-766.

Daikoku, T., Hirota, Y., Tranguch, S., Joshi, A. R., DeMayo, F. J., Lydon, J. P., Ellenson, L. H. and Dey, S. K. (2008). Conditional loss of uterine Pten unfailingly and rapidly induces endometrial cancer in mice. *Cancer Res.* **68**, 5619-5627.

Ellenson, L. H. and Wu, T. C. (2004). Focus on endometrial and cervical cancer. *Cancer Cell* **5**, 533-538.

Frese, K. K. and Tuveson, D. A. (2007). Maximizing mouse cancer models. *Nat. Rev. Cancer* **7**, 645-658.

Gallardo, T., Shirley, L., John, G. B. and Castrillon, D. H. (2007a). Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre. *Genesis* **45**, 413-417.

Gallardo, T. D., John, G. B., Shirley, L., Contreras, C. M., Akbay, E. A., Haynie, J. M., Ward, S. E., Shidler, M. J. and Castrillon, D. H. (2007b). Genomewide discovery and classification of candidate ovarian fertility genes in the mouse. *Genetics* **177**, 179-194.

Gargett, C. E., Chan, R. W. and Schwab, K. E. (2007). Endometrial stem cells. *Curr. Opin. Obstet. Gynecol.* **19**, 377-383.

Gu, H., Zou, Y. R. and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73**, 1155-1164.

Gurumurthy, S., Hezel, A. F., Berger, J. H., Bosenberg, M. W. and Bardeesy, N. (2008). LKB1 deficiency sensitizes mice to carcinogen-induced tumorigenesis. *Cancer Res.* **68**, 55-63.

Hezel, A. F. and Bardeesy, N. (2008). LKB1; linking cell structure and tumor suppression. *Oncogene* **27**, 6908-6919.

Hezel, A. F., Gurumurthy, S., Granot, Z., Swisa, A., Chu, G. C., Bailey, G., Dor, Y., Bardeesy, N. and Depinho, R. A. (2008). Pancreatic LKB1 deletion leads to acinar polarity defects and cystic neoplasms. *Mol. Cell. Biol.* **28**, 2414-2425.

Hong, S. H., Nah, H. Y., Lee, J. Y., Lee, Y. J., Lee, J. W., Gye, M. C., Kim, C. H., Kang, B. M. and Kim, M. K. (2004). Estrogen regulates the expression of the small proline-rich 2 gene family in the mouse uterus. *Mol. Cells* **17**, 477-484.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J. and Thun, M. J. (2007). Cancer statistics, 2007. *CA Cancer J. Clin.* **57**, 43-66.

Jeong, J. W., Lee, H. S., Franco, H. L., Broaddus, R. R., Taketo, M. M., Tsai, S. Y., Lydon, J. P. and DeMayo, F. J. (2009). beta-catenin mediates glandular formation and dysregulation of beta-catenin induces hyperplasia formation in the murine uterus. *Oncogene* **28**, 31-40.

Ji, H., Ramsey, M. R., Hayes, D. N., Fan, C., McNamara, K., Kozlowski, P., Torrice, C., Wu, M. C., Shimamura, T., Perera, S. A. et al. (2007). LKB1 modulates lung cancer differentiation and metastasis. *Nature* **448**, 807-810.

Li, D., Ambrogio, L., Shimamura, T., Kubo, S., Takahashi, M., Chirieac, L. R., Padera, R. F., Shapiro, G. I., Baum, A., Himmelsbach, F. et al. (2008). BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* **27**, 4702-4711.

Lu, K. H., Wu, W., Dave, B., Slomovitz, B. M., Burke, T. W., Munsell, M. F., Broaddus, R. R. and Walker, C. L. (2008). Loss of tuberous sclerosis complex-2 function and activation of mammalian target of rapamycin signaling in endometrial carcinoma. *Clin. Cancer Res.* **14**, 2543-2550.

Lu, P., Li, Y., Gorman, A. and Chi, Y. I. (2006). Crystallization of hepatocyte nuclear factor 1beta in complex with DNA. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **62**, 525-529.

McDermott, U., Sharma, S. V., Dowell, L., Greninger, P., Montagut, C., Lamb, J., Archibald, H., Raudales, R., Tam, A., Lee, D. et al. (2007). Identification of genotype-correlated sensitivity to selective kinase inhibitors by using high-throughput tumor cell line profiling. *Proc. Natl. Acad. Sci. USA* **104**, 19936-19941.

McDermott, U., Sharma, S. V. and Settleman, J. (2008). High-throughput lung cancer cell line screening for genotype-correlated sensitivity to an EGFR kinase inhibitor. *Methods Enzymol.* **438**, 331-341.

Meric-Bernstam, F. and Gonzalez-Angulo, A. M. (2009). Targeting the mTOR signaling network for cancer therapy. *J. Clin. Oncol.* **27**, 2278-2287.

Milam, M. R., Celestino, J., Wu, W., Broaddus, R. R., Schmeler, K. M., Slomovitz, B. M., Soliman, P. T., Gershenson, D. M., Wang, H., Ellenson, L. H. et al. (2007). Reduced progression of endometrial hyperplasia with oral mTOR inhibition in the Pten heterozygote murine model. *Am. J. Obstet. Gynecol.* **196**, 247 e1-5.

Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003). Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H. and Sawyers, C. L. (2001). Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *PNAS* **98**, 10314-10319.

Oda, K., Stokoe, D., Taketani, Y. and McCormick, F. (2005). High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma. *Cancer Res.* **65**, 10669-10673.

Oza, A. M., Elit, L., Biagi, J., Chapman, W., Tsao, M., Hedley, D., Hansen, C., Dancey, J. and Eisenhauer, E. (2006). Molecular correlates associated with a phase II study of temsirolimus (CCI-779) in patients with metastatic or recurrent endometrial cancer-NCIC IND 160. *J. Clin. Oncol. (Meeting Abstracts)* **24**, 3003.

Patel, S., Kartasova, T. and Segre, J. A. (2003). Mouse Sprr locus: a tandem array of coordinately regulated genes. *Mamm. Genome* **14**, 140-148.

Pearson, H. B., McCarthy, A., Collins, C. M., Ashworth, A. and Clarke, A. R. (2008). Lkb1 deficiency causes prostate neoplasia in the mouse. *Cancer Res.* **68**, 2223-2232.

Shackelford, D. B. and Shaw, R. J. (2009). The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat. Rev. Cancer* **9**, 563-575.

Shackelford, D. B., Vasquez, D. S., Corbeil, J., Wu, S., Leblanc, M., Wu, C. L., Vera, D. R. and Shaw, R. J. (2009). mTOR and HIF-1{alpha}-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome. *Proc. Natl. Acad. Sci. USA* **106**, 11137-11142.

Song, H. J., Poy, G., Darwiche, N., Lichti, U., Kuroki, T., Steinert, P. M. and Kartasova, T. (1999). Mouse Sprr2 genes: a clustered family of genes showing differential expression in epithelial tissues. *Genomics* **55**, 28-42.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Strazisar, M., Mlakar, V., Rott, T. and Glavac, D. (2009). Somatic alterations of the serine/threonine kinase LKB1 gene in squamous cell (SCC) and large cell (LCC) lung carcinoma. *Cancer Invest.* **27**, 407-416.

Susini, T., Amunni, G., Molino, C., Carriero, C., Rapi, S., Branconi, F., Marchionni, M., Taddei, G. and Scarselli, G. (2007). Ten-year results of a prospective study on the prognostic role of ploidy in endometrial carcinoma: dNA aneuploidy identifies high-risk cases among the so-called 'low-risk' patients with well and moderately differentiated tumors. *Cancer* **109**, 882-890.

Tan, Y. F., Li, F. X., Piao, Y. S., Sun, X. Y. and Wang, Y. L. (2003). Global gene profiling analysis of mouse uterus during the oestrous cycle. *Reproduction* **126**, 171-182.

Tan, Y. F., Sun, X. Y., Li, F. X., Tang, S., Piao, Y. S. and Wang, Y. L. (2006). Gene expression pattern and hormonal regulation of small proline-rich protein 2 family members in the female mouse reproductive system during the estrous cycle and pregnancy. *Reprod. Nutr. Dev.* **46**, 641-655.

Tashiro, H., Blazes, M. S., Wu, R., Cho, K. R., Bose, S., Wang, S. I., Li, J., Parsons, R. and Ellenson, L. H. (1997). Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. *Cancer Res.* **57**, 3935-3940.

Wei, C., Amos, C. I., Zhang, N., Wang, X., Rashid, A., Walker, C. L., Behringer, R. R. and Frazier, M. L. (2008). Suppression of Peutz-Jeghers polyposis by targeting mammalian target of rapamycin signaling. *Clin. Cancer Res.* **14**, 1167-1171.

Wei, C., Amos, C. I., Zhang, N., Zhu, J., Wang, X. and Frazier, M. L. (2009). Chemopreventive efficacy of rapamycin on Peutz-Jeghers syndrome in a mouse model. *Cancer Lett.* **277**, 149-154.

Wingo, S. N., Gallardo, T. D., Akbay, E. A., Liang, M. C., Contreras, C. M., Boren, T., Shimamura, T., Miller, D. S., Sharpless, N. E., Bardeesy, N. et al. (2009). Somatic LKB1 mutations promote cervical cancer progression. *PLoS ONE* **4**, e5137.