Lkb1 Deficiency Causes Prostate Neoplasia in the Mouse

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Abstract

Mutation of LKB1 is the key molecular event underlying Peutz-Jeghers syndrome, a dominantly inherited condition characterized by a predisposition to a range of malignancies, including those of the reproductive system. We report here the use of a Cre-LoxP strategy to directly address the role of Lkb1 in prostate neoplasia. Recombination of a LacI-flanked Lkb1 allele within all four murine prostate lobes was mediated by spontaneous activation of a p450 CYP1A1-driven Cre recombinase transgene (termed AhCre). Homozygous mutation of Lkb1 in males expressing AhCre reduced longevity, with 100% manifesting atypical hyperplasia and 83% developing prostate intraepithelial neoplasia (PIN) of the anterior prostate within 2 to 4 months. We also observed focal hyperplasia of the dorsolateral and ventral lobes (61% and 56% incidence, respectively), bulbourethral gland cysts associated with atypical hyperplasia (100% incidence), hyperplasia of the urethra (39% incidence), and seminal vesicle squamous metaplasia (11% incidence). PIN foci overexpressed nuclear β-catenin, p-GSK3β, and downstream Wnt targets. Immunohistochemical analysis of foci also showed a reduction in Pten activation and up-regulation of both p-PDK1 (an AMPK kinase) and phosphorylated Akt. Our data are therefore consistent with deregulation of Wnt and phosphoinositide 3-kinase/Akt signaling cascades after loss of Lkb1 function. For the first time, this model establishes a link between the tumor suppressor Lkb1 and prostate neoplasia, highlighting a tumor suppressive role within the mouse and raising the possibility of a similar association in the human.

Introduction

Prostate cancer is the second most common malignancy next to lung cancer in men (1). A central limitation to studying prostate cancer has been the lack of suitable animal models that recapitulate all the stages of human disease progression. This has at least in part been alleviated by the generation of a range of mouse strains with prostate phenotypes, including those mutant for RB, PTEN, AR, AKT, and, more recently, β-catenin (2). However, it remains of paramount importance to develop novel models of prostate cancer to further our understanding of the molecular mechanisms and genetic events underlying prostate cancer.

LKB1 encodes a serine-threonine kinase that was first identified as a gene whose multiple germ line mutations abrogate enzymatic function and are associated with familial Peutz-Jeghers syndrome (PJS; ref. 3). The disorder is characterized by melanin deposits on the buccal mucosa, lips, and digits and the risk of intestinal hamartomas and extraintestinal cancers, such as stomach, pancreas, thyroid, and those of the reproductive organs is at least 10-fold higher than the general population (4). The molecular mechanisms underlying this enhanced tumor predisposition remain to be fully elucidated, but LKB1 has been implicated in the regulation of multiple pathways associated with tumor prevention (3–7). These include chromatin remodeling, angiogenesis, p53-dependent apoptosis, cell cycle arrest, energy metabolism, fatty acid biosynthesis, Wnt signaling, proliferation, polarity, and differentiation (4, 8–11). Recently, somatic deletion of Lkb1 has been linked to lung tumorigenesis and mutation screening of human lung cancer patients revealed LKB1 inactivation is a common event in lung adenocarcinomas (34%) and squamous cell carcinomas (19%), further implicating Lkb1 as a tumor suppressor (12). In addition, somatic mutation of Lkb1 has been reported in pancreatic and biliary cancers (13), as well as malignant melanomas (14).

LKB1 is a member of the Snf1 family of kinases and has been shown to phosphorylate at least 13 members of the AMPK subfamily, many of which play a fundamental role in metabolic regulation (7). In particular, Lkb1 can activate AMPK by phosphorylating Thr172 within the T-loop (11, 15). This leads to mTOR inhibition via TSC2 (tuberin) to suppress cell growth and proliferation (16), as well as down-regulating fatty acid and cholesterol biosynthesis and enhancing glucose uptake and glycolysis (17, 18). However, to date, there is little published data to support a direct role for AMPK deregulation in prostate tumorigenesis (19).

With respect to the Wnt pathway, it has been reported that loss of function of LKB1 elevates Wnt signaling via its regulation of MARK3 (Par1A, cTAK). In the absence of Lkb1, Par1A is unphosphorylated and available to participate in the Wnt cascade, instigating translocation of β-catenin into the nucleus where it instructs transcription of target genes to stimulate proliferation (8). This gives a direct mechanism, whereby mutation of LKB1 may lead to activated Wnt signaling. The potential relevance of such deregulation to prostate cancer has been indicated through studies of human prostate cancer that have identified both mutations in β-catenin and aberrant β-catenin expression (20). Further evidence in support of such a link is derived from Cre-Lox–based models, which develop high-grade PIN and squamous metaplasia after Cre-mediated activation of a constitutive β-catenin mutation (20, 21). Most recently, the rat probasin promoter (PB-Cre4) has been used to drive Cre-mediated deletion of Apc in the prostate, and this has been shown to predispose to adenocarcinoma of the prostate (22).

LKB1 has also been shown to interact with the tumor suppressor PTEN and thereby the phosphoinositide 3-kinase (PI3K)/Akt and mTOR pathways. The most direct evidence for this interaction was...
derived from in vitro studies that have shown LKB1 to bind and phosphorylate PTEN (3, 23). This interaction has been speculated to result in PTEN stabilization and activation (23). Further indirect evidence for an interaction comes from the observation that ~70% of PJS patients also harbor mutation or display loss of at least one allele of PTEN (24). Any potential interaction between PTEN and LKB1 is of particular relevance to prostate neoplasia, as PTEN is well established as a tumor suppressor within this tissue. In humans, PTEN has been reported to be frequently deleted in prostate adenocarcinomas (24). In mice, homozygous inactivation of Pten leads to embryonic lethality (25), whereas heterozygosity predisposes to prostate carcinoma within 9 to 16 months (50%). This phenotype can be accelerated by additional homozygous deletion of Cdkn1b, with carcinomas occurring within 3 months on this background (26). Prostate-specific deletion of Pten has also been achieved using the PB-Cre4 construct to drive recombination of a LoxP-flanked Pten allele. This model recapitulates the full spectrum of human prostate cancer progression from hyperplasia, low-grade PIN, high-grade PIN, carcinoma, and metastasis (27). Finally, it has also been argued that progressive depletion of Pten levels correlates with a more aggressive prostate phenotype and elevated Akt signaling (24), frequently associated with human prostate cancer (28).

There is currently little in the literature to directly link LKB1 mutation with human or murine prostate neoplasia. However, the fact that somatic LKB1 mutations have been observed in human lung cancer and the observation of Lkb1-driven lung tumorigenesis in the mouse suggests that Lkb1 may have a broad tumor suppressive role in epithelial tissues (12).

In the prostate, LKB1 protein has been detected in the cytoplasm of luminal cells using immunohistochemistry (29) and low levels of Lkb1 mRNA are detectable (30). Sequencing studies have reported that LKB1 is mutated in one of five sequenced human prostate carcinoma cell lines, with a frame-shift deletion (p.K178fs*86) within the kinase domain of DU145 cells (31). Further support for a tumor suppressive role for Lkb1 comes from a whole-genome scan study that identified an association between chromosome segments 19q12-q13.11 and prostate cancer aggressiveness (32). The chromosome segment 19p13.3 harbors the LKB1 tumor suppressor gene and also contains members of the kalikrein family (such as KLK3, PSA) and the MUC16 gene that encodes the ovarian cancer antigen CA125 (32). Deletions, amplifications, and structural rearrangements of chromosome 19 have also been reported in a variety of tumors, including pancreatic adenocarcinomas, both benign and anaplastic thyroid tumors and stomach cancers (reviewed in ref. 32). Interestingly, prostate cancer has also been anecdotally reported in a PJS patient (66 years old) after the development of colon cancer (6). The presence of prostate cancer in PJS patients is not a frequent occurrence, possibly as a consequence of the normal age of onset of disease. Prostate cancer is typically diagnosed in the seventh decade of life, whereas PJS patients have an average life span of 57 years (4).

Together with the biochemical evidence of pathway interaction, these observations suggest a potential role for deregulated LKB1 signaling in prostate cancer. To directly assess this possibility in vivo, we have characterized the phenotype of mice deficient for Lkb1 within the prostate. Mice engineered to carry floxed (B) Lkb1 alleles (Lkb1fl/fl; ref. 33), where the kinase domain has been replaced by a DNA cassette encoding exons 5 to 7, enabled Lkb1 deletion within the prostate by using the AhCre promoter to drive expression of Cre recombinase (34). By applying this strategy, we show that loss of Lkb1 predisposes to atypical hyperplasia (AH) that progressed to PIN in the anterior lobe and focal hyperplasia associated with nuclear atypia of the dorsolateral and ventral lobes within 2 to 4 months. Within PIN lesions, we observe elevation of both the PI3K/Akt and Wnt signaling pathways, supporting the concept that loss of Lkb1 promotes neoplasia through deregulation of these pathways.

Materials and Methods

Generation of AhCre\(^{+/loxp}\) mice. All animal studies and breeding were carried out under a UK Home Office project license. Lkb1fl/fl mice and the AhCre transgenic mice have been described previously (33, 34). The Lkb1 and AhCre alleles were backcrossed six times onto a C57BL/6 background. Mice containing one wild-type Lkb1 allele and one floxed allele (Lkb1\(^{f/f}\)) were mated with AhCre-positive mice carrying the Rosa26 reporter allele (35). The Lkb1 homozygous progeny from this cross was intercrossed to generate AhCre\(^{+}\)Lkb1\(^{f/f}\) mice (as Lkb1\(^{f/f}\) males are sterile; ref. 33). Cohorts were aged, and the male genitourinary (GU) tract was harvested when mice developed symptoms. Mice were genotyped by PCR using DNA isolated from tail biopsies. The wild-type and LoxP-flanked Lkb1 alleles were detected using the primers Lkb1fwd, 5’-GATTCCCGAGCGCT-GATTGCA-3’ and Lkb1rev, 5’-AGTGTGACCCCAGCTGACCA-3’, producing 320-bp (wild-type) and 280-bp (floxed) PCR fragments. Recombined Lkb1 was detected using Lkb1frev1 5’-CAGAATACATACCTGCTGTTG-3’ and Lkb1rev2, 5’TCCCTCTCCTGTCGAGAT-3’, producing a PCR product of 500 bp. Cre recombinase activity was induced in control mice by four i.p. injections of 80 mg/kg 1-naphthoflavone within 24 h. Recombined liver tissue was harvested 7 d later.

\(\beta\)-Galactosidase analysis.\] To determine the pattern of recombination at the Rosa26R reporter locus, sectioned material was analyzed as previously described, except X-gal staining was reduced to 1 h at 37°C (34).

Tissue isolation. Tissue was harvested as described previously (36) and fixed for no longer than 24 h in 10% neutral buffered formaldehyde at 4°C before being embedded in paraffin and sectioned at 5 μm. Frozen sections were prepared by snap-freezing in liquid nitrogen, embedded in OCT on dry ice, and sectioned at 10 μm.

Histology, immunohistochemistry, and immunofluorescence. For histology, sections were stained with H&E. For immunohistochemistry, antigen retrieval was performed by incubating the slides in 1× citrate buffer (pH 6.0) in the microwave on full power for 15 min, and endogenous peroxidase activity was inactivated in a solution containing 1.5% H2O2 in deionized water. Detection and visualization was carried out using the 3,3-diaminobenzidine chromagen (DAKO Cytomation) according to the manufacturer’s protocol. Images were taken at 40× magnification using “AnalySIS” software (Olympus Soft Imaging System GMBH), and scale bars were added to represent 50 μm. Control slides known to be positive for each antibody were incorporated. Primary antibodies were obtained from the following sources: anti-androgen receptor 1:100 dilution (Lab Vision Corporation), anti-β-catenin 1:50 dilution (Transduction Laboratories), CD44 1:50 dilution (PharMingen), anti-Foxa1 1:800 dilution (Clone 2F83, Seven Hills Bioreagents), anti-androgen receptor 1:100 dilution (Lab Vision Corporation), anti–p-Gsk3β (Ser9) 1:50 dilution (Cell Signaling Technology), anti–Keratin-5 1:1000 dilution (Covance), anti–Ki-67 1:200 dilution (Vector Laboratories), anti-p63 1:50 dilution (Lab Vision Corporation), anti–p-PTEN (Ser380/Thr382/383) 1:25 dilution (Cell Signaling Technology), anti–p-ERK1/2 (Thr202/Tyr204) 1:50 dilution (Cell Signaling Technology), anti–p-PI3K (Ser473) 1:50 dilution (Cell Signaling Technology), anti–p-PI3K (Ser473) 1:50 dilution (Cell Signaling Technology), anti–p-PDK1 (Ser 241) 1:50 dilution (Cell Signaling Technology), anti–p-Akt (Ser473) 1:50 dilution (Cell Signaling Technology), anti–p-Akt (Ser473) 1:50 dilution (Cell Signaling Technology), anti–p-AKT (Ser473) 1:50 dilution (Cell Signaling Technology), anti–p-AMPK (Thr172) 1:100 dilution (Cell Signaling Technology), anti–p-mTOR (Ser2486) 1:100 dilution (Cell Signaling Technology), anti–p-p70S6K (Thr389/Ser405) 1:100 dilution (Cell Signaling Technology), and anti–p-S6 ribosomal protein (p-Ps6, Ser240/244) 1:100 dilution (Cell Signaling Technology). For immunofluorescence, the frozen sections were treated with pepsin solution (Zymed) and incubated with the primary rabbit polyclonal anti-azona occulendes 1 (ZO-1) antibody 1:20 dilution (Zymed). The primary was detected using the AlexaFluo-488 Nanogold Fab fragment of goat anti-rabbit.
IgG (Molecular Probes) 1:200 dilution. Slides were mounted with Vectashield HardSet + 4,6-diamidino-2-phenylindole mounting medium (Vector Laboratories), and fluorescence was detected using the Leica TCS SP2 AOBS confocal microscope.

**In situ hybridization.** The Qiagen midi-prep kit was used to produce large-scale preparation of the Lkb1 insert, an Lkb1 full-length cDNA clone inserted in the pEX-Asc vector (IRAPv68E05125D, BDZPD). Template were prepared by linearization with EcoRI or NotI (Promega); DNA phenol chloroform was extracted, and ethanol was precipitated. The plasmid sequence was checked by automated sequencing, confirming 100% identity of the Lkb1 clone with the National Center for Biotechnology Information Sequence (accession number BC052379). Anti-sense and sense RNA probes were then obtained by *in vitro* transcription using T3 and T7 RNA polymerases (Roche) and labeled using the DIG RNA labeling kit (BM 1175025, Roche). In *situ* hybridization was carried out as described previously on 10% formalin-fixed paraffin-embedded sections (37). Briefly, sections were fixed in 4% paraformaldehyde at 4°C for 15 min, treated with proteinase K (20 mg/mL), and hybridized with the probes overnight at 65°C. The sections were washed at 65°C and adsorbed with alkaline phosphatase-conjugated anti-DIG antibody (Roche) overnight at 4°C. Alkaline phosphatase activity was detected by using BM purple AP solution (Roche).

**Laser capture microdissection and DNA isolation and amplification.** Frozen tissue was dissected and sectioned (15-20 μm) on polystyrene naphthal membrane-coated glass slides (PALM Microlaser Technologies) and lightly stained with 1% cresyl violet acetate. Laser capture microdissection (LCMD) was performed immediately and did not exceed 30 min. DNA was isolated with the QIAamp DNA microkit (Qiagen) and amplified using the GenomiPh DNA amplification kit (Amersham). PCR reactions were performed in the log phase of amplification using 100 ng of LCMD–amplified DNA in a 50-μl reaction.

### Results

**AhCre** Lkb1+/− mice have a reduced life span. AhCre mice were intercrossed with mice carrying a LoxP-flanked Lkb1 allele and the Rosa26 reporter allele. Cohorts of wild-type (AhCre−/−, Lkb1+/−), AhCre+/−, AhCre Lkb1+/−, and AhCre Lkb1−/− mice were generated and aged. Each cohort contained a minimum of 20 males. Animals were then monitored for signs of illness and killed when they became symptomatic of disease (Fig. 1A). Wild-type, AhCre Lkb1+/−, and AhCre Lkb1−/− cohorts showed average survival times exceeding 450 days and did not significantly differ from each other (χ² test). However, all AhCre Lkb1−/− mice became ill by 200 d, with a significantly reduced average survival of 83 d compared with wild-type (P < 0.0001, χ² = 39.85).

**AhCre Lkb1−/− mice develop multiple GU phenotypes, including PIN.** Histologic analysis of the GU tract was performed in accordance with the consensus report from the Bar Harbor meeting of the mouse models of human cancer consortium prostate pathology committee (36). No gross phenotype was observed in wild-type (n = 21), AhCre Lkb1+/− (n = 18), and AhCre Lkb1−/− (n = 19) mice, whereas the AhCre Lkb1−/− cohort (n = 20) was predisposed to a number of GU phenotypes.

The anterior prostate from AhCre Lkb1−/− mice between 2 to 4 months of age revealed atypical hyperplastic foci (100% incidence) and progression to prostate neoplasia predominantly in the proximal region of the duct (Fig. 2A). PIN was observed as early as 8 weeks when mice became sick (83% incidence). Within these lesions, solid and cribriform intraluminal proliferation of markedly atypical epithelial cells was accompanied by cytologic (nuclear) atypia, such as nuclear enlargement, pleomorphism, chromatin abnormalities, and an increased prominence of nucleoli, along with apparent focal rosetting that contained mitotic bodies (38). This process was also coupled to thickened stroma surrounding the acini and scattered interstitial infiltrate of lymphocytes and plasma cells. Sparse lymphocytes were also observed within some AH and PIN lesions and in severe cases obstructing prostate acini, resulting in gross tubular dilation. Dorsal and ventral lobes displayed a less severe phenotype, where cells exhibited nuclear atypia associated with focal epithelial hyperplasia with 61% and 56% incidence, respectively (Fig. 2B).

In addition to PIN, AhCre Lkb1+/− mice displayed other GU phenotypes. The cohort could be identified visually from 6 to 8 weeks of age because 100% of the mice developed swellings at the base of the tail. Histologic analysis from this region revealed bulbourethral gland (BUG) cysts associated with atypical hyperplasia (Fig. 2C). The acinar mucosal epithelium of these cysts was characterized by a loss of cell polarity and was composed primarily of ductal cells, consistent with reduced secretory function indicative of the depleted foamy cytoplasm, similar to an Nkx3.1 deficiency (39, 40). The presence of interstitial, AH, and PIN inflammatory cells is probably linked to disruption of the cystic BUGs. It is unlikely that the inflammation predisposes to the prostate phenotype, because lesions were not regenerative. We also identified urethral gland hyperplasia in 39% of AhCre Lkb1+/− mice (Fig. 2D,ii). Nodular hyperplasia of the membranous urethra transitional epithelium (UGH) within the lamina propria and a predisposition to cytologic atypia was apparent. There were also two cases (11%) of seminal vesicle squamous metaplasia (SV SqM; Fig. 2D,iii–iv). Interestingly, somatic mutation of Lkb1 in the lung has been shown to predispose to squamous metaplasia (12). Finally, all Lkb1−/− mice were sterile, correlating with previous reports (33).

PIN is not a likely cause of our observed reduced longevity. Health of the AhCre Lkb1+/− male cohort deteriorated, owing to a combination of phenotypes. These include the development of both cystic BUGs (susceptible to rupture) and subsequent infection.
of the GU tract. We assume these other phenotypes prevent progression of PIN to more advanced stages of prostate cancer, such as adenocarcinoma and metastasis. The fact that the AhCre + Lkb1fl/fl lesions are not regenerative and also that not all PIN lesions were associated with inflammation strongly suggests that infection does not drive the development of PIN in this model.

The AhCre transgene mediates recombination in the GU. The observation of a GU phenotype in the AhCre + Lkb1fl/fl mice implied recombination was occurring within these tissues, with subsequent loss of Lkb1 function. To characterize this pattern, we used mice bearing both the AhCre transgene and the Rosa26R reporter locus (35). Using this approach, AhCre-mediated excision, as reported by LacZ expression, was observed in all four lobes of the prostate and in a mosaic pattern in the urethral glands (Fig. 3A). Further LacZ analysis revealed that uninduced AhCre-mediated recombination also occurred in the kidney (41), as well as in the BUG, seminal vesicle, testis, epididymis, and vas deferens (not shown). Histologic analysis of the kidney from the AhCre + Lkb1fl/fl cohort determined no abnormalities, whereas the testis, epididymis, and vas deferens showed male hypospermatogenesis and aspermia, respectively. Interestingly, Lkb1 expression in the testis of Lkb1fl/fl hypomorphic male mice is dramatically reduced in the testis. The common 50-kDa isoform is 10-fold lower, whereas the 48-kDa form is completely absent (33).

Recombination of the Lkb1 occurs within the GU and is associated with PIN. To confirm that the Lkb1 kinase domain had been deleted in the neoplastic prostate tissue, LCMD and PCR amplification of extracted DNA samples was carried out. This approach was taken as insufficient tissue was available for Western blots and immunohistochemistry using anti-Lkb1 antibodies did not detect a product in control tissue and was therefore inappropriate to show loss of function. For PCR amplification, positive controls were derived from AhCre + Lkb1fl/fl mice which had been exposed to δ-naphthoflavone. This protocol delivers near
100% recombination of the target allele in the liver. Laser capture microdissected DNA from the anterior prostate epithelia of both normal (N) regions from AhCre\textsuperscript{+}\textsuperscript{Lkb1fl/fl} and AhCre\textsuperscript{+}\textsuperscript{Lkb1fl/fl} mice served as a negative control for a PCR specific for the recombinated Lkb1 allele (Fig. 3B). Semiquantitative densitometry of PIN in the anterior prostate from AhCre\textsuperscript{+}\textsuperscript{Lkb1fl/fl} mice showed 64% recombination (when compared with fully recombined liver controls). This correlated with a reduction in the level of the unrecombined LoxP-flanked Lkb1 allele. The fact that recombination was below 100% presumably reflects the observed stromal content in the PIN lesions. These observations establish that recombination of the LoxP-flanked Lkb1 allele is associated with PIN, but cannot discriminate between loss of function and haploinsufficiency of Lkb1.

To confirm depletion of Lkb1 transcripts in neoplastic tissue, we performed an in situ hybridisation specific for Lkb1 mRNA transcripts. Lkb1 mRNA was detected in all wild-type prostate lobes (ventral prostate not shown) but was not present in AhCre\textsuperscript{+}\textsuperscript{Lkb1fl/fl} mice (Fig. 3C).

Characterization of Lkb1-deficient PIN. To characterize the prostate lesions at a molecular level, immunohistochemistry was performed. The proliferation marker Ki-67 was rarely expressed in control (AhCre\textsuperscript{+}\textsuperscript{Lkb1fl/fl}) anterior prostate epithelium; however, it was significantly elevated in epithelial cells throughout the acini of AhCre\textsuperscript{+}\textsuperscript{Lkb1fl/fl} mice (Fig. 4A). Basal cells were detected using antibodies against p63 and Keratin-5 in the epithelial lining of anterior acini, indicating a marked accumulation and clustering of...
these cells within the lesions (Fig. 4B). This pattern of expression was also recently observed in lung tumors from mice bearing Lkb1 inactivation (12). Keratin-18 is a luminal cell marker. Here, we show an elevation in Keratin-18 expression in PIN foci, mimicking human prostate cancer (Fig. 4C). We also show increased expression of the androgen receptor within the lesions, suggesting that PIN development in the context of mutant Lkb1 is probably androgen sensitive (Fig. 4D).

**mTOR signaling is decreased in AhCre-Lkb1fl/fl PIN after p-AMPK activation.** Lkb1 is known to mediate mTOR signaling by the phosphorylation of AMPK under low-energy conditions within the small intestine and skeletal muscle (4, 7, 11, 15). Consequently, in the absence of Lkb1, the extent of this phosphorylation event is expected to decrease concomitantly with mTOR signaling activation. To establish on a molecular level whether the loss of Lkb1 deregulates mTOR signaling in PIN foci, we used immunohistochemistry to stain for active p-AMPKα, p-mTOR, p-S6K (ribosomal protein S6 kinase, 70kDa), an mTOR downstream target, and its substrate p-Rps6 (Fig. 5A). We observed an increase in cytoplasmic p-AMPKα expression in association with loss of nuclear p-mTOR in Lkb1-deficient PIN lesions, contradicting S6K activation and phosphorylation of its substrate p-Rps6. This suggests that an alternative AMPK kinase (AMPKK) compensates for the absence of Lkb1, inhibiting mTOR production and stimulating S6K via an alternative mechanism or that Lkb1 does not regulate AMPK in prostatic epithelia.

**Wnt signaling is deregulated in AhCre-Lkb1fl/fl PIN.** Considering mTOR signaling is not stimulated in Lkb1-deficient prostate epithelium, we investigated alternative pathways mediated by Lkb1. To this end, we monitored the expression of a number of Wnt signaling components and downstream transcriptional targets using immunohistochemistry. The β-catenin (CTNNB1) oncogene plays a dual role in cells by participating in both Wnt signaling, essential for normal mammalian development, polarity, and migration, as well as forming adheren junctions at the cell surface membrane together with E-cadherin (42). We observed elevated nuclear β-catenin in PIN foci compared with control tissue, indicating activation of the Wnt signal cascade (Fig. 5Bi,ii). Aberrant Wnt signaling was further shown through overexpression of a number of β-catenin transcriptional targets. We detected elevated levels of the migration marker CD44 in PIN foci, a known immediate transcriptional target of Wnt signaling (ref. 43; Fig. 5Biii,iv). Foxa1, a Forkhead box factor involved in prostate development and an indirect Wnt target via Sox17 (44), was also up-regulated in PIN lesions (Fig. 5Bv,vii). Interestingly, we also observed high-cyttoplasmic expression of inactivated p-Gsk3β (Ser9), a negative regulator of β-catenin (Fig. 5Bvii,viii), which further implies perturbation of the Wnt cascade.
Considering the role Lkb1 plays in organizing cellular polarity via Par1A regulation, it is rational for Lkb1-deficient prostate epithelial cells to have undergone not only an elevation in Wnt signaling, but also to have lost cell polarity (11, 45). To this end, we analyzed the expression pattern of the tight junction protein ZO-1 using immunofluorescence. This revealed highly organized tight junctions located on the surface of the luminal cells in control tissues. In contrast, AhCre′Lkb1fl/fl mice showed aberrant expression of ZO-1, being either principally lost completely or perturbed in a small subset of cells. In addition, reduced ZO-1 expression correlated with nuclear accumulation of ZO-1 (Fig. 5Bix,x), a phenomenon previously hypothesized to reflect altered regulation of cell polarity events (46).

Deregulation of Pten and Akt in AhCre′Lkb1fl/fl PIN.

Recently, Lkb1 has been linked to the Pten/PI3K/Akt pathway (3). To determine whether the PI3K/Akt pathway is deregulated upon loss of Lkb1, we used an antibody directed against total Pten (Fig. 6Ai,ii) and one that only recognizes inactive/phosphorylated (Ser380/385 and Thr382) p-Pten (Fig. 6Ai,iv) to show that inactive Pten is elevated in Lkb1-deficient PIN. This suggests loss of Pten function/stability is a direct consequence of losing Lkb1 function, ultimately facilitating a predisposition to PIN. Lkb1 activation and coincident stabilization of Pten has been shown by in vitro studies, supporting our data (3, 23). Consistent with this, the Lkb1 mutant mice also exhibited increased activation of phosphorylated Akt and the AMPKK p-PDK1 (47) within PIN foci (Fig. 6Av–viii). This indicates that the PI3K/Akt pathway is deregulated after Lkb1 mutation.

Discussion

Murine prostate cancer models are becoming increasingly powerful in elucidating the mechanisms underlying prostate intraepithelial neoplasia, the most established precursor to ...
Here, we show, for the first time, a role for the tumor suppressor Lkb1 in prostate cancer using Cre-LoxP technology to derive a conditional knockout of Lkb1 within the prostate epithelial cells. Loss of Lkb1 reduced male longevity and predisposed to hyperplasia, which progressed to high-grade PIN in the anterior lobe and mild hyperplasia, was also observed in the dorsolateral and ventral glands (within 2–4 months). This positively correlated with β-catenin nuclear translocation and up-regulation of the Wnt and PI3K/Akt signaling cascades within the prostate epithelium. Our immunohistochemical analysis suggests that mTOR signaling seems to decrease after an unexpected surge of p-AMPK in PIN lesions. It is feasible that either Lkb1 does not regulate AMPK within prostate tissue or that an alternative AMPKK compensates for the loss of Lkb1, resulting in suppression of the mTOR pathway. Our data indicate PDK1 is up-regulated in Lkb1 deficient PIN and may therefore play a role in the observed AMPK phosphorylation and activation of pS6K (47). A speculative schematic of events occurring in the presence and absence of Lkb1 in prostate epithelium is depicted in Fig. 6. One caveat of our studies is that we were limited to immunohistochemical analysis of these proteins, owing to the size of the lesions identified.

Figure 6. Loss of Lkb1 stimulates the PI3K/Akt signaling cascade. A, immunohistochemistry of control and AhCre-Lkb1+/− anterior prostate revealed that total Pten (i and ii), inactive p-Pten (Ser380/Thr382/383; iii and iv), active phosphorylated Akt kinase (Ser473; v and vi), and p-PDK1 (Ser241; vii and viii) are all overexpressed in PIN lesions. Images were taken at 40× magnification, and scale bars represent 50 μm. B, a speculative schematic for signaling events mediated by Lkb1 (top) and those under Lkb1 deficient conditions (bottom) within prostatic epithelia. Firstly, Lkb1 typically acts to phosphorylate AMPK to suppress mTOR signaling. Once Lkb1 is lost, mTOR signaling may proceed. Our investigations indicate that an alternative AMPK kinase acts on behalf of Lkb1 (e.g., p-PDK1, which stimulates S6K) to sustain mTOR signaling inhibition. Secondly, Lkb1 phosphorylates Par1A to maintain cellular polarity. Upon loss of Lkb1 function, Par1A is redirected to stimulate Disheveled (Dsh) to inhibit the APC/Axin/Gsk3β complex, allowing β-catenin to translocate into the nucleus. Here, it stimulates transcription of downstream Wnt target genes and induces growth and proliferation, as well as androgen signaling (51). Finally, although the role of Lkb1 interaction and phosphorylation of Pten is still undefined, our data suggest that Lkb1 maintains Pten stability, inhibiting Akt activation. In the absence of Lkb1, we observed inactivation of Pten function, a common precursor to prostate cancer. Pten loss results in activated Akt and ultimately results in p-Gsk3β expression, which can act to maintain Wnt signaling and drive tumorigenesis.
Our results from the AbCre\textsuperscript{Lkb1\textsuperscript{f/f}} mice parallel those of previous studies which have monitored the effects of aberrant Wnt signaling in the prostate, demonstrating an association with prostate tumorigenesis (20–22). Upon Lkb1 loss, Par1A can propagate the translocation of β-catenin into the nucleus to initiate transcription of Wnt target genes, instigating tumorigenesis accompanied with loss of cellular polarity (8). This gives a direct mechanism whereby mutation of Lkb1 may lead to activated Wnt signaling. The phenotype we observe is somewhat less severe than that reported for either constitutive activation of β-catenin or conditional deletion of Apc (20–22). This difference probably reflects differences in gene function (between Lkb1 and either Apc or β-catenin) within the prostate, but may also reflect differences in the experimental approaches used, such as the pattern of Cre-mediated recombination.

The Lkb1\textsuperscript{-deficient prostate phenotype also parallels the phenotypic characteristics of Pten\textsuperscript{-deficient mice (25–27), although again being somewhat less severe. Loss of Pten results in HG-PIN which may progress into carcinoma, where tumorigenesis is positively correlated with the overexpression of p-PDK1, phosphorylated Akt, and its downstream targets, such as pGsk3β (24, 27). Lkb1 mutant mice paralleled this pattern, and the elevation of both total Pten and inactive Pten was previously observed in the TRAMP model of prostate neoplasia where elevated Pten protein levels, as well as phosphorylation of stabilization sites, associated with inactivation (Ser\textsuperscript{380}, Ser\textsuperscript{385}, and Thr\textsuperscript{382}) correlated with progression (48). Our data therefore suggest loss of Lkb1 function impairs Pten function, possibly as a consequence of directly interacting and regulating Pten stability (3), leading to enhanced PDK1 and Akt activity, and ultimately predisposing to PIN. This is consistent with an \textit{in vitro} study that speculates LKB1 phosphorylates PTEN to stabilize its function (23). Deregalration of the P38/Akt signaling pathway also offers a potential explanation for the observed deregalration of the Wnt pathway via the inactivation of Gsk3β, which maintains Wnt signaling (49, 50).

In summary, we describe here a transgenic mouse model which provides the first link between mutation of the tumor suppressor gene Lkb1 and prostate neoplasia. Conditional biallelic loss of Lkb1 leads to the development of a PIN phenotype and to other lesions within the GU. The mechanism underlying this predisposition to PIN involves deregulation of both the Wnt and PI3K/Akt/Pten pathways. Indeed, the phenotype of Lkb1 deficiency mirrors aspects of both Pten loss and Wnt deregalration. Surprisingly, we observed a decrease in mTOR signaling, which we hypothesize may occur as a consequence of a negative feedback mechanism whereby AMPK is activated by an alternate AMPKK to Lkb1, possibly PDK1, to suppress the mTOR pathway. Mechanisms for such deregalration and pathway crosstalk have already been described (Fig. 6b), both through the phosphorylation of Par1A and by altering Pten activity/stabilization.

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