Altered LKB1/AMPK/TSC1/TSC2/mTOR signaling causes disruption of Sertoli cell polarity and spermatogenesis

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Male patients with Peutz–Jeghers syndrome (PJS) have defective spermatogenesis and are at increased risk of developing Sertoli cell tumors. Mutations in the Liver Kinase B1 (LKB1/STK11) gene are associated with the pathogenesis of PJS and have been identified in non-PJS patients with sporadic testicular cancers. The mechanisms controlled by LKB1 signaling in Sertoli cell functions and testicular biology have not been described. We have conditionally deleted the Lkb1 gene (Lkb1cko) in somatic testicular cells to define the molecular mechanisms involved in the development of the testicular phenotype observed in PJS patients. Focal vacuolization in some of the seminiferous tubules was observed in 4-week-old mutant testes but germ cell development appeared to be normal. However, similar to PJS patients, we observed progressive germ cell loss and Sertoli cell only tubules in Lkb1cko testes from mice older than 10 weeks, accompanied by defects in Sertoli cell polarity and testicular junctional complexes and decreased activation of the MAP/microtubule affinity regulating and focal adhesion kinases. Suppression of AMP kinase and activation of mammalian target of rapamycin (mTOR) signaling were also observed in Lkb1cko testes. Loss of Tsc1 or Tsc2 copies the progressive Lkb1cko phenotype, suggesting that dysregulated activation of mTOR contributes to the pathogenesis of the Lkb1cko testicular phenotype. Ptencko mice had a normal testicular phenotype, which could be explained by the comparative lack of mTOR activation detected. These studies describe the importance of LKB1 signaling in testicular biology and the possible molecular mechanisms driving the pathogenesis of the testicular defects observed in PJS patients.

INTRODUCTION

Peutz–Jeghers syndrome (PJS) is a hereditary autosomal dominant, cancer prone disease (1,2), linked to inactivating mutations in the Liver Kinase B1/Serine Threonine Kinase 11 (LKB1/STK11) gene (3,4). PJS patients develop hyper-pigmented skin spots and hamartomatous polyposis of the gastrointestinal tract (2). These patients also have increased prevalence of cancers in various organs including reproductive tract cancers, such as uterine, ovarian and testicular cancers (1). LKB1 is activated after forming a heterotrimeric complex with STE20-related adaptor proteins and mouse 25 proteins, to regulate the activity of 14 different kinases including AMP-activated protein kinases (AMPK) (1,5). The LKB1–AMPK signaling cascade negatively regulates the mammalian target of rapamycin (mTOR) by both phosphorylating and activating tuberin (TSC2)-induced Ras homolog enriched in brain (RHEB)-GTPase activity and by phosphorylating and inhibiting Raptor (regulatory-associated protein of mTOR) (6).

Human PJS patients (7–9) have defective spermatogenesis and often develop Sertoli cell tumors. Histological examination of human PJS testes revealed severe loss of germ cells, vacuolated Sertoli cell cytoplasm and a Sertoli cell only seminiferous tubular phenotype (7), suggesting that Sertoli cell functions are compromised in human PJS patients. Sertoli cells, which are the only somatic cells present inside the seminiferous tubules of mammalian testes, provide the necessary...
microenvironment for normal germ cell development and self-renewal of spermatogonial stem cells, both of which are essential for spermatogenesis and male fertility (10). In adult testes, Sertoli cells form the blood–testis barrier with Sertoli–Sertoli and Sertoli–germ cell junctional complexes to provide an immuno-protective environment for postmeiotic germ cells (11,12). All the stages of germ cell development are in contact with the Sertoli cells and only the most mature differentiated germ cells are released into the lumen of seminiferous tubules and the male ductal system (12). Disruption of these Sertoli cell functions causes a premature release of germ cells, leading to disrupted spermatogenesis and infertility (13).

How mutations in \textit{Lkb1} might contribute to the development of testicular abnormalities in PJS patients is currently not well understood. The first evidence showing a role for LKB1 in spermatogenesis came from an \textit{Lkb1} hypomorphic allele (14,15) in which the middle five exons of the \textit{Lkb1} gene were replaced with a cDNA of exons 5-7 to produce the long isoform of LKB1 only. In contrast to testicular defects of male PJS patients (7,9), histological examination of testes from these \textit{Lkb1} mutant mice revealed normal testicular size and showed normal germ cell development and spermatogenesis. These mice were infertile due to defects in spermatozoa suggesting for abnormalities in spermigenesis, the final process of sperm maturation (16,17). These studies were unable to shed light on the role of \textit{Lkb1} in Sertoli cell functions, which is important because PJS patients develop Sertoli cell only tubules and Sertoli cell tumors (7,9).

We have previously shown that maintenance of Sertoli cell polarity and microtubule integrity are essential for normal germ cell development and spermatogenesis (13). Other studies have shown the importance of LKB1 in maintaining cell polarity and normal tissue architecture (1). We hypothesized that loss of LKB1 signaling affects the Sertoli cell polarity and causes early germ cell loss in PJS patients. To determine dysregulated mechanisms induced by mutant LKB1 in the pathogenesis of testicular defects observed in PJS patients, we conditionally deleted \textit{Lkb1} in somatic cells of the testis and showed that its loss causes disruption of Sertoli cell polarity and leads to the development of the Sertoli cell only tubule phenotype in mice. Loss of \textit{Tsc1} or \textit{Tsc2} phenocopies the defects observed in \textit{Lkb1} mutant mice suggesting that dysregulated mTOR signaling also contributes to the pathogenesis of PJS testicular anomalies.

**RESULTS**

**Expression of mTOR and AMPK in mouse testis**

AMPK and mTOR are the well-known targets of LKB1 (5). In normal adult mouse testes, communostaining of mTOR with β-catenin [a marker for adherens junctions; arrowheads], vimentin [a marker for Sertoli cells apical extensions; arrowheads] and tyrosinated α-tubulin [a marker for Sertoli cell microtubules (13,18)] showed that mTOR is highly expressed at the site of the blood–testis barrier formed by adherens, tight and desmosomal junctions, and by Sertoli cell apical extensions and microtubules (Fig. 1A–a–c), suggesting a role for this signaling pathway in the maintenance of Sertoli cell polarity and testicular junctions. AMPKα is highly expressed in the germ cells and weakly expressed in Sertoli cells (Supplementary Material, Fig. S1A and B). Colocalization of phosphorylated form of AMPKα (pAMPKα) and germ cell nuclear antigen [GCNA, a germ cell marker (19)] revealed that pAMPKα protein is focally expressed at the intercellular junctions (Supplementary Material, Fig. S1C–F). We hypothesized that disruption of the AMPK–mTOR pathway by deletion of LKB1 activity might be causing the testicular phenotype observed in PJS patients.
We examined whether the loss of LKB1 in somatic cells of the testis affected testicular development by conditionally deleting Lkb1 using anti-Müllerian hormone type II receptor (Amhr2)-Cre, which is expressed in both Sertoli and Leydig cells of the testes (13,20–22). However, Amhr2-Cre driven recombination is mainly observed in Sertoli cells and very weak or no recombination is usually detected in Leydig cells (13,21–24). In normal adult testes, LKB1 is expressed in the Sertoli and germ cells (Fig. 1B-a). Loss of Lkb1 in the Sertoli but not in germ cells was observed in Lkb1+/ko testes (n = 3; Fig. 1B-b). Low levels of LKB1 expression appeared unchanged in the Leydig cells of control and mutant testes. Western blotting of LKB1 confirmed a significant decrease in its expression in mutants compared with controls (Fig. 1B-c). Gross examination of testes showed significant decreases in size and weight of testes from Lkb1+/ko mice (Fig. 1B-d and e). However, no difference in seminal vesicle weight was observed between control and Lkb1+/ko mice (Fig. 1B-f), suggesting that testosterone levels and Leydig cell functions were normal in these mice.

**Conditional deletion of Lkb1, Tsc1 and Tsc2, but not Pten, causes germ cell loss and a Sertoli cell only tubules phenotype**

Histological examination of testes from control and mutant mice at different developmental stages was performed to determine the cause of the Lkb1 mutant testicular phenotype (Fig. 2). At 4 weeks, vacuolization of seminiferous epithelium, indicating unhealthy and defective Sertoli cells (25), was observed in a few seminiferous tubules (N = 5; Fig. 2A). However, no differences in testicular weight were observed at this stage of development (control, 111.1 ± 6.3, N = 6; mutant, 150.9 ± 32.0, N = 3). By 10 weeks, normal germ cell arrangement in the seminiferous tubules was disrupted and prominent vacuolization of seminiferous epithelium was observed in most of the tubules (N = 5; Fig. 2D). Decreased tubular width accompanied by progressive germ cell loss and Sertoli cells only tubules were observed in testes of older (5 months) Lkb1+/ko mice (N = 15; Fig. 2F). In contrast, the full complement of germ cells and normal tubular cytoarchitecture was observed in age-matched controls (N = 10; Fig. 2A, C and E). In seminiferous tubules, spermatogonial stem cells are located close to the basement membrane in the basal compartment and meiotic germ cells are located in the adluminal compartment (26). All stages of germ cells are in contact with Sertoli cells for their proper development and only the most differentiated germ cells are released into the male ductal system (epididymides and vasa deferens) (12). Premature release of germ cells from the seminiferous epithelium because of abnormal Sertoli cell function or defects in testicular junctional complexes leads to infertility (13). We observed immature round germ cells in epididymides from Lkb1+/ko mutant mice, whereas only mature elongated germ cells (spermatozoa) were observed in controls (Fig. 2G and H).

Tumors in PJS patients share features with other hereditary disorders such as Cowden’s disease and tuberous sclerosis complex (TSC), which are associated with mutations in the gene for phosphatase and tensin homolog (Pten) and Tsc1 or Tsc2, respectively (1,27,28). Disruptions in Lkb1, Pten, Tsc1 and Tsc2 genes lead to upregulation of mTOR activity and causes tumorigenesis in various organs (29–32). LKB1–AMPK signaling negatively regulates mTOR signaling in part by increasing phosphorylation of TSC2 (30,33). We observed decreased levels of pTSC2 protein in Lkb1 mutant testes compared with controls (Supplementary Material, Fig. S2A), suggesting that mTOR signaling is also affected in our model system.

To examine the contribution of dysregulated mTOR signaling to development of the Lkb1cko testicular phenotype, we created three additional mouse models with conditional deletion of Pten, Tsc1 and Tsc2 using the Amhr2-Cre, the same Cre we used for Lkb1 deletion (Fig. 2). Western blot analyses of TSC1, TSC2 and PTEN showed decreased expression in Tsc1cko, Tsc2cko and Ptencko testes, respectively (Supplementary Material, Fig. S2A). No significant differences in testicular weight (control, 140.9 mg ± 10.9, N = 4; mutant 127.7 mg ± 32.0, N = 3) and histology (Supplementary Material, Fig. S2B) were observed between 4-week-old Tsc1cko and controls. Histological examination of 12-week-old Tsc1cko testes showed vacuolization of seminiferous epithelium (N = 1/3; Fig. 2I and J). Similar to Lkb1cko, by 5 months of age, we observed complete or partial loss of germ cells and Sertoli cell only tubules phenotype in 100% of the Tsc1 mutant animals (N = 16; Fig. 2K–N). Adult Tsc1cko mice testes were much smaller compared with controls (Fig. 2O) and the testicular weight was significantly decreased in Tsc1cko adult (>5 months) mice (Fig. 2P), which is again similar to Lkb1cko testes. Staining with GCNA, a germ cell marker, confirmed a progressive germ cell loss phenotype in Lkb1cko and Tsc1cko adult mice (Supplementary Material, Fig. S3). Deletion of Tsc2 phenocopies abnormal testicular changes observed in Tsc1cko mice (N = 10; Fig. 2P–T), showing the importance of TSC1/2 signaling in Sertoli cells for the maintenance of germ cells and spermatogenesis. Because we observed abnormal round germ cells in the epididymides of Lkb1cko mice (Fig. 2H), we collected epididymides from Lkb1cko, Ptencko, Tsc1cko and Tsc2cko adult mice for comparative analyses. GCNA-positive immature round germ cells were present in epididymides of Lkb1cko, Tsc1cko and Tsc2cko mice (N = 3/each; Supplementary Material, Fig. S4A) indicating defects in Sertoli cell functions and testicular junctions. In contrast to the Lkb1, Tsc1 and Tsc2 loss of function testicular phenotype, no abnormalities were observed in testes of Ptencko mice (N = 5; Fig. 2U–X) and no round germ cells were observed in Ptencko mice epididymides (Supplementary Material, Fig. S4A).

**Overactivation of mTOR signaling in the Sertoli cells of Lkb1cko, Tsc1cko and Tsc2cko but not in Ptencko mice**

We analyzed the expression of mTOR pathway members to gain more insights into mechanisms involved in the development of testicular phenotypes observed in these mouse models. Increased expression of mTOR, pmTOR and phosphorylated riboprotein S6, pS6, a downstream target of mTOR, was observed by immunohistochemistry (IHC) in Lkb1cko (Fig. 3B, G, L and Q), Tsc1cko (Fig. 3 C, H, M and R) and Tsc2cko (Fig. 3D, I, N and S) Sertoli cells (N = 3/each) compared with
Figure 2. Premature germ cell loss and Sertoli cell only tubules of Lkb1<sup>fl/fl</sup>, Tsc1<sup>fl/fl</sup> and Tsc2<sup>fl/fl</sup> but not in Pten<sup>fl/fl</sup> mice testes. (A–F) Histology of control and Lkb1<sup>cko</sup> mutant testes by H&E. (G and H) Epididymides from control and mutant mice. Histology of control and Tsc1<sup>cko</sup> (I–N) testes. (O) Gross picture of control and Tsc1<sup>cko</sup> mutant testes. Arrows in (B and J) indicate vacuolated seminiferous epithelium. Arrows in (E), (F), (M), and (N) are pointing towards Sertoli cells. (P) Weight of adult Tsc1<sup>cko</sup>, Tsc2<sup>cko</sup> and control testes. Columns represent the mean in n = 3 tests, bars equal the SEM and asterisks indicate P < 0.05. Histology of control, Tsc2<sup>cko</sup> (Q–T), and Pten<sup>cko</sup> (U–X) mutant testes. (M), (N), (S), (T), (W) and (X) are higher magnification images of the boxed areas in K, L, Q, R, U and V, respectively. Arrowheads in (S), (T), (W) and (X) indicate Sertoli cells. Insets in (Q and U) show adult testes from control and mutant mice. Bars: 50 μm.
controls (Fig. 3A, F, K and P). However, no qualitative change in expression of these proteins was observed in Ptencko (Fig. 3E, J, O and T), suggesting that mTOR signaling is not stimulated and acting downstream of PTEN signaling in the mutant testes.

To determine if dysregulated activation of mTOR in Sertoli cells contributes to the development of defective germ cells, we performed analyses of pS6 expression in testes from Lkb1cko, Tsc1cko and Tsc2cko mice (Fig. 3U–Z).
Sertoli cells polarity and testicular junction integrity is compromised in \(Lkb1^{cko}\), \(Tsc1^{cko}\) and \(Tsc2^{cko}\) testes

Sertoli cells provide the necessary microenvironment and signals for spermatogenesis and provide the structural framework for migration of differentiated germ cells from the basal to adluminal compartment (13,34). Sertoli cells also participate in the formation of the blood–testis barrier (comprised of tight, adherens and desmosomal junctions), which provides an immune-privileged microenvironment for the germ cells (12). LKB1 regulates cell polarity, adhesion, motility and size largely by regulating AMPK (35,36), mTOR (37,38), MARK (39), SNF1-related kinase (SNRK) (40), and P21-activated kinase (41) signaling. LKB1-dependent phosphorylation of AMPK has also been shown to regulate epithelial tight junction complexes (35). We examined the activation of AMPK in these model systems and observed that pAMPK levels are only suppressed in \(Lkb1^{cko}\) mice but not in \(Tsc1^{cko}\), \(Tsc2^{cko}\) and \(Pten^{cko}\) mice (Fig. 4A). The testis-expressed gene 14 (TEX14), is expressed at germ cell intercellular bridges and its loss leads to defective spermatogenesis (42). A normal TEX14 expression pattern was observed in control and \(Pten^{cko}\) mutant mice, whereas defective localization of TEX14 was present in \(Lkb1^{cko}\), \(Tsc1^{cko}\) and \(Tsc2^{cko}\) testes (Fig. 4B). The integrity of testicular junction complexes was examined by analyzing the expression of the adherens junction proteins β-catenin and N-Cadherin (12), and ZO-1, a component of tight junctions (12). Compared with controls and \(Pten^{cko}\), mislocalization of β-catenin, N-Cadherin and ZO-1 was observed in \(Lkb1^{cko}\), \(Tsc1^{cko}\) and \(Tsc2^{cko}\) mice (Fig. 4C–E), suggesting defects in testicular junctional complexes. Extracellular-signal-regulated-kinase (ERK1/2) and focal adhesion kinase (FAK) signaling are dysregulated in \(Lkb1\) mutant mouse models (43,44) and are known to regulate testicular junction complexes (45,46). We observed increased levels of phospho-ERK1/2 and decreased levels of phospho-FAK(925) in \(Lkb1^{cko}\) testes compared with controls (Supplementary Material, Fig. S2). No change was observed in ERK1/2 and FAK levels between \(Lkb1\) control and mutants (Supplementary Material, Fig. S2). In testes, ERK signaling is involved in germ-Sertoli cells adhesion and its activation is associated with germ cell loss (47,48). FAK is expressed at the blood–testis barrier site in seminiferous tubules and its downregulation compromises the integrity of blood–testis barrier (46). Collectively, these studies suggest that LKB1-mediated effects on testicular junctions could also involve ERK and FAK signaling.

Sertoli cells are highly polarized mesoepithelial cells (49) that extend from the basement membrane to the lumen of seminiferous tubules (25). The long apical extensions of Sertoli cells are in constant contact with differentiating germ cells, directing their migration towards the lumen of the seminiferous tubules; the loss of these apical extensions leads to premature germ cell loss (13). Sertoli cells also have an extensive network of microtubules arranged in a spoke-like pattern that is also required for germ cell migration. Disruption of this microtubular network using microtubules-specific toxins or by genetic alterations similarly leads to germ cell loss (13,18). LKB1 is a known regulator of cell polarity and microtubular assembly (1,6). We examined Sertoli cell apical extensions (marked by vimentin) and microtubules (stained with tyrosinated tubulin) in the mouse models developed in this study. Disruption of the spoke-like pattern of microtubules (Fig. 4F) and loss of apical extensions (Fig. 4G) were observed in \(Lkb1^{cko}\), \(Tsc1^{cko}\) and \(Tsc2^{cko}\) testes, whereas normal looking apical extensions and microtubular arrangement were present in control and \(Pten^{cko}\) mice (Fig. 4F and G). Examination of actin by phalloidin showed disrupted arrangement of actin filaments in \(Lkb1^{cko}\), \(Tsc1^{cko}\) and \(Tsc2^{cko}\) (Fig. 4H). LKB1 has been shown to regulate microtubule dynamics by activating MARK (39) and we also observed decreased expression of pMARK in \(Lkb1^{cko}\) testis compared with controls (Supplementary Material, Fig. S2). SNRK is an AMPK-related kinase and a substrate for LKB1 that is mainly expressed in testes and thought to play a role in spermatogenesis (40). We observed no change in the expression of SNRK between \(Lkb1\) mutant and controls, suggesting that SNRK is not involved in the development of \(Lkb1^{cko}\) testicular phenotype (Supplementary Material, Fig. S2).

Disruption of adult Sertoli cell cycle quiescence in \(Lkb1^{cko}\), \(Tsc1^{cko}\) and \(Tsc2^{cko}\) mice

In adult mammalian testes, Sertoli cells do not normally proliferate (13,25). Testicular biopsies collected from young PJS human patients show germ cell loss and Sertoli cell only tubules (7,9). Proliferating Sertoli cells crowd the seminiferous tubules of these patients; this pathological condition is described as “intratubular Sertoli cell proliferation” (7,9). However, long-term (up to 20 years) clinical follow-up revealed that very few PJS patients develop testicular tumors (7,9). To determine the proliferation status of Sertoli cells in the mouse models developed in this study, we performed colocalization of SOX9 (a Sertoli cell marker) and proliferating cell nuclear antigen (PCNA, a marker for proliferating cells) (Fig. 5). At 4 weeks, no combined PCNA/SOX9-positive cells were observed in either control or mutant animals from all the genotypes (Supplementary Material, Fig. S4C), suggesting that Sertoli cells undergo normal differentiation and that alterations in Sertoli cell polarity and cytoskeleton are the earliest pathological changes in \(Lkb1\), \(Tsc1\) and \(Tsc2\)
Figure 4. Disruption of the Sertoli cell polarity and testicular junctions in Lkb1<sup>fl/fl</sup>, Tsc1<sup>fl/fl</sup> and Tsc2<sup>fl/fl</sup> mice. Expression of pAMPKα (A), TEX14 (B; a marker of testicular intercellular junctions), β-catenin (C), N-cadherin (D; markers of adherens junctions), ZO-1 (E; a marker for tight junctions), tyrosinated α-tubulin (F; a marker for Sertoli cell microtubules), SOX9 (a Sertoli cell nuclear marker)/vimentin (G; a marker for Sertoli apical extensions) and Phalloidin (H) in Lkb1<sup>fl/fl</sup>, Lkb1<sup>cko</sup>, Tsc1<sup>cko</sup>, Tsc2<sup>cko</sup> and Pten<sup>cko</sup> testes. Arrowheads in (C), (D), (E) indicate positive staining for β-catenin, N-Cadherin and ZO-1 at the site of the blood-testis barrier. Arrowheads in (F) indicate Sertoli cell microtubules. White-dotted lines mark the basement membrane of the seminiferous tubules. Arrowheads in (G) indicate the Sertoli cells apical extensions. Bars: 50 μm. Nuclei are stained with DAPI.
mutant testes. However, examination of older testes (≥4 months, N = 3/each) revealed that Sertoli cells that are positive for both PCNA and SOX9 are present in Lkb1cko, Tsc1cko and Tsc2cko mice (Fig. 5B–E), showing that Sertoli cell cycle quiescence is disrupted in these mutant testes. In control testes (N = 3), PCNA-positive and SOX9-negative germ cells were present in the basal compartment of the seminiferous tubules. However, SOX9-positive cells were negative for PCNA, confirming normal Sertoli cell quiescence in control testes (Fig. 5A). Even though we observed germ cell loss and proliferation of Sertoli cells in Lkb1cko, Tsc1cko and Tsc2cko testes, testicular

Figure 5. Proliferation of Sertoli cells in Lkb1cko, Tsc1cko and Tsc2cko adult murine testes. Colocalization of PCNA (a marker for proliferating cells) and SOX9 (a nuclear marker for Sertoli cells) in Lkb1flo flo (A) Lkb1cko, (B) Tsc1cko, (C) Tsc2cko, (D) and Ptencko (E) testes. Arrowheads in (A–E) represent Sertoli cells. Whitened lines indicate the basement membranes of the seminiferous tubules. Bars: 50 μm.
tumors were not observed up to 10 months in all the genotypes (N = 10/each) examined in this study. To confirm that increased Sertoli cell death is not hampering the initiation of testicular tumorigenesis, we performed a terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick end labeling (TUNEL) assay and observed no evidence of Sertoli cell apoptosis in Lkb1\(^{cko}\), Tsc1\(^{cko}\), Tsc2\(^{cko}\) and Pten\(^{cko}\) mice (Supplementary Material, Fig. S4B). However, TUNEL-positive germ cells were observed in all the animals examined (Supplementary Material, Fig. S4B). Sertoli cell proliferation is regulated by thyroid hormone (50,51). To examine whether changes in thyroid hormone signaling cause resumption of Sertoli cell proliferation in adult mutant testes, we examined thyroid hormone receptor \(\alpha_1\) (TR\(\alpha_1\)) expression. TR\(\alpha_1\) is highly expressed in Sertoli cells of neonatal or prepubertal testes but weaker expression is observed in adult Sertoli cells (50). Consistent with a previous report (50), we also observed intense staining for TR\(\alpha_1\) in Sertoli but not in germ cells of 1-day-old testes, which was used as a positive control. No change in expression of TR\(\alpha_1\) was observed between adult control and mutant testes (Supplementary Material, Fig. S5), suggesting that thyroid hormone receptor expression is not affected in the testes of the mouse models developed in this study.

**DISCUSSION**

PJS patients develop cancers in various organs including ovary, testis and endocervix (52). Mutations in the LKB1 gene have been associated with PJS in patients and studies using mouse models have provided compelling evidence for the involvement of mutated Lkb1 in the pathogenesis of phenotypic PJS characteristics (1,53). Mutations in the LKB1 gene are observed in 60% of familial PJS-associated and in 50% of the sporadic testicular patients (54); another 11% of testicular cancer patients also show LKB1 promoter hypermethylation (55). Younger PJS male patients present with defective testicular functions, including premature germ cell loss and Sertoli cell only tubules, and only after a long latency period do some of these patients develop Sertoli cell tumors (7–9). Ulbright et al. followed male PJS patients for 5 years after first observing defects in initial testicular biopsies and observed no progression to cancer in all five patients (7). In a similar study (9), in which six male PJS patients were followed for 20 years after initial diagnosis, only two out of six developed Sertoli cell tumors, suggesting that testicular cancer development is not a common occurrence in male PJS patients. We observed premature germ cell loss and a Sertoli cell only phenotype in Lkb1\(^{cko}\), Tsc1\(^{cko}\) and Tsc2\(^{cko}\) mice, suggesting the importance of LKB1–mTOR signaling in maintaining normal Sertoli cell functions and spermatogenesis. However, no testicular cancer formation was observed in these mouse models through the 10-month observation period. Similar results were also observed upon loss of Lkb1 in the mammary glands, where only defects in epithelial junctional complexes and mammary glands ductal branching but no tumorigenesis were observed up to 1 year (56). These studies in human PJS patients and in the murine mouse models we have described here suggest that LKB1 mutation alone does not drive Sertoli cell transformation.

LKB1 signaling is known to control cell polarity and junctional complexes by targeting AMPK, MARK and mTOR signaling (1–6). For example, dysregulated activation of AMPK signaling adversely affects tight junction assembly and polarity in MDCK epithelial cells (35,36), and inhibition of mTOR rescues that phenotype, suggesting a role for mTOR in regulating junctional dynamics (36). In support of this hypothesis, hyperactivation of AMPK regulates neuronal polarization and development by controlling TSC1/2-mTOR signaling (38) and loss of Tsc1 or Tsc2 functions causes defects in neuronal polarity by modulating mTOR (57). Lastly, defects in renal cell polarity are also observed in Tsc1 and Tsc2 heterozygous mice (58). Because activation of mTOR is usually associated with cell proliferation (59), it could be argued that changes in cell polarity and junctional complexes are the side-effects of excessive proliferation. However, we observed defects in Sertoli cell polarity and junctional complexes (Figs. 4 and 5), but no changes in proliferation in younger mice (Supplementary Material, Fig. S4C). Proliferating Sertoli cells were only observed in older animals (>4 months), suggesting that Sertoli cytoskeletal changes are occurring earlier than proliferation in Lkb1\(^{cko}\), Tsc1\(^{cko}\) and Tsc2\(^{cko}\) testes.

The mechanisms controlled by the LKB1–AMPK–mTOR signaling axis during gonadogenesis, as well as in testicular carcinogenesis, have not been thoroughly described. Studies using germ cell-specific expression of an Lkb1 hypomorphic allele only showed defects in sperm maturation but normal germ cell development and spermatogenesis (16,17), suggesting a limited role for germ cell LKB1 signaling in spermatogenesis. However, male PJS patients show complete germ cell loss and a Sertoli cell only tubule phenotype, indicative of severe defects in spermatogenesis (7,9). In this study, we showed that Sertoli cell-specific loss of Lkb1 causes severe defects in spermatogenesis including a Sertoli cell only tubule phenotype. TSC1/2-mTOR signaling is negatively regulated by LKB1 and loss of LKB1 causes upregulation of mTOR (30). We showed that loss of Tsc1 and Tsc2 phenocopies the testicular defects observed in Lkb1 mutant mice. Mutation of the TSC1/2 genes is associated with human TSC (27). Although neuronal, respiratory and cardiac problems predominate in TSC patients, testicular cancer has also been reported in these patients (60,61), supporting the importance of this signaling pathway in testicular biology. mTOR signaling is also a downstream target of the PI3K/PTEN pathway (62). We hypothesized that Pten loss would phenocopy Tsc1\(^{cko}\) and Tsc2\(^{cko}\), and by extension the Lkb1\(^{cko}\) testicular phenotype. However, no aberrant phenotype was observed in Pten\(^{cko}\) mice (Fig. 2). Examination of pS6 showed very little activation of mTOR (Fig. 4) and correspondingly very few tubules (mean 3.8 defective tubules/100, N = 3 mice) showed defects in germ cell development by 8–12 months in Pten\(^{cko}\) mice (Supplementary Material, Fig. S2C). Similar observations are also made in kidneys, where loss of Tsc1 but not Pten causes polycystic kidney disease (63). Lack of a phenotype upon Pten loss was attributed to the unappreciable changes in mTOR signaling (63).

In this study, we observed fewer defects in germ cell development in 4-week-old Lkb1 mutant mice compared with
adults, even though Amhr2-Cre is expressed in Sertoli cells of both pre- and post-pubertal mouse testes (20). Spermatogenesis in younger mice followed by permanent germ cell loss in older testes has also been observed in other complete gene knockout or conditional gene deletion mouse models (23,64). Previous studies have shown that the first wave of mammalian spermatogenesis is unique and different from later stages (64,65). It has been proposed that the first wave of spermatogenesis is directly initiated by the gonocytes and undifferentiated spermatogonia and that spermatogonial stem cells are not required for this phase of spermatogenesis (65). Massive apoptosis of germ cells also occurs during the first wave of spermatogenesis and inhibition of cell death at this stage leads to defective spermatogenesis at later stages (66). Collectively, these studies confirm that a unique signaling program operates during the first wave of spermatogenesis and LKB1–mTOR signaling plays a limited role during this phase of spermatogenesis.

In summary, we have shown the importance of LKB1 signaling in Sertoli cell biology and spermatogenesis. Loss of Lkb1 causes defects in Sertoli cell polarity and testicular junctional complexes by regulating multiple kinases including AMPK, MARK and mTOR. Deletion of Tsc1 and Tsc2, but not Pten, phenocopies Lkb1cko mice, showing the importance of mTOR activation in the development of the Lkb1 mutant phenotype. In these studies, we have not only shown a critical need for homeostatic LKB1–mTOR pathway signaling in testicular biology but also contributed to understanding the pathogenesis of testicular defects in PJS patients.

MATERIAL AND METHODS

Mouse genetics and husbandry

The Institutional Animal Care and Use Committee at Massachusetts General Hospital approved animal experimentation protocols for this study. Mice were maintained under standard housing conditions and maintained on a mixed genetic background (C57BL/6;129/SvEv). The following mice strains were mated to produce offspring for the current study: C57BL/6;129/SvEv, (67), Stk11tm1Rdp [Lkb1fl/fl], obtained from Dr Nabeel Bardeesy (68), Tsc2fl/fl [National Cancer Institute (29)], Tsc22fl/fl [obtained from Dr Michael J. Gambello (69)], and Ptenfl/fl [Jackson laboratory (70)] were mated to produce Amhr2tm3(cre)Bhr [Amhr2-Cre, obtained from Dr Richard Behringer, (67)], Stk11tm1Rdp [Lkb1fl/fl], obtained from Dr Nabeel Bardeesy (68), Tsc1fl/fl;Tsc1cko, Tsc2fl/fl;Tsc2cko, Ptenfl/fl;Ptencko [National Cancer Institute (29)], Tsc12fl/fl [National Cancer Institute (29)], and Ptenfl/fl [National Cancer Institute (29)]. Western blot analyses

Testes from Lkb1fl/fl and Lkb1cko mice were collected and protein extracts were prepared using RIPA buffer as described previously (13). Protein concentrations were calculated by Bradford assays and equal amounts of protein were loaded on NuPAGE gels (Invitrogen). The following antibodies are used: phosphorylated form of TSC2 (pTSC2), pMARK (phosphorylated MAP/microtubule affinity regulating kinase), phosphorylated 44/42 mitogen-activated protein kinases/phosphorylated extracellular-signal-regulated-kinases 1/2 (p44/42MAPK/pERK1/2), phosphorylated focal adhesion kinase (pFAK), FAK, AMPK, TSC1, PTEN (Cell Signaling Technology), Sucrose non-fermenting protein-related Kinase (SNRK), TSC2; β-actin (Neomarkers, Fremont, CA, USA). Semi-quantitative measurements of band intensities were made by scanning and normalizing to actin.

Statistical analyses

Statistical analyses were performed using Prism software (GraphPad Software, La Jolla, CA, USA) as previously described (13). The unpaired t-test was used to calculate differences between groups, and a P-value of ≤ 0.05 was considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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