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

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ABSTRACT
Male patients with Peutz-Jeghers syndrome (PJS) have defective spermatogenesis and are at increased risk for 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 sporadic testicular cancers from non-PJS patients. 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 development of the testicular phenotype observed in PJS patients. Focal vacuolization in some of the seminiferous tubules was observed in four week old mutant testes but germ cell development appeared normal. However, similar to PJS patients, we observed progressive germ cell loss and Sertoli cell only tubules in Lkb1cko testes from mice older than ten 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 increase 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 (STRAD) and mouse 25 proteins (MO25), to regulate the activity of 14 different kinases including AMP-activated protein kinases (AMPK) (1, 5). The LKB1-AMPK signaling cascade negatively regulates 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 patient’s 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 cells development are in contact with the Sertoli cells and only the most mature differentiated germ cells are release into the lumen of seminiferous...
tubules and the male ductal system (11). Disruption of these Sertoli cell functions causes premature release of germ cells, leading to disrupted spermatogenesis, and infertility (13).

How mutations in 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 the Lkb1 hypomorphic allele developed by Sakamoto et al (14, 15) in which middle five exons of Lkb1 gene were deleted and replaced with only long form of Lkb1. In contrast to testicular defects of male PJS patients (7, 9), histological examination of testes from these Lkb1 mutant mice had normal testicular size and showed normal germ cell development and spermatogenesis. These mice were infertile due to defects in spermatozoa suggesting for abnormalities in spermiogenesis, the final process of sperm maturation (16, 17). These studies were unable to shed light on the role of 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 Lkb1 in somatic cells of the testis and showed that its loss causes disrupts Sertoli cell polarity and leads to the development of the Sertoli cell only tubule phenotype in mice. Loss of Tsc1 or Tsc2 phenocopy the defects observed in 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, coimmunostaining of mTOR with β-catenin (a marker of testicular adherens junction (11)), vimentin (a marker of Sertoli cell apical extensions (13)), tyrosinated α-tubulin (a marker for Sertoli cell microtubules (13, 14)) showed that mTOR is highly expressed at the site of blood testis barrier formed by adherens, tight and desmosomal junctions, and by Sertoli cell apical extensions and microtubules (Fig. 1Aa-c), suggesting a role for this signaling pathway in maintenance of Sertoli cell polarity and testicular junctions. AMPKα is highly expressed in the germ cells and weakly expressed in Sertoli cells (SFig.1 A&B). Colocalization of phosphorylated form of AMPKα (pAMPKα) and GCNA (a germ cell marker (15)) revealed that pAMPKα protein is focally expressed at the intercellular junctions (SFig.1 C-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 loss of LKB1 in somatic cells of the testis affected testicular development by conditionally deleting Lkb1 using antiMüllerian hormone type II receptor (Amhr2)-Cre, which is expressed in both Sertoli and Leydig cells of the testis (13, 16-18). However, Amhr2-Cre driven recombination is mainly observed in Sertoli cells and very weak or no recombination is usually detected in Leydig cells (13, 17-20). In normal adult testes, LKB1 is expressed in the Sertoli and germ cells (Fig.1Ba). Loss of Lkb1 in the Sertoli but not in germ cells was observed in Lkb1cko testes (n=3; Fig. 1Bb). Low levels of LKB1 expression appeared unchanged the Leydig cells of control and mutant testes, suggesting inefficient Lkb1 deletion. Western blotting of Lkb1 confirmed a significant decrease in LKB1 expression in mutants.
compared to controls (Fig. 1Bc). Gross of examination of testes showed significant decreases in testicular size and weight of $Lkb1^{cko}$ mice (Fig. 1Bd&e). However, no difference in seminal vesicle weight between control and $Lkb1^{cko}$ mice was observed (Fig. 1Bf), 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 animals 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 (21), was observed in a few seminiferous tubules (N=5; Fig. 2B). 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 cells loss and Sertoli cells only tubules were observed in testes of older (5 months) $Lkb1^{cko}$ testes (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. 2 A, C&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 (22). All the stages of germ cells are in contact with Sertoli cells for their proper development and only most differentiated germ cells are released in male ductal system (epididymides and vasa deferens) (11). Premature release of the germ cells from the seminiferous epithelium because of
abnormal Sertoli cells function or defects in testicular junction complexes leads to infertility (13). We observed immature round germ cells in epididymides from \( Lkb1^{cko} \) mutant mice, whereas mature elongated germ cells (spermatozoa) were observed in controls (Fig. 2 G&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, 23, 24). Disruptions in \( Lkb1, Pten, Tsc1, \) and \( Tsc2 \) genes lead to upregulation of mTOR activity and causes tumorigenesis in various organs (25-28). LKB1-AMPK signaling negatively regulates mTOR signaling in part by increasing phosphorylation of TSC2 (25, 29). We observed decreased levels of pTSC2 protein in \( Lkb1 \) mutant testes compared to controls (SFig. 2A), suggesting that mTOR signaling is also affected in our models system.

To examine the contribution of dysregulated mTOR signaling to development of the \( Lkb1^{cko} \) 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 \( Tsc1^{cko}, Tsc2^{cko}, \) and \( Pten^{cko} \) testes, respectively (SFig. 2A). No significant differences in weight (control, 140.9 ± 10.9, N=4; mutant 127.7 ± 32.0, N=3) and histology (SFig. 2B) were observed between 4 week-old \( Tsc1^{cko} \) and controls. Histological examination of 12 week-old \( Tsc1^{cko} \) testes showed vacuolization of seminiferous epithelium (N=1/3; Fig. 2I&J). Similar to \( Lkb1^{cko} \), by 5 months of age, we observed complete or partial loss of germ cell loss and Sertoli cell only tubules phenotype in 100% of the \( Tsc1 \) mutant animals (N=16; Fig. 2K-N). Adult \( Tsc1^{cko} \) mice testes were much smaller compared to controls (Fig. 2O) and testicular weight was significantly decreased in \( Tsc1^{cko} \) adult (>5mo) mice (Fig. 2P), which is again similar to \( Lkb1^{cko} \) testes.
Staining with GCNA, a germ cell marker, confirmed progressive germ cell loss phenotype in \( Lkb1^{cko} \) and \( Tsc1^{cko} \) adult mice (SFig. 3). Deletion of \( Tsc2 \) phenocopies abnormal testicular changes observed in \( Tsc1^{cko} \) mice (N=10; Fig. 2 P-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 epididymis of \( Lkb1^{cko} \) (Fig. 2H), we collected epididymides from \( Lkb1^{cko}, \ Tsc1^{cko}, \ Tsc2^{cko} \) adult mice for comparative analyses. GCNA positive immature round germ cells were present in epididymides of \( Lkb1^{cko}, \ Tsc1^{cko} \) and \( Tsc2^{cko} \) mice (N=3/each; SFig. 4A) indicating defects in Sertoli cells functions and testicular junctions. In contrast to the \( Lkb1, \ Tsc1, \) and \( Tsc2 \) loss of function testicular phenotype, no abnormalities were observed in testes of \( Pten^{cko} \) mice (N=5; Fig. 2U-X) and no round germ cells were observed in \( Pten^{cko} \) mice epididymides (SFig. 4A).

**Overactivation of mTOR signaling in the Sertoli cells of \( Lkb1^{cko}, \ Tsc1^{cko} \) and \( Tsc2^{cko} \) but not in \( Pten^{cko} \) mice**

We analyzed the expression of mTOR pathway members to gain more insight into mechanisms involved in 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 in \( Lkb1^{cko} \) (Fig. 3 B,G,L&Q), \( Tsc1^{cko} \) (Fig. 3 C,H,M&R) and \( Tsc2^{cko} \) (Fig. 3 D,I,N&S) Sertoli cells (N=3/each) compared to controls (Fig. 3 A,F,K&P). However, no qualitative change in expression of these proteins was observed in \( Pten^{cko} \) (Fig. 3 E,J,O&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 defective germ cells development, we performed analyses of pS6 expression in testes from Lkb1cko, Tsc1cko, and Tsc2cko mice (Fig. 3 U-Z). We intentionally selected younger Lkb1cko (10 weeks; N=3) and Tsc1cko (16 weeks; N=3) mice testes with some seminiferous tubules containing full a complement of germ cells to see if increased expression of pS6 in the Sertoli cells will correlate with defective germ cell differentiation (absence of elongated spermatids) or loss of germ cells. We observed no elongated spermatids in tubules where most of the Sertoli cells showed increased expression of pS6 in Lkb1cko and Tsc1cko mice, whereas elongated spermatids were clearly present in tubules with few or no Sertoli cells staining for pS6 (Fig. 3 U,V,X&Y), suggesting that well regulated mTOR signaling is required for normal Sertoli cell functions. Sertoli cells in a few tubules also showed upregulation of pS6, accompanied by loss of elongated spermatids in Ptencko mutant mice (Fig. 3 W&Z; N=3), indicating that PTEN loss is able to stimulate mTOR signaling but not at levels equivalent to deletion of Lkb1 and Tsc1/2 genes, which could explain lack of an obvious phenotype in Ptencko mice.

Sertoli cells polarity and testicular junctions integrity is compromised in Lkb1cko, Tsc1cko and Tsc2cko 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, 30). Sertoli cells also participate in formation of the blood-testis barrier (comprised of tight, adherens and desmosomal junctions), which provides an immune-privileged microenvironment for the germ cells (11). LKB1 regulates cell polarity, adhesion, motility, and size largely by regulating AMPK (31, 32), mTOR (33, 34), MARK (35), SNRK
LKB1-dependent phosphorylation of AMPK has also been shown to regulate epithelial tight junction complexes (31). We examined the activation of AMPK in these model systems and observed that pAMPK levels are only suppressed in Lkb1 mutants but not in Tsc1cko, Tsc2cko and Ptencko mice (Fig. 4A). The testis expressed gene 14 (TEX14), is expressed at germ cell intercellular bridges and its loss leads to defective spermatogenesis (38). Normal TEX14 expression pattern was observed in control and Pten mutant mice, whereas defective localization of TEX14 was present in Lkb1cko, Tsc1cko and Tsc2cko testes (Fig. 4B). The integrity of testicular junction complexes was examined by analyzing expression of the adherens junction proteins β-catenin and N-Cadherin (11), and ZO-1, a component of tight junctions (11). Compared to controls and Ptencko, mislocalization of β-catenin, N-Cadherin, and ZO-1 was observed in Lkb1cko, Tsc1cko and Tsc2cko mice (Fig. 4 C-E), suggesting defects in testicular junctional complexes.

ERK1/2 and FAK signaling are dysregulated in Lkb1 mutant mouse models (39, 40) and are known to regulate testicular junction complexes (41, 42). We observed increased levels of phospho-ERK1/2 and decreased levels of phospho-FAK(925) in Lkb1cko testes compared to controls (SFig.2). No change was observed in ERK1/2 and FAK levels between Lkb1 control and mutants (SFig.2). In testes, ERK signaling is involved in germ-Sertoli cells adhesion and its activation is associated with germ cell loss (43, 44). FAK is expressed at blood-testis barrier site in seminiferous tubules and its downregulation compromises the integrity of blood-testis barrier (42). 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 (45) that extend from the basement membrane to the lumen of seminiferous tubules (21). 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 and disruption of this microtubular network using microtubules-specific toxins or by genetic alterations similarly leads to germ cell loss (13, 14). LKB1 is a known regulator of cell polarity and microtubular assembly (1, 46). 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) was observed in Lkb1cko, Tsc1cko and Tsc2cko testes, whereas normal looking apical extensions and microtubular arrangement was present in control and Ptencko mice (Fig. 4F&G). Examination of actin by phalloidin showed disrupted arrangement of actin filaments in Lkb1cko, Tsc1cko and Tsc2cko (Fig. 4H). LKB1 has been shown to regulate microtubule dynamics by activating MARK (35) and we also observed decreased expression of pMARK in Lkb1cko testis compared to controls (SFig. 2). SNF1-related kinase (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 (36). We observed no change in expression of SNRK between Lkb1 mutant and controls, suggesting SNRK is not involved in development of Lkb1cko testicular phenotype (SFig. 2).

**Disruption of adult Sertoli cell cycle quiescence in Lkb1cko, Tsc1cko and Tsc2cko mice**

In adult mammalian testes, Sertoli cells do not proliferate (13, 21). Testicular biopsies collected from young PJS human patients show germ cell loss and Sertoli cell only tubules (9, 47). Proliferating Sertoli cells crowd the seminiferous tubules of these patients; this pathological condition is described as “intratubular Sertoli cell proliferation” (9, 47). However, long term (up
to 20 yrs) clinical follow up revealed that very few PJS patients develop testicular tumors (9, 47). To determine the cell cycle status of Sertoli cells in mouse models developed in this study, we performed colocalization of SOX9 (a Sertoli cell marker) and 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 (SFig. 4C), suggesting that Sertoli cells undergo normal differentiation and alterations in Sertoli cell polarity and cytoskeleton are the earliest pathological changes in Lkb1, Tsc1, and Tsc2 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, but not in Ptencko 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 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 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 TUNEL assay and observed no evidence of Sertoli cell apoptosis in Lkb1cko, Tsc1cko, Tsc2cko, and Ptencko mice (SFig. 4B). However, TUNEL positive germ cells were observed in all the animals examined (SFig. 4B). Sertoli cell proliferation is regulated by thyroid hormone (48, 49). To examine if changes in thyroid hormone signaling cause resumption of Sertoli cell proliferation in adult mutant testes, we examined thyroid hormone receptor α 1 (TRα1) expression. TRα1 is highly expressed in Sertoli cells of neonatal or prepubertal testes and weaker expression is observed in adult Sertoli cells. (50). Consistent
with a previous report (50), we also observed intense staining for TRα1 in Sertoli but not in germ cells of 1-day-old testes, which was used as a positive control. No change in expression of TRα1 was observed between adult control and mutant testes (SFig. 5), suggesting that thyroid hormone receptor expression is not affected in testes of the mouse models developed in this study.

DISCUSSION

PJS patients develop cancers in various organs including ovary, testis and endocervix (51). 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, 52). Mutations in the LKB1 gene are observed in 60% of familial PJS-associated and in 50% of the sporadic testicular patients (53); another 11% of testicular cancer patients also show LKB1 promoter hypermethylation (54). 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 (8, 9, 47). 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 (47). In a similar study (9), in which 6 male PJS patients were followed for 20 years after initial diagnosis, only 2 out of 6 developed Sertoli cells 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 Lkb1cko, Tsc1cko and Tsc2cko 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 \textit{Lkb1} in the mammary glands, where only defects in epithelial junctional complexes and mammary glands ductal branching but no tumorigenesis was observed up to 1 year (55). Combined, these studies in human PJS patients and in the murine mouse models we have described here suggest that, although phenotypic Sertoli cell only tubule were always observed, \textit{LKB1} mutation alone does not drive Sertoli cell transformation.

\textit{LKB1} signaling is known to control cell polarity and junctional complexes by targeting AMPK, MARK, and mTOR signaling (1, 46). For example, dysregulated activation of AMPK signaling adversely affects tight junction assembly and polarity in MDCK epithelial cells (31, 32), and inhibition of mTOR rescues that phenotype, suggesting a role for mTOR in regulating junctional dynamics (32). In support of this hypothesis, hyperactivation of AMPK regulates neuronal polarization and development by controlling TSC1/2-mTOR signaling (34) and loss of \textit{Tsc1} or \textit{Tsc2} functions causes defects in neuronal polarity by modulating mTOR (56). Lastly, defects in renal cell polarity are also observed in \textit{Tsc1} and \textit{Tsc2} heterozygous mice (57).

Because activation of mTOR is usually associated with cell proliferation (58), 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&5), but no changes in proliferation in younger mice (SFig. 4C). Proliferating Sertoli cells were only observed in older animals (>4 mo), suggesting that Sertoli cytoskeleton changes are happening earlier than proliferation in \textit{Lkb1}^{cko}, \textit{Tsc1}^{cko} and \textit{Tsc2}^{cko} testes.

The mechanisms controlled by \textit{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 \textit{Lkb1} hypomorph allele only showed defects
in sperm maturation but normal germ cell development and spermatogenesis (59, 60), 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 (9, 47). 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 (25). We showed that loss of Tsc1 and Tsc2 phenocopy the testicular defects observed in Lkb1 mutant mice. Mutation of the TSC1/2 genes is associated with human TSC (23). Although neuronal, respiratory, and cardiac problems predominate in TSC patients, testicular cancer has also been reported in these patients (61, 62), supporting the importance of this signaling pathway in testicular biology. mTOR signaling is also a downstream target of the PI3K/PTEN pathway (63). We hypothesized that Pten loss would phenocopy Tsc1cko and Tsc2cko, and by extension the Lkb1cko testicular phenotype. However, no aberrant phenotype was observed in Ptencko 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 Ptencko mice (SFig. 2C). Similar observation are also made in kidney, where loss of Tsc1 but not Pten causes polycystic kidney disease (64). Lack of a phenotype upon Pten loss was attributed to the unappreciable changes in mTOR signaling (64).

In this study, we observed fewer defects in germ cell development of four week old Lkb1 mutant mice compared to adults, even though Amhr2-Cre is expressed in Sertoli cells of both pre-and post-pubertal mice testes (16). 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 (19, 65). Previous studies have shown that first wave of mammalian spermatogenesis is unique and different from the later stages (66, 67). 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 (66). Massive apoptosis of germ cells also occurs during first wave of spermatogenesis and inhibition of cell death at this stage leads to defective spermatogenesis at later stages (67). Collectively, these studies confirm that a unique signaling programs 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 Lkb1<sup>cko</sup> mice, showing importance of mTOR activation in 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 have 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 in standard housing conditions and maintained on a mixed genetic background (C57BL/6;129/SvEv). The following mice strains were bred by standard husbandry techniques to obtain conditional knockout for Lkb1, Tsc1, Tsc2, and Pten genes: Amhr2^{tm3(cre)Bhr} (Amhr2-Cre, obtained from Dr Richard Behringer, (68)), and Stk11^{tm1Rdp} (Lkb1^{β/β}, obtained from Dr Nabeel Bardeesy (69)), Tsc1^{β/β} (National Cancer Institute (27)), Tsc2^{β/β} (obtained from Dr. Michael J. Gambello (70)), Pten^{β/β} (Jackson laboratory (71)) were mated to produce Amhr2^{tm3(cre)Bhr} /+;Stk11^{Δ/Δ}, Amhr2^{tm3(cre)Bhr} /+;Tsc2^{Δ/Δ}, Amhr2^{tm3(cre)Bhr} /+;Tsc1^{Δ/Δ}, Amhr2^{tm3(cre)Bhr} /+;Pten^{Δ/Δ} hereafter referred to as Lkb1^{cko}, Tsc2^{cko}, Tsc1^{cko}, and Pten^{cko} respectively. Tail biopsies were used for genotyping as previously described (70-73). The primers and PCR conditions to detect wt and flox Lkb1 alleles: 5'-GGG CTT CCA CCT GGT GCC AGC CTG T, 5'-GAT GGA GAA CCT CTT GGC CGG CTC A-3' and 35 cycles of 94 C 30 sec, 65 C 1 min, 72 C 1 min. Testes were collected from mutant mice at different stages of development and gross pictures were taken using a Nikon SMZ1500 microscope with an attached Spot camera (Diagnostic Instruments, Sterling Heights, MI).

Histological, Immunohistochemistry (IHC), Immunofluorescence (IF) staining

Testes were fixed in Bouin’s fixative and 4% paraformaldehyde as previously described (13, 16). Detailed protocols for IHC and IF are previously described by Tanwar et al. (16, 74). Defective tubules were counted on 2 separate sections for each of 3 mice and divided by the total number of tubules. The following primary and secondary antibodies are used in this study: β-catenin (BD Transduction Laboratories, San Jose, CA); LKB1, vimentin, Proliferating Cell Nuclear
Antigen (PCNA) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Tyrosinated Tubulin (Sigma, St. Louis, MO); Zonula Occludens-1/Tight Junction Protein-1 (ZO-1/TJP-1), N-cadherin (Developmental Studies Hybridoma Bank, Iowa City, IA); p-S6 ribosomal protein (pS6), pmTOR, S6, mTOR, AMPK (Cell Signaling Technology, Danvers, MA); SRY (sex determining region Y)-box 9 (SOX9) (Millipore, Billerica, MA); AMPK (Abcam, Cambridge, MA); Germ Cell Nuclear Antigen (GCNA; A gift from Dr George Enders); Phalloidin 568, AlexaFluor second antibodies (Invitrogen, Carlsbad, CA); and Biotinylated donkey antimouse or antirabbit F(ab)2 (Jackson ImmunoResearch, West Grove, PA). Terminal deoxynucleotidyl transferase 2’-deoxyuridine, 5’-triphosphate nick end labeling (TUNEL) (Roche, Indianapolis, IN) staining was performed according to the manufacturer’s instructions.

**Western blot analyses**

Testes from *Lkb1*^{fl/fl} and *Lkb1*^{cko} mice were collected and protein extracts were prepared using RIPA buffer as described (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, Danvers, MA); Sucrose Non-fermenting protein-Related Kinase (SNRK), TSC2, and thyroid receptor alpha 1 (TR) (Abcam, Cambridge, MA); β-actin (Neomarkers, Fremont, CA). 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) as previously described (13). The unpaired t test was used to calculate differences between groups, and P value of less than or equal to 0.05 were considered statistically significant.

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CONFLICT OF INTEREST

The authors have no conflicts to declare.
REFERENCES


FIGURE LEGENDS

Fig. 1. Conditional deletion of LKB1 in mouse testes. (A) Colocalization of mTOR with ßcatenin (Aa; a marker for adherens junctions; arrowheads), vimentin (Ab; a marker for Sertoli cells apical extensions; arrowheads) and tyrosinated αtubulin (Ac; a marker for Sertoli cells microtubules; arrowheads) by immunofluorescence. (Ba&b) Localization of LKB1 in control and Lkb1cko mutant testes (Bc) by immunofluorescence. Arrowheads indicate Sertoli cell nuclei and asterisks indicate interstitial Leydig cells. Western blot and semiquantitative analyses of LKB1 in control and mutant testes (Bc). Gross testes from Lkb1 control and mutant mice (Bd). Weight of Lkb1cko mutant and control testes (Be) and seminal vesicles (Bf). Columns represent the mean in n=3 testes, bars equal the SEM, and asterisks indicate p<0.05. Bars: 50 um. Nuclei are stained with DAPI.

Fig. 2. Premature germ cell loss and Sertoli cell only tubules of Lkb1cko, Tsc1cko and Tsc2cko but not in Ptencko mice testes. (A-F) Histology of control and Lkb1cko mutant testes by H&E. (G&H) Epididymides from control and mutant mice. Histology of control and Tsc1cko (I-N) testes,. (O) Gross picture of control and Tsc1cko mutant testes. Arrows in Panel B and J are marking vacuolated seminiferous epithelium. Arrows in Panel E, F, M, and N are pointing towards Sertoli cells. (P) Weight of adult Tsc1cko, Tsc2cko and control testes. Columns represent the mean in n=3 testes, bars equal the SEM, and asterisks indicate p<0.05. Histology of control, Tsc2cko (Q-T), and Ptencko (U-X) mutant testes. Panels 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 Panels S, T, W, and X indicate Sertoli cells. Insets in Panels Q and U show adult testes from control and mutant mice. Bars: 50 um.
Fig. 3. Overactivation of mTOR signaling in Lkb1cko, Tsc1cko and Tsc2cko testes. IHC for mTOR (A-E), pmTOR (F-J), S6 (K-O), and pS6 (P-T) in Lkb1fl/fl, Lkb1cko, Tsc1cko, Tsc2cko and Ptencko testes. Black arrowheads in Panel A, F, K, P are pointing towards Sertoli cells. (U&X) Vacuolation (black arrowheads) of seminiferous epithelium next to Sertoli cell positive (white arrowhead) for pS6 immunostaining. Panels X, Y, and Z are higher magnification images of the boxed areas in U, V, and W, respectively. Seminiferous tubules in Panel U to Z with elongated spermatids (black arrow) are marked by asterisks. Bars: 50 um.

Fig. 4. Disruption of the Sertoli cell polarity and testicular junctions in Lkb1cko, Tsc1cko and Tsc2cko 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 Lkb1fl/fl, Lkb1cko, Tsc1cko, Tsc2cko and Ptencko testes. Arrowheads in Panel C, D, E are marking positive staining for β-catenin, N-Cadherin, and ZO-1 at the site of the blood testis barrier. Arrowheads in Panel F indicate Sertoli cell microtubules. White dotted lines mark basement membrane of the seminiferous tubules. Arrowheads in Panel G are marking the Sertoli cells apical extensions. Bars: 50 um. Nuclei are stained with DAPI.

Fig. 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 Lkb1fl/fl (A), Lkb1cko (B), Tsc1cko (C), Tsc2cko (D) and Ptencko (E) testes. Arrowheads in Panel A to E represent Sertoli cells. White dotted lines indicate the basement membranes of the seminiferous tubules. Bars: 50 um.
ABBREVIATIONS

LKB1, Liver kinase b1; STK11, serine/threonine kinase 11; PJS, Peutz-Jeghers Syndrome; mTOR, mammalian target of rapamycin; TSC, tuberous sclerosis complex; PTEN, phosphatase and tensin homolog; MAP, microtubule associated protein; MARK, AMPK, AMP kinase; GCNA, germ cell nuclear antigen; AMHR2, antiMüllerian hormone receptor type II; TEX14, testis expressed gene 14; SOX9, SRY (sex determining region Y)-box 9; PCNA, proliferating cell nuclear antigen; FAK, focal adhesion kinase; MAPK/ERK, Mitogen-Activated Protein Kinases/Extracellular-signal-Regulated-Kinases; pS6, phosphorylated form of S6 ribosomal protein; TSC1/2, Tuberous Sclerosis protein1/2; TUNEL, Terminal deoxynucleotidyl transferase 2’-deoxyuridine, 5’-triphosphate nick end labeling; SNRK, Sucrose Non-fermenting protein-Related Kinase; PAK, P21-activated kinase; TRα1, thyroid hormone receptor α 1