

Specific deletion of *LKB1/Stk11* in the Müllerian duct mesenchyme drives hyperplasia of the periurethral stroma and tumorigenesis in male mice

Jitu W. George^{a,1}, Amanda L. Patterson^{a,1}, Pradeep S. Tanwar^{b,1}, André Kajdacsy-Balla^c, Gail S. Prins^d, and Jose M. Teixeira^{a,2}

^aDepartment of Obstetrics, Gynecology, and Reproductive Biology, College of Human Medicine, Michigan State University, Grand Rapids, MI 49503; ^bSchool of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan 2308, New South Wales, Australia; ^cDepartment of Pathology, School of Medicine, University of Illinois at Chicago, Chicago, IL 60612; and ^dDepartment of Urology, School of Medicine, University of Illinois at Chicago, Chicago, IL 60612

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Nearly all older men will experience lower urinary tract symptoms associated with benign prostatic hyperplasia (BPH), the etiology of which is not well understood. We have generated *Stk11^{CKO}* mice by conditional deletion of the liver kinase B1 (LKB1) tumor suppressor gene, *Stk11* (serine threonine kinase 11), in the fetal Müllerian duct mesenchyme (MDM), the caudal remnant of which is thought to be assimilated by the urogenital sinus primordial mesenchyme in males during fetal development. We show that MDM cells contribute to the postnatal stromal cells at the dorsal aspect of the prostatic urethra by lineage tracing. The *Stk11^{CKO}* mice develop prostatic hyperplasia with bladder outlet obstruction, most likely because of stromal expansion. The stromal areas from prostates of *Stk11^{CKO}* mice, with or without significant expansion, were estrogen receptor positive, which is consistent with both MD mesenchyme-derived cells and the purported importance of estrogen receptors in BPH development and/or progression. In some cases, stromal hyperplasia was admixed with epithelial metaplasia, sometimes with keratin pearls, consistent with squamous cell carcinomas. Mice with conditional deletion of both *Stk11* and *Pten* developed similar features as the *Stk11^{CKO}* mice, but at a highly accelerated rate, often within the first few months after birth. Western blot analyses showed that the loss of LKB1 and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) induces activation of the phospho-5' adenosine monophosphate-activated protein kinase and phospho-AKT serine/threonine kinase 1 signaling pathways, as well as increased total and active β -catenin. These results suggest that activation of these signaling pathways can induce hyperplasia of the MD stroma, which could play a significant role in the etiology of human BPH.

benign prostatic hyperplasia | mouse model | LUTS | Müllerian duct | liver kinase B1

Benign prostatic hypertrophy/hyperplasia (BPH) is one of the most commonly observed proliferative diseases in older men and is characterized by hyperplasia of stromal and epithelial cells of the prostate gland (1). Urinary obstruction resulting from the anatomical enlargement of the prostate in the periurethral region is one of the clinical hallmarks of BPH and the primary indication for medical intervention (2). Several studies investigating the pathophysiology of BPH indicate that the bioavailability of androgens, race, obesity, and other risk factors can contribute to progression of the disease, but age is the most prognostic factor, with estimates of up to 90% incidence in men in their ninth decade (2, 3). However, the precise mechanisms driving BPH have not been completely elucidated. The evidence does suggest that dysregulated stromal cell proliferation is a major component in symptomatic disease (1). Also, BPH appears to develop most often in the transition zone and in the periurethral region, both of which are near the base of the verumontanum (4), a remnant of the fused ends of the caudal Müllerian ducts (MDs). The tendency of BPH to develop in this anatomically distinct region of the prostate suggests the stroma has some unique qualities characteristic of its embryonic origin, which is often ascribed to the MD mesenchyme (MDM) (4–6).

Speculation that the etiology of BPH might be the result of reawakening of embryonic or dedifferentiated nonprostatic stromal cell activity is consistent with this hypothesis (4, 5, 7).

Shortly after commitment of the bipotential embryonic mammalian gonadal ridge to male development, Sertoli cells differentiate in the testes and produce Müllerian inhibiting substance (also known as anti-Müllerian hormone, or AMH), a TGF- β family member that causes MD regression in males (reviewed in ref. 8). The embryonic female gonads do not produce Müllerian inhibiting substance; thus, the MDs differentiate into the oviduct, uterus, cervix, and anterior vagina. However, the caudal MD remnant, which is commonly referred to as the prostatic utricle, is not completely regressed in males and has been thought to participate in the development of the rodent and human prostate (5, 9). Prostatic stromal cells have been shown to express estrogen receptor (10, 11), particularly in BPH (12), which also suggests that estrogen-responsive cells from the MDs could contribute to prostate development and the etiology of BPH.

One of the best-studied mouse models of prostate cancer is the TRAMP mouse (13). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor that inhibits the receptor tyrosine kinase-activated PI3K/AKT (RAC-alpha serine/threonine-protein kinase) signaling pathway, and its loss or deletion is frequently associated with a variety of cancers, including prostate cancer in humans (14, 15). Conditional deletion of *Pen* in prostate epithelia leads to high-grade neoplasia with complete penetrance and early onset in mice (16), supporting its essential role as a driver

Significance

Symptoms of benign prostatic hyperplasia (BPH) affect many older men, but the etiology of the disease is largely unknown. We show that male mice develop prostatic stromal hyperplasia accompanied by lower urinary tract symptoms that appear similar to BPH with conditional homozygous deletion of the tumor suppressor gene, *Stk11* (serine threonine kinase 11), in the Müllerian duct mesenchyme (MDM), which regresses in males during early fetal development. Cell lineage tracing studies confirmed that cells from the caudal MDM contribute to the stromal cell population of the dorsal periurethral area, which has been compared with the transition zone of the human prostate. These studies show that some of the stromal cells of the prostate are MD-derived, and that their mutation can lead to BPH.

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¹J.W.G., A.L.P., and P.S.T. contributed equally to this work.

²To whom correspondence should be addressed. Email: jose.teixeira@hc.msu.edu.

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of prostate carcinogenesis. PTEN loss has not been reported to play a role in BPH. Another mouse model of prostate cancer has been developed by conditional deletion of *Stk11* (serine threonine kinase 11), the gene for liver kinase B1 (LKB1), which is a gene linked to Peutz-Jeghers syndrome (PJS), a human hereditary cancer-prone disorder characterized by hamartomatous polyposis. Conditional deletion was accomplished in these mice by using the *Cyp11A1* promoter driving Cre recombinase expression in the prostate epithelium. These mice developed intraepithelial neoplasia with nearly 100% penetrance in many of the prostate lobes examined (17).

Recent studies from our laboratory showed that conditional deletion of *Stk11*, along with *Pten* from the MDM, led to high-grade serous epithelial ovarian cancer, endometrial hyperplasia and carcinomas, and oviductal and cervical adenomas in females (18, 19). Strikingly, we observed significant expansion of the stromal compartment admixed with glandular hyperplasia in the lower reproductive tracts of all of the female mice examined in that study, a phenotype that was consistent with adenoma malignum/minimal deviation adenocarcinoma (MDA), requiring euthanasia in most cases because of tumor growth-related exstrophy. In this report, we show that male mice with conditional deletion of *Stk11* in the embryonic MDM develop periurethral stromal hyperplasia, accompanied by urinary obstruction, suggesting an important etiological link between the MD remnant and BPH.

Materials and Methods

Mouse Genetics and Animal Husbandry. All animal experimentation protocols used in this study were approved by the Institutional Animal Care and Use Committees at Massachusetts General Hospital and Michigan State University and are in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Mice used in this study were kept in standard housing conditions and were maintained on a mixed genetic background (C57BL/6;129/SvEv). *Amhr2^{tm3(cre)Bhr}* (*Amhr2-Cre*) (20), *Stk11^{tm1Rdp}* (*Stk11^{fl/fl}*) (21), and *Pten^{tm1Hwu}* (*Pten^{fl/fl}*) (22) were mated to produce *Amhr2-cre⁺;Stk11^{Δ/Δ}*, *Amhr2-cre⁺;Pten^{Δ/Δ}* and *Amhr2-cre⁺;Stk11^{Δ/Δ};Pten^{Δ/Δ}*, hereafter referred to as *Stk11^{CKO}*, *Pten^{CKO}*, and *Stk11^{CKO};Pten^{CKO}*, respectively. *Amhr2^{tm2(lacZ)Bhr}* (*Amhr2-LacZ*) mice for real-time *Amhr2* promoter-driven *LacZ* expression and *Amhr2-Cre⁺* crosses with *Rosa26;EYFP* *Rosa26;LacZ^{fl/fl}* reporter mice have been previously described (23, 24). Tail biopsies were collected for genotyping, and PCR conditions for *Amhr2-Cre*, *Stk11*, and *Pten* alleles have been described (21, 22, 25, 26). *Amhr2-LacZ* mice were genotyped with 5'-TGC GTG ACT ACC TAC GGG TAA C and 5'-GAT CGA CAG ATT TGA TCC AGC G primers, using standard PCR amplification conditions; the presence of a 900-bp band by gel electrophoresis was considered positive for the *Amhr2-LacZ* allele. Gross pictures of the animals or the tumors were taken using a Nikon SMZ1500 microscope with an attached Spot camera (Diagnostic Instruments) or with a Nikon D60 digital camera and macro lens.

MDM Lineage Tracing. *TRE-H2b-GFP* (*Tg(tetOHIST1H2BJ/GFP)47Efu*) mice and *Rosa26-flox-stop-tTA* (*Gt(ROSA)26Sor^{tm1(tTA)Roostl}*) mice were purchased from The Jackson Laboratory and mated to *Amhr2-Cre* mice to generate triple transgenic offspring (*Amhr2-Cre;Rosa26-tTA;TRE-H2b-GFP*). Cre is expressed in the MDM on embryonic day 13.5 in both male and female fetuses, leading to expression of H2B-GFP, which becomes stably incorporated into nucleosomes during DNA replication, thereby indelibly labeling MDM-derived cells. Urogenital systems from 7–9-wk-old *Amhr2-Cre;Rosa-tTA;TRE-H2b-GFP* and control (*Rosa26-flox-stop-tTA;H2b-GFP*) mice were fixed in 4% (vol/vol) PFA on ice for 30 min. After three washes in ice-cold PBS, tissues were incubated overnight at 4 °C in 15% (wt/vol) sucrose buffered in PBS. The following day, tissues were incubated at 37 °C for 1 h in gelatin [15% sucrose (wt/vol), 7.5% (wt/vol) gelatin in PBS], embedded in gelatin, then frozen at –50 °C to –65 °C in isopentane and stored at –80 °C until sectioning. Tissues were cryosectioned at 6 μm and thaw mounted. Gelatin was removed from tissue sections by incubating slides in 37 °C PBS for 10 min. After gelatin removal, slides were counterstained with DAPI (Vector Laboratories) mounting medium and viewed directly using fluorescence microscopy.

Histology and Western blot analyses are presented in *SI Materials and Methods*.

Results

Caudal MD-Derived Cells in the Male Reproductive Tract. The involvement of the caudal MD remnant in male urogenital tissue

development has been debated (9, 27). To study the contribution, if any, of the MDM to prostate organogenesis, we mated mice with the *Amhr2-Cre* allele, which is expressed in the embryonic MDM cells, with mice carrying *EYFP*, *LacZ*, or *GFP* floxed reporter alleles. AMH type 2 receptor (AMHR2), a bona fide receptor for AMH (28), is only expressed in the mesenchyme surrounding the fetal MDs and in the gonads of male urogenital ridges, but not in the Wolffian duct (29–32). We bred mice to contain the *Amhr2-Cre;Rosa26-YFP* alleles to determine whether any MD-derived tissues persisted in males and directly observed YFP activity in the dorsal prostatic region of sexually immature male mice (Fig. S1 A and B), but not in control littermates (Fig. S1 C and D). As expected, females showed YFP activity in the MD-derived tissues, the oviducts, uterine horns, cervix, and ovaries, which express *Amhr2* on the surface epithelium (33) and granulosa cells (29–32) (Fig. S1 E and F). As further confirmation of the presence of MDM cells in male urogenital tissue, β-galactosidase activity was assessed in adult mice with floxed *Rosa26-LacZ* alleles (Fig. S1 G–N). In control littermates, variable levels of endogenous β-galactosidase activity were observed in the urogenital tracts (Figs. S1 G, I, K, and M and S2A), whereas in *Amhr2-Cre;Rosa26-LacZ* mice, in addition to endogenous β-galactosidase activity, strong β-galactosidase activity was observed in the ampullary glands and in stromal cells at the base of the seminal vesicles (Fig. S1 H, J, L, and N) and in the stroma and smooth muscle of the vasa deferens (Fig. S2A). Furthermore, cells expressing real-time β-galactosidase activity in which *LacZ* is directly under the control of the *Amhr2* promoter and is only expressed in cells actively expressing AMHR2 [*Amhr2-LacZ* mice (23)] were observed at the base of the ampullary glands and seminal vesicles (Fig. S2 B and C), suggesting the *Amhr2* locus was still active in adult mice.

Because of the caveats associated with *LacZ* reporter mice, specifically the confounding endogenous β-galactosidase activity, we used a more reliable and robust reporter mouse model to further substantiate the presence of MDM cells in the male urogenital tract. *Amhr2-Cre;Rosa26-tTA;TRE-H2b-GFP* mice, which express nuclear GFP, showed similar gross anatomical results to that of the *YFP* and *LacZ* reporter mice with robust GFP expression in the dorsal prostatic region of adult male mice, which was absent from control littermates (Fig. 1 A, B, E, and F). To assess whether MDM cells contribute to the male prostatic stroma, we examined frozen sections of the male urogenital tissues for direct GFP activity and observed nuclear GFP activity in the dorsal periurethral stroma of *Amhr2-Cre;Rosa26-tTA;TRE-H2b-GFP*, but not in control littermates (Fig. 1 C and G). As discussed earlier, the *Amhr2* promoter was still active in adult *Amhr2-LacZ* mice (Fig. S2). Because of this, we wanted to confirm that the GFP-positive cells in *Amhr2-Cre;Rosa26-tTA;TRE-H2b-GFP* mice were in fact derived from MDM that persisted in the male after MD regression and were not mature periurethral stromal cells that had reactivated *Amhr2* expression, and thus might not be MDM-derived. To demonstrate this, we collected postnatal day (PND) 0.5 male urogenital tracts from control and *Amhr2-Cre;Rosa26-tTA;TRE-H2b-GFP* mice (Fig. 1 D and H) and showed the presence of GFP-positive MDM cells in the periurethral stroma shortly after MD regression (23) and during prostate formation, which was confirmed with colocalization with NKX3.1, a prostate epithelial marker (34, 35) (Fig. 1 I and J), indicating that the MD remnant contributed to postnatal prostate development. These results reveal that AMHR2-expressing cells derived from the caudal MDM persist in the male reproductive tract, well after MD regression has occurred, and contribute to the development of these male genitourinary tissues.

Loss of LKB1 in the MDM. We have shown here that MDM cells contribute to the stroma of male reproductive tract tissues in mice, including the vasa deferens and the periurethral stroma. We have previously shown that deletion of tumor suppressor genes in uterine stroma can lead to hyperplasia and tumorigenesis (18). In

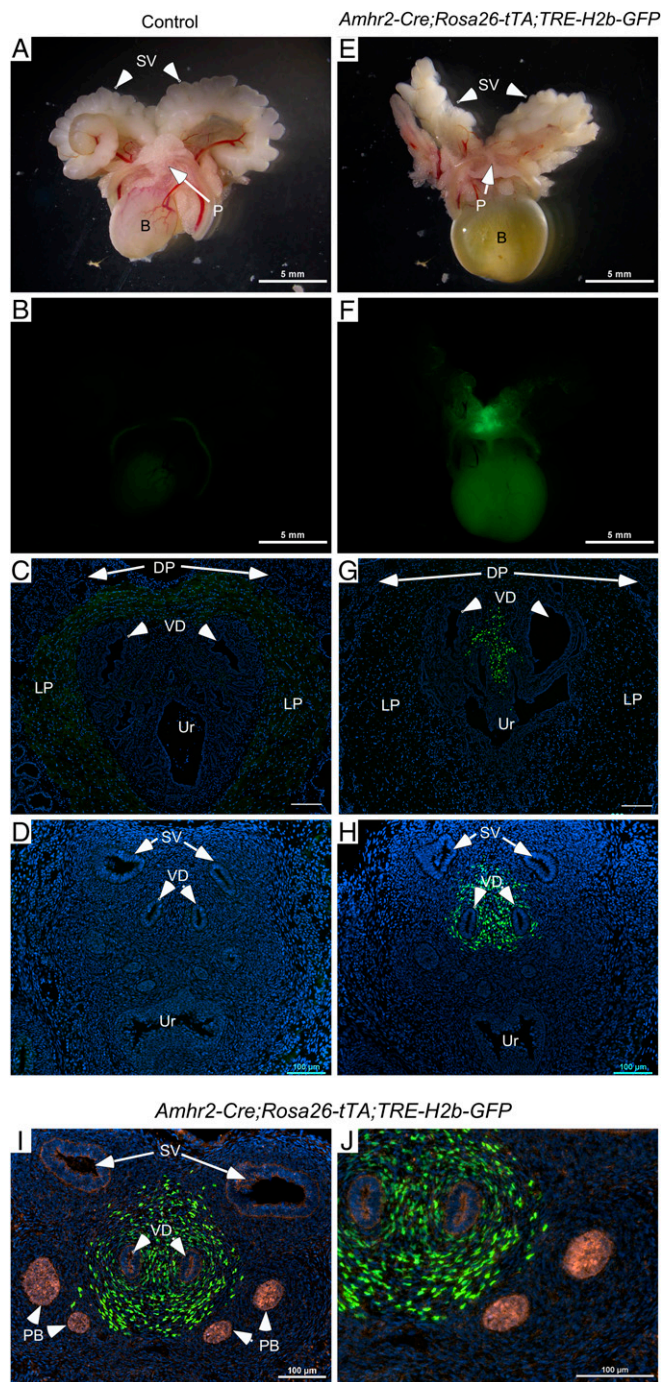


Fig. 1. Caudal MDM-derived cells persist in the male reproductive tract. The reproductive tracts of adult (A–G) and PND 0.5 (D–J) progeny of *Amhr2-Cre* mice mated with GFP reporter mice (*Amhr2-Cre;Rosa26-tTA;TRE-H2b-GFP*) were examined grossly for fluorescence. Fluorescence was not observed in any of the control littermates examined (A–D). (E and F) *Amhr2-Cre*-driven fluorescence is detected in the prostatic or genitourinary (P) region of adult males. The bladder (B) and seminal vesicles (SV) did not fluoresce. MDM-derived cells were detected by *Amhr2-Cre*-driven nuclear GFP expression in the dorsal stroma of the periurethral (Ur) area between the vasa deferens (VD) in both adult (C and G) and PND 0.5 (D and H). *Nkx3.1*, a prostate epithelial marker, was used to show the developing prostate buds (PB) in PND 0.5 tissue (I and J). Dorsal and lateral prostate (DP, LP) are indicated in C and G for orientation. Nuclei are stained with DAPI in C, D, and G–J. [Scale bars, 5 mm (A, B, E, and F) or 100 μ m (C, D, and G–J).]

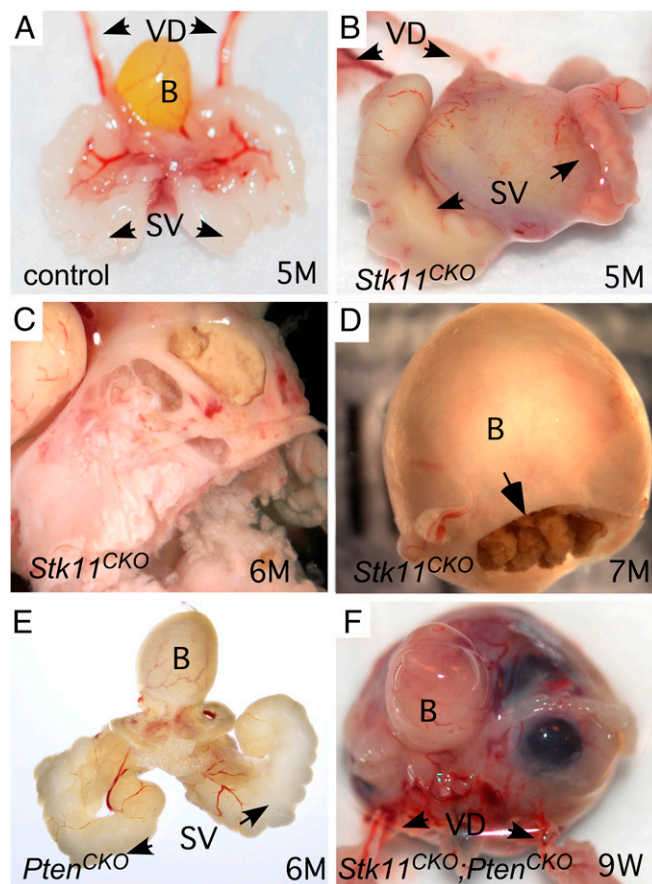


Fig. 2. Gross analyses of male reproductive tracts in mice with *Amhr2-Cre*-driven loss of LKB1 and PTEN. Control littermate male reproductive tract at 5 mo shown in A. Representative reproductive tract of similarly aged mice with loss of LKB1 in the MDM cells shown in B, with another bisected in C. Calcified debris was often observed in the bladders of *Stk11^{CKO}* mice (D). Mice with loss of PTEN alone had no overt phenotype (E). Development of stromal tumors in mice with *Amhr2-Cre*-driven deletion of both *Stk11* and *Pten* was accelerated (F). B, bladder; SV, seminal vesicles; VD, vasa deferens.

particular, we have observed that deletion of the *Stk11* gene in the MDM-derived female reproductive tract leads to significant cervical tumor burden in most mice. These tumors are stromal and resemble human adenoma malignum/MDA, and often require euthanasia of the mouse because of associated morbidities. We thus hypothesized that loss of LKB1 in the retained caudal MDM-derived stromal cells could similarly lead to the development of BPH or prostate carcinomas. To test this hypothesis, we examined the reproductive tracts of male *Stk11^{CKO}* mice at various ages. From 2 to 6 wk postnatal, no gross or histological differences were observed between control and *Stk11^{CKO}* mice, but by 12 wk, gross analysis of *Stk11^{CKO}* mice showed that some of the mice examined had notable enlargement of the periurethral area compared with controls, which was clearly evident by 5 and 6 mo (Fig. 2 A–C). These were often accompanied by accumulation of calculi in the bladder (Fig. 2D), a diagnostic of urinary stasis and lower urinary tract obstruction in men with BPH. Additional deletion of the *Pten* gene, which encodes for the PTEN tumor suppressor, resulted in faster and more severe tumorigenesis (Fig. 2F). Mice with deletion of *Pten* alone did not show any periurethral phenotype (Fig. 2E) by 6 mo, indicating that PTEN loss accelerates tumorigenesis only in cells already prone to developing cancer.

More thorough analyses of the periurethral tissues were performed by histology (Fig. 3). The periurethral area of

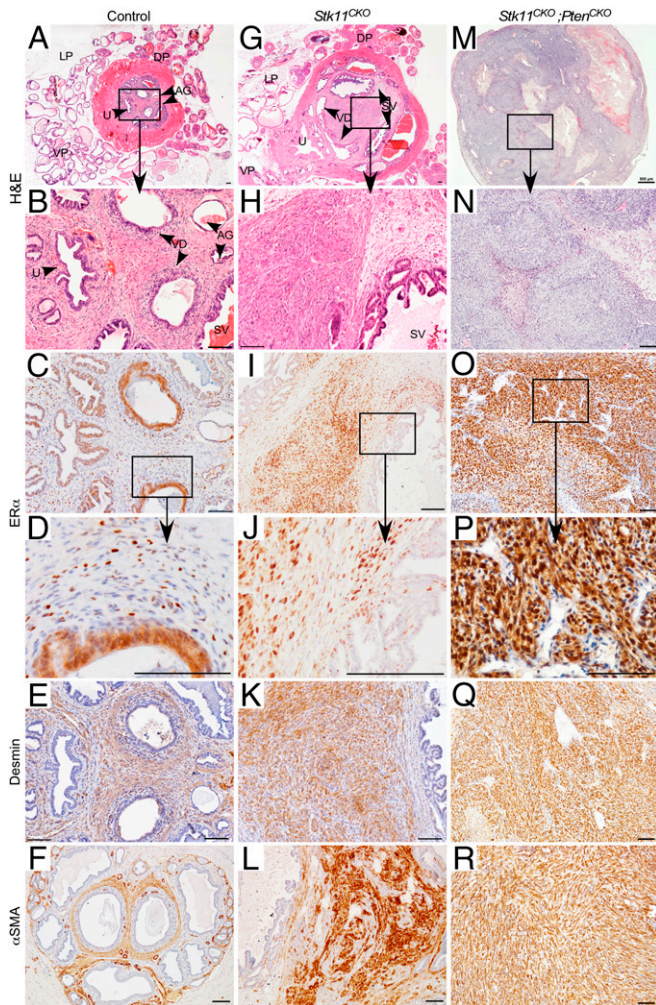


Fig. 3. Histological and immunohistochemistry analyses of prostatic/periurethral region in mice with Amhr2-Cre-driven loss of LKB1 and PTEN. H&E of prostatic area of controls (A) and mice with *Stk11* deletion (G) or *Stk11* and *Pten* (M) deletion in the MDM cells shows typical stromal expansion (G) and tumor formation (M) observed over time compared with control littermates (A). Boxed areas in A, G, and M are shown at higher magnification in B, H, and N. Nuclear ER α is observed in fewer than 50% of the stromal cells in the prostatic/periurethral area of control mice by immunohistochemistry (C). Nearly all the stromal cells in the *Stk11*^{CKO} (I) and *Stk11*^{CKO};*Pten*^{CKO} (O) prostatic/periurethral area are positive for nuclear ER α . Boxed areas in C, I, and O are shown at higher magnification in D, J, and P. Desmin and α SMA are expressed by immunohistochemistry in the control (E and F) and mutant (K, Q, L, and R) stromal areas. AG, ampullary glands; DP, dorsal prostate; LP, lateral prostate; SV, seminal vesicles; U, urethra; VD, vasa deferens; VP, ventral prostate. Nuclei counterstained with hematoxylin. [Scale bars, 100 μ m; 500 μ m (M).]

control littermates ($n = 13$) between 3 and 8 mo postnatal showed normal histology (36) with well-differentiated ducts, glands, and stroma (Fig. 3 A and B). In contrast, most mice with loss of LKB1 in MDM showed either expansion of the stroma between 3 and 7 mo postnatal (11/17; Fig. 3 G and H), often accompanied by deformation of the ducts and glands, or frank stromal tumorigenesis (4/17; Fig. S3 A and B). In the periurethral expansions or smaller tumors, the histology of prostatic and accessory glands and ducts appeared normal in mutant mice (Fig. S4), suggesting the mutant stroma was not affecting adjacent epithelial cell homeostasis, at least during the early stages. The bladder urothelium of some of the mice appeared abnormally thickened and/or edematous (Fig. S4H),

probably as a result of the urethral obstruction caused by adjacent stroma. Histological analyses of the periurethral tissues of *Stk11*^{CKO};*Pten*^{CKO} mice was more challenging because of the rapid growth of these tumors and their total overgrowth of adjacent accessory ducts and glands. The stroma appeared as greatly expanded (Fig. 3 M and N and Fig. S5A) versions of larger tumors from older *Stk11*^{CKO} mice, as shown above (Fig. S3 A and B). In a minority of both large *Stk11*^{CKO} tumors and *Stk11*^{CKO};*Pten*^{CKO} tumors, various forms of epithelial metaplasia were observed (Fig. S5B), including stratified squamous epithelia with and without keratin pearls and transitional epithelium that resembles the urothelium. Invasive and phyllodermal epithelial metaplasia were also observed in some tumors (Fig. S5 C and D). All this suggests the expansion of the mutant stroma can induce tumorigenesis in the epithelial compartment.

The most caudal MD-derived tissue in females is the vagina, which expresses estrogen receptor- α (ER α) in both the epithelial and stromal compartments (37, 38) and has been observed in both compartments of the prostate as well (10, 11). We examined whether the periurethral stromal cells of control male mice similarly expressed ER α , and whether expression was altered in the stromal cells of *Stk11*^{CKO} and *Stk11*^{CKO};*Pten*^{CKO} mice by immunohistochemistry. In control mice ($n = 7/9$),

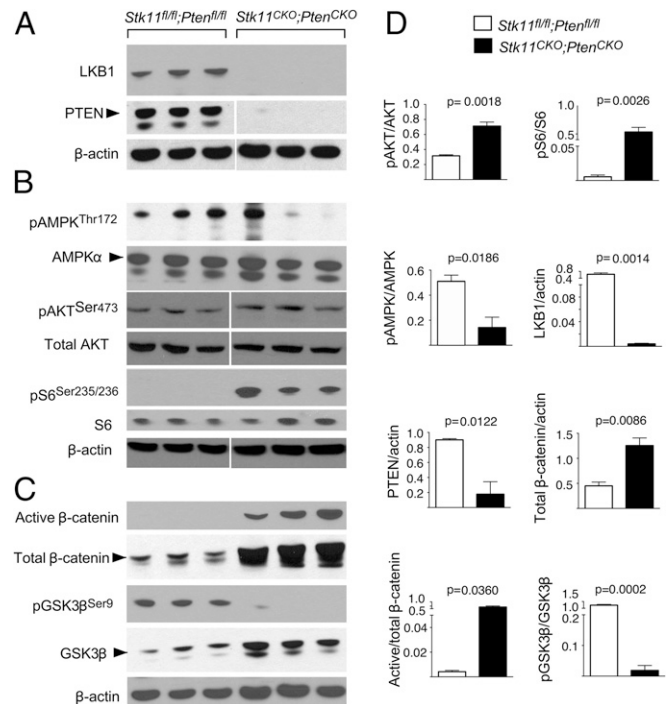


Fig. 4. Conditional loss of LKB1 and PTEN leads to activation of mTOR and Wnt signaling. (A) Western blots of protein lysate from the male urogenital tissues of *Stk11*^{+/+};*Pten*^{+/+} controls and *Stk11*^{CKO};*Pten*^{CKO} mutants were performed in triplicate. Strong reductions in LKB1 and PTEN were observed in the double mutant. (B) Western blots show increased levels of downstream signaling partners of pAKT^{Ser473}, pS6^{Ser235/236}, and pAMPK^{Thr172} for PTEN and LKB1, respectively, in the double mutants. No change was observed in total AKT and total AMPK. (C) Alternative LKB1 signaling was also observed with increased total and active β -catenin, as well as reduced pGSK3 β ^{Ser9} in the double mutants. Control and tumor extracts were immunoblotted with indicated antibodies. β -Actin was used as loading control. Arrows indicate the correct band. Phosphorylation is indicated by p, and corresponding residues are identified. (D) Semiquantification of the bands in A, B, and C was performed, averaged, and graphed. P values were calculated by the unpaired t test. Error bars represent SEM.

strong ER α expression was observed in the ductal, glandular, and urethral epithelial cells (Fig. 3C) and in scattered stromal cells (Fig. 3D). In contrast, strong ER α expression was observed in nearly all the stromal cells in half the *Stk11*^{CKO} mice ($n = 4/8$) (Fig. 3I and J and Fig. S3D and E), and in all the *Stk11*^{CKO}; *Pten*^{CKO} mice (Fig. 3O and P) that were examined. Weaker or scattered expression was observed in the adjacent epithelial cells in *Stk11*^{CKO} mice ($n = 8/8$) (Fig. 3I and J) compared with controls (Fig. 3C and D), suggesting the mutant stromal cells could be controlling the fate of the nearby, unmutated epithelium. Expression of desmin and α -smooth muscle actin, smooth muscle cell markers, was observed throughout the stroma of control, *Stk11*^{CKO}, and *Stk11*^{CKO}; *Pten*^{CKO} mice (Fig. 3E, F, K, L, Q, and R and Fig. S3A–C and F), indicating that the affected cells were more likely a myofibroblast phenotype. In addition, the stromal cells of control and *Stk11*^{CKO}; *Pten*^{CKO} mice showed the presence of collagen by trichrome staining (Fig. S3B), which was more abundant in the *Stk11*^{CKO}; *Pten*^{CKO} mice as a result of tumorigenesis, further substantiating the likelihood of these cells being myofibroblast-like. Taken together, these data are consistent with what is observed in human BPH (39).

Disrupted Signaling in the *Stk11*^{CKO}; *Pten*^{CKO} Stroma. To investigate the mechanisms that could be driving tumorigenesis in the periurethral stroma, we assessed activation of known signaling cascades downstream of LKB1 and PTEN tumor suppressors. Western blot analysis confirmed gene deletion and protein reduction of both LKB1 and PTEN in *Stk11*^{CKO}; *Pten*^{CKO} prostatic/periurethral tissues (Fig. 4A). LKB1 mediates its tumor suppressor activity by phosphorylating AMPK at Thr172 (40). In contrast to *Stk11*^{+/+}; *Pten*^{+/+} control tissues, phosphorylated AMPK was reduced in *Lkb1*^{CKO}; *Pten*^{CKO} mice (Fig. 4B), indicating a loss in control of the mTOR pathway. PTEN antagonizes PI3K activity by dephosphorylating PIP3 and sequestering AKT from the membrane, thus inhibiting phosphorylation and activation of the kinase (41). Levels of Ser473-phosphorylated AKT were elevated in tumors, consistent with activation of AKT (Fig. 4B). On the basis of these data, we assayed phosphorylation of S6 at Ser235/236 and saw increased levels, consistent with hyperactive mTOR signaling (Fig. 4B). Deletion of *Stk11* alone affected both AMPK and S6 phosphorylation compared with control tissues, confirming the role of LKB1 in these pathways (Fig. S6).

In addition to suppression of the mTOR pathway, LKB1 is also known to regulate wingless-related integration site (WNT)/ β -catenin signaling, and conditional deletion of *Stk11* in mouse prostate has been reported to cause an increase in WNT signaling (17, 42, 43). Increased expression of both active and total β -catenin, the canonical downstream effector protein in the WNT signaling cascade, was observed in the *Stk11*^{CKO}; *Pten*^{CKO} stromal tumor (Fig. 4C). Interestingly, we observed loss of phosphorylation at Ser9 of pGSK3 β (Fig. 4C) and increased total GSK3 β levels (Fig. 4C) in the periurethral stroma, indicating that LKB1 and/or PTEN are important for maintaining GSK3 β phosphorylation levels in these tissues. Analyses of *Stk11*^{CKO} prostate tissues compared with controls suggests that LKB1 affected β -catenin and GSK3 β phosphorylation and expression (Fig. S6).

Discussion

LKB1 has multiple functions in cells. It is best known as a tumor suppressor whose kinase is active during cellular metabolic stress conditions such as low ATP. Mutations of the gene (*STK11*) for LKB1 are responsible for PJS, an autosomal dominant disease that leaves patients at an increased risk of developing benign and malignant tumors with age (44). Patients typically present with hamartomatous polyps. LKB1 would normally phosphorylate and activate AMPK family kinases in

response to stress, which subsequently activates TSC2 and inhibits mTOR, the master regulator of proliferation. Thus, loss of PTEN, another tumor suppressor upstream of mTOR, would be expected to synergize with loss of LKB1 and lead to uncontrolled cellular proliferation, which is what we observe in our *Stk11*^{CKO}; *Pten*^{CKO} mice, as shown by accelerated tumorigenesis. LKB1 appears to also have an evolutionary conserved role in regulating cellular polarity and structure (45), which can also be important for constraining any effects of mitogens on proliferation, and possibly tumorigenesis. We have shown that mTOR activity is induced in the tumors of *Stk11*^{CKO} mice (Fig. 4), which suggests that unrestrained proliferation is an immediate trigger to tumorigenesis in the MDM, but we cannot rule out an effect on cellular organization contributing to the phenotype.

The loss of LKB1 in the MDM results in a male phenotype that is characterized by the growth of periurethral stroma in mice, which is akin to BPH in humans, a disease whose etiology is essentially unknown (1). These results in mice suggest two relatively understudied areas that could be important for understanding how BPH develops in humans. First, it is clear that MD regression is not complete in males and that some of the MD mesenchyme persists postnatally and differentiates into the periurethral stroma. The implications on BPH biology of this observation are notable. MD mesenchyme in females develops into the stroma of the female reproductive tract, which is a highly estrogen-responsive tissue. In BPH, the prostatic stroma is thought to become more estrogen-responsive, which would suggest that the stroma derived from the MDM could be de-differentiating or “re-awakening” in a more primitive state (4, 5, 7). Although hypothesized for some time, we believe our results shown here provide strong evidence that this indeed might be the case.

We have also observed that β -catenin levels are elevated in the stromal tumor tissues (Fig. 4B), which is consistent with previous reports that loss of LKB1 can induce β -catenin (17, 43, 46, 47). LKB1 physically associates with GSK3 β and regulates WNT signaling pathway by modulating phosphorylation of pGSK3 β ^{Ser9} (43). In our mice with conditional deletion of *Stk11* and *Pten*, we observed that loss of LKB1 led to down-regulation of pGSK3 β ^{Ser9} and increased expression of both total and active forms of β -catenin. This indicates that LKB1 antagonizes the stability of β -catenin by activating GSK3 β , decreasing protein levels of β -catenin, and turning off WNT target genes. Deregulation of β -catenin is associated with a number of cancers and other diseases (48). This inverse correlation highlights the multifunctional role of LKB1 and confirms its role in regulation of β -catenin and its contribution to tumorigenesis in the MDM.

Another important aspect of our study is that although we have deleted many different genes, including other tumor suppressor genes using *Amhr2*-Cre, we have observed this BPH-like phenotype only with conditional deletion of *Stk11*, the gene that is linked with PJS. In patients with PJS, the incidence of adenoma malignum of the uterine cervix (MDA), a usually rare form of cervical cancer, is estimated at between 15% and 30%, and patients with PJS account for 10% of the total cases (49). In another study of 11 MDA tumors from patients without PJS, six had mutations in *STK11* with loss of heterozygosity. MDA was observed in our female mice with conditional deletion of *Stk11* and *Pten* with 100% penetrance (18), indicating that, as with humans, *Stk11* mutation renders the caudal MD highly susceptible to MDA tumorigenesis in mice. In this context, development of the periurethral stromal tumors from the caudal remnant of the MDM with *Stk11* deletion in male mice makes sense and strengthens the hypothesis that dysregulation of *STK11* expression, or more likely downstream signaling events, could be involved in the human caudal MDM developing into BPH. Determining whether the LKB1/AMPK signal transduction cascade is dysregulated in BPH, although challenging, will be needed to confirm this hypothesis.

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