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Uterine-specific knockout of Tsc-2: a mouse model for lymphangioleiomyomatosis

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14. ABSTRACT Our goal was to develop and characterize a mouse model for the disease lymphangioleiomyomatosis (LAM). This disease is found almost exclusively in women and is due to a mutation in one of the two TSC suppressor genes. Women with LAM develop smooth muscle-like tumors in the lungs that grow progressively, often leading to lung failure. Evidence suggested that these tumors do not originate in the lungs but instead are metastatic from another location in the body. Our hypothesis was that lung LAM tumors were actually metastatic from smooth muscle myometrial cells in the uterus, thus explaining both the appearance of the LAM cells as well as the overwhelming female prevalence. Thus, we created a uterine-specific TSC-2 knockout mouse. These mice developed uterine tumors that were completely dependent on estrogen for growth. Importantly, as mice aged above 30 weeks, 75% of them developed myometrial lung tumors that shared many features consistent with LAM. These data were just published in and featured on the cover of the September, 2013 issue of "Molecular Endocrinology." This is an exciting new model, and we hope to use it to both better understand LAM and to help test novel treatment options for LAM.					
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Uterine-specific knockout of Tsc-2: a mouse model for lymphangioleiomyomatosis

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Introduction:

Lymphangioleiomyomatosis (LAM) is a rare disease that consists of smooth muscle cell tumors within the lungs that progressively grow, often leading to pulmonary failure and sometimes even lung transplantation. Interestingly, even after transplantation, LAM tumors often return to the lungs, suggesting that LAM cells do not originate in the lung. Two other unusual features of LAM are: 1) LAM is found almost exclusively in women; and 2) LAM cells contain mutations in one of the two TSC genes. To explain all of these observations, we proposed that LAM cells might metastasize from smooth muscle myometrial cells in the uterus that have sustained mutations in one of the TSC genes. To test this theory, we proposed to make a uterine-specific TSC-2 knockout mouse by mating a mouse expressing Cre driven by the progesterone receptor promoter with a mouse containing a floxed TSC-2 gene. Our goal was to: 1) test our hypothesis; 2) create a viable mouse model for LAM that can be used to study LAM physiology as well as to test LAM treatment options; and 3) create a TSC-2 null myometrial cell line that can be used to study molecular mechanisms of LAM proliferation.

Body:

This project has been remarkably successful in the first year of funding from the DOD. Our first task was to mate TSC-2 floxed and PR-Cre mice to create a uterine-specific TSC-2 null mouse. Subtasks included examination of estrus cycling, measurement of hormone levels, examination of the uterine phenotype, examination of the ovarian, pituitary, and hypothalamic regions, examination of the lungs, and a look at the dependence of mTOR signaling for tumor growth. In fact, as seen in the accompanying manuscript, we have completed all of these tasks. We have used quantitative PCR and Western blot to confirm that we have a uterine-specific TSC-2 null mouse. These mice are chronically in estrus and have normal estradiol levels. Their uteri are markedly enlarged, and older mice develop myometrial tumors (leiomyomas). These tumors are mTORC1-dependent, as treatment with the mTORC1 inhibitor rapamycin abrogate myometrial proliferation. The ovaries, hypothalamus, and pituitary of these mice are normal. Finally, 75% of older uterine-specific TSC-2 null mice develop metastatic myometrial lung tumors, confirming that LAM might indeed originate from the uterus. All of these accomplishments are described in detail in the attached publication (see appendix).

Task 2 was to remove the ovaries from uterine-specific TSC2 null mice and then add back estrogen versus progesterone to see which steroid, if any, promotes myometrial overgrowth. In fact, removal of the ovaries completely abrogated leiomyoma formation, and addition of estradiol, but not progesterone, pellets completely restored tumor formation. Thus, like LAM, myometrial tumor formation in the TSC-2 null uterus requires estradiol signaling. Again, these accomplishments are described in detail in the attached publication (see appendix).

Tasks 3 and 4 were to create a myometrial cell line that can be tested in-vitro and then in-vivo using xenograft models. These studies are currently underway.

Key Research Accomplishments:

- Created a uterine-specific TSC-2 null mouse
- Demonstrated the presence of myometrial overgrowth and leiomyomas in 100% of uterine-specific TSC-2 null mice.
- Demonstrated upregulation of mTORC1 signaling in these leiomyomas.
- Demonstrated that this mTORC1 signaling is required for myometrial growth in the TSC-2 null mice.
- Demonstrated that estradiol, but not progesterone, is required for myometrial growth in the TSC-2 null mice.
- Demonstrated that the lungs of most older TSC-2 null mice develop myometrial LAM-like tumors.

Reportable Outcomes:

To date, this work has resulted in one publication that was featured on the cover of the September, 2013 issue of *Molecular Endocrinology*: Prizant et. al. (2013), *Molecular Endocrinology* 27, 1403-14. Please see appendix.

Conclusion:

To summarize, in the first year of our two-year project we have finished the first half of our proposed studies, creating and characterizing a uterine-specific TSC-2 null mouse. In addition, we have data to support our hypothesis that TSC-2 null LAM lesions can indeed originate in the uterus and metastasize to the lungs. Our future work will focus on isolating myometrial cells from these uteri and creating a cell line that can be used to study signaling and proliferation both in-vitro and in-vivo using xenograft models. We have successfully isolated myometrial cells two times now, and are in the process of passaging these cells to create a stable immortalized cell line. Once we have this cell line, we will proceed with the proposed signaling and xenograft studies.

References:

None.

Uterine-Specific Loss of Tsc2 Leads to Myometrial Tumors in Both the Uterus and Lungs

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Lymphangioleiomyomatosis (LAM) is a rare disease characterized by proliferation of abnormal smooth-muscle cells in the lungs, leading to functional loss and sometimes lung transplantation. Although the origin of LAM cells is unknown, several features of LAM provide clues. First, LAM cells contain inactivating mutations in genes encoding Tsc1 or Tsc2, proteins that limit mTORC1 activity. Second, LAM tumors recur after lung transplantation, suggesting a metastatic pathogenesis. Third, LAM is found almost exclusively in women. Finally, LAM shares features with uterine leiomyomas, benign tumors of myometrial cells. From these observations, we proposed that LAM cells might originate from uterine leiomyomas containing Tsc mutations. To test our hypothesis, and to develop mouse models for leiomyoma and LAM, we targeted Tsc2 deletion primarily in uterine cells. In fact, nearly 100% of uteri from uterine-specific Tsc2 knockout mice developed myometrial proliferation and uterine leiomyomas by 12 and 24 weeks, respectively. Myometrial proliferation and mTORC1/S6 activity were abrogated by the mTORC1 inhibitor rapamycin or by elimination of sex steroid production through ovariectomy or aromatase inhibition. In ovariectomized Tsc2 null mice, mTORC1/S6 activity and myometrial growth were restored by estrogen but not progesterone. Thus, even without Tsc2, estrogen appears to be required for myometrial mTORC1/S6 signaling and proliferation. Finally, we found Tsc2 null myometrial tumors in lungs of older Tsc2 uterine-specific knockout females, suggesting that lung LAM-like myometrial lesions may indeed originate from the uterus. This mouse model may improve our understanding of LAM and leiomyomas and might lead to novel therapeutic strategies for both diseases. (*Molecular Endocrinology* 27: 1403–1414, 2013)

Pulmonary lymphangioleiomyomatosis (LAM) is a life-threatening, rare disease characterized by proliferation of abnormal smooth muscle cells in the lungs (1–4). With time, patients with LAM develop pulmonary cyst formation, pneumothorax, and progressive pulmonary failure. LAM has 3 unique characteristics: 1) LAM demonstrates strong gender specificity, affecting almost exclusively reproductive-age women; 2) LAM cells express markers of melanocyte differentiation, including gp100 and melanoma-antigen-recognized-by-T-cells (also known as protein melan-A) (5); and 3) LAM cells contain inactivating mutations in tumor suppressor genes Tsc1 or

Tsc2. GTPase activating protein function within a protein complex containing Tsc1, Tsc2, and recently discovered TBC1D7 (6), inhibits a small GTPase called Rheb (Ras-homologue-enriched-in-brain), which is a key activator of mammalian target of rapamycin complex 1 (mTORC1). Thus, the Tsc1/Tsc2/TBC1D7 protein complex suppresses mTORC1 signaling pathway and thereby cell growth. In LAM, mutations in the genes encoding either Tsc1 or Tsc2 render this protein complex nonfunctional; therefore, Rheb and downstream mTORC1 are activated, leading to increased cell growth. Interestingly, only 40% of female patients with tuberous sclerosis complex (also with mutations

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Abbreviations: ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; LAM, lymphangioleiomyomatosis; mTORC1, mammalian target of rapamycin complex 1; OVX, ovariectomies; PR, progesterone receptor; SMA α , α -smooth muscle actin.

in either the Tsc1 or Tsc2 gene) have lung LAM lesions (7), suggesting that, although Tsc mutations are important in LAM, other “second hits” might be needed for complete development of the disease.

A great mystery in LAM revolves around the origin of the lung LAM cell. Evidence suggests that LAM cells are not derived within the lung, but in fact migrate to the lung from elsewhere in the body through lymphatics or possibly the bloodstream (8–10). For example, many women with LAM who have required lung transplantation develop recurrent LAM tumors in their new lungs that are genetically identical to those in the original lungs (11, 12), suggesting that the new lung LAM cells must have come from elsewhere in the body. Further proof comes from genetic analysis of lung LAM lesions showing that Tsc mutations in lung LAM cells are identical to those found in angiomyolipomas, but not in normal kidney cells, again suggesting an extrapulmonary origin of LAM cells (13–15).

If lung LAM is indeed metastatic, where does it come from? To address this question, one must re-examine the sexual dimorphism of LAM. Notably, LAM progression is greatest in women during their reproductive years (16–18). Furthermore, estradiol appears to be able to promote LAM cell proliferation and possibly metastasis in vitro (19, 20). Together, these observations suggest that estradiol in women might explain the sexual dimorphism of LAM. However, men still produce estradiol. Furthermore, although LAM progression often slows after menopause or ovariectomy (21–23), many anovulatory women still have significant advancement of their disease (24), indicating that estradiol cannot be the only answer.

Another potential explanation for the sexual dimorphism of LAM is the unique presence of a uterus in women. Lung LAM tumors and uterine leiomyomas share many features, including similar-appearing smooth muscle cells, expression of estrogen and progesterone receptors (ERs and PRs), and growth sensitivity to estrogens. Interestingly, a recent study looking for the microscopic presence of LAM cells in the uteri of patients with lung LAM revealed that every patient had leiomyomas, and all but one had actual LAM lesions in the uterus (25). Furthermore, LAM shares features with another rare disease called benign metastasizing leiomyoma, a less aggressive lung disease where it is well established that uterine leiomyomas metastasize to the lungs (26–29). Together, these data suggest that LAM might originate from Tsc2 null myometrial cells, and that estradiol may then promote metastasis and proliferation of these myometrial cells.

To test this hypothesis, and to create a novel mouse model for both leiomyoma and LAM, we generated a

primarily uterine-specific Tsc2 knockout mouse by crossing mice containing a floxed Tsc2 gene with mice expressing Cre-recombinase driven by the PR promoter. In fact, in these PR-Cre/Tsc2 null mice, we saw significant loss of Tsc2 expression only in the uterus. Knockout of Tsc2 in the uterus resulted in progressive uterine enlargement and myometrial overgrowth starting at 6 to 12 weeks of age, with leiomyoma formation by 24 weeks of age. Uterine and myometrial growth were prevented by treatment with the mTORC1 inhibitor, rapamycin, or by ovariectomy. Repletion of ovariectomized uterine-specific Tsc2 null mice with estradiol, but not progesterone, restored abnormal uterine growth. Finally, we discovered myometrial Tsc2 null tumors in the lungs of 31-week and older Tsc2 uterine-specific knockout females, suggesting that Tsc2 null myometrial cells, and therefore possibly LAM cells, can indeed migrate from the uterus to the lungs.

Materials and Methods

Animals and sample preparation

Mouse studies were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and were approved by the University Committee on Animal Resources at the University of Rochester. Mice were generated by crossing Tsc2-floxed mice (30), with mice expressing Cre recombinase driven by the PR promoter (31). Tail DNA was isolated for genotyping using appropriate primers (30, 31). Animals were killed between 3 and 36 weeks as indicated. Whole blood was collected via cardiac puncture. Serum was isolated and analyzed for hormone levels at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Uteri were removed and weighed, followed by gross dissection and fixation with 10% formalin. For uterine histological analysis sections from paraffin-embedded tissue were stained with either hematoxylin and eosin (H&E) or immunohistochemistry (Immunohistochemistry). The fourth mammary glands were used for whole mount staining using carmine alum (Sigma-Aldrich, St Louis, Missouri). Myometrial thickness was measured in longitudinal uterine sections from at least 3 animals per genotype. Four measurements of the distance between the most outer layer of the myometrium and the endometrium were obtained from each uterine wall and the average of the values was calculated. Lungs were inflated and fixed with 10% formalin.

Western blot

Uteri and lungs were homogenized in RIPA lysis buffer (1×TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, supplemented with PMSF, sodium orthovanadate and protease inhibitor cocktail solutions; Santa Cruz, Santa Cruz, California). Protein lysates were separated on SDS-polyacrylamide gels. Western blots were conducted by using 1:1000 rabbit anti-Tsc2 (Epitomics, Burlingame, California), anti-phospho-S6 (Ser 235/236), anti-S6 and 1:5000 anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling, Danvers, Massachusetts), primary antibodies.

Quantitative real-time PCR

RNA samples were isolated from uterus, ovary, hypothalamus, pituitary, and mammary glands by RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Expression levels of *Tsc2*, *MLANA*, and *GAPDH* mRNA were validated by $\Delta\Delta C_t$ method using Taqman primers (Mm00441991_m1, Mm01195780_m1, and Mm03302249_g1, respectively) and a StepOne Plus PCR machine (Applied Biosystems, Foster City, California).

Immunohistochemistry

Uterine sections from paraffin-embedded tissue (5 μ m) were deparaffinized and rehydrated in a graded alcohol series. Sections were incubated overnight with 1:1000 anti-smooth-muscle- α -actin (Epitomics) or 1:500 anti-PR (Dako, Glostrup, Denmark), 1:4000 anti-ER α , 1:400 antiphospho S6 (Cell Signaling), 1:300 anti-Ki-67 (Thermo Scientific, Rockford, Illinois), and 1:1000 anti-NKX2.1 (Santa Cruz) antibodies (all from rabbits). On the following day, sections were washed in PBS and incubated with 1:200 anti-rabbit secondary antibody (5 μ L/mL; Vector Laboratories, Burlingame, California) for 1 hour at room temperature. Immunoreactivity was detected using the Vectastain Elite ABC kit (Vector Laboratories). Sections were counterstained by hematoxylin, dehydrated, and mounted using cytooseal 60 (Thermo Scientific).

Rapamycin treatment

Rapamycin (LC Laboratories, Woburn, Massachusetts) was dissolved at 20 mg/mL in ethanol and 1 mg/mL solution was prepared with 5% Tween-80 and 5% polyethylene glycol 400 (Sigma-Aldrich) in PBS. Four-week-old uterine-specific *Tsc2* knockout mice and littermate controls were injected with Rapamycin (5 mg/kg, 3 d/wk) ip for 8 weeks. Mice were killed and analyzed as described above.

Letrozole treatment

Letrozole was dissolved in 0.3% hydroxypropyl cellulose (both from Sigma-Aldrich). Four-week-old uterine-specific *Tsc2* knockout mice and littermate controls were injected daily with Letrozole (10 μ g/mouse/d) sc for 8 weeks. Mice were killed and analyzed as described above.

Ovariectomy and rescue

Four-week-old uterine-specific *Tsc2* knockout mice and littermate controls were ovariectomized. Briefly, a dorsal midline incision was made; ovaries were removed, and uterine horns were placed back into the body cavity. The incision was sutured and mice recovered under a heating lamp. Ovariectomized mice were treated with 90-day-release pellet of estradiol (0.5 mg/pellet), progesterone (25 mg/pellet), estradiol + progesterone (Innovative Research of America, Sarasota, Florida), or no pellet for 8 weeks. Mice were killed and analyzed as described above.

Results

Generation of uterine-specific *Tsc2* knockout mice

Global deletion of *Tsc2* in mice leads to early embryonic lethality (30). Therefore, to study the role of *Tsc2* in

the mouse uterus, we generated a primarily uterine-specific *Tsc2* knockout mouse strain. Mice with floxed *Tsc2* (*Tsc2*^{flox/flox}) (30) were crossed with mice expressing Cre recombinase driven by the PR promoter (PR^{Cre/Cre}) (31) to generate heterozygous PR^{Cre/+}/*Tsc2*^{flox/+} mice. These heterozygous mice were then crossed with *Tsc2*^{flox/flox} females or males, after which PR^{Cre/+}/*Tsc2*^{flox/flox} (uterine-specific *Tsc2* knockout) females were identified by PCR (Figure 1A). Littermates that were heterozygotes or that did not express Cre (referred to as wild-type) were used as controls. In the mouse uterus, low-level PR promoter activity occurs in luminal and glandular epithelium (reflecting less than 20% of total uterine volume) after birth. As mice enter puberty (5–6 wk of age), PR promoter activity increases dramatically in the myometrium (31). Accordingly, uteri of prepubertal 3- to 4-week-old PR-Cre/*Tsc2* knockout mice expressed similar levels of *Tsc2* mRNA as heterozygote and wild-type littermates (Figure 1B). In contrast, at 6 (Figure 1C) and 12 (Figure 1D) weeks of age, *Tsc2* mRNA expression in *Tsc2* knockout uteri was significantly reduced. As expected, *Tsc2* protein expression was essentially undetectable in uteri from these knockout mice (Figure 1, C and D). Notably, *Tsc2* mRNA expression in the pituitary, hypothalamus, and ovary (Figure 1E), as well as the mammary gland (Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>), all of which contain low-level PR promoter activity (31), was not significantly reduced in any of the genotypes, demonstrating uterine-specific knockout. In fact, pituitary expression of *Tsc2* mRNA may be slightly elevated in the PR-Cre/*Tsc2* knockout mouse. Finally, no abnormalities or tumors were seen in whole mounts of PR-Cre/*Tsc2* mammary glands (Supplemental Figure 1), again confirming the uterine specificity of our knockout model.

Postpubertal uterine-specific *Tsc2* knockout mice develop myometrial proliferation and leiomyomas

No significant morphological changes or differences in uterine weight were observed among the different genotypes in prepubertal mice (Figure 2A). However, starting at 6 weeks of age, uterine-specific *Tsc2* knockout mice demonstrated a strong trend toward uterine enlargement, with increased endometrial and myometrial proliferation compared with wild-type and heterozygotic littermates (Figure 2B). At 12 weeks of age, the mTORC1/S6 pathway was constitutively active in *Tsc2* knockout uteri, as evidenced by the presence of phosphorylated S6 protein (Figure 2D). Furthermore, uterine size and weight in uterine-specific *Tsc2* knockout mice were significantly larger

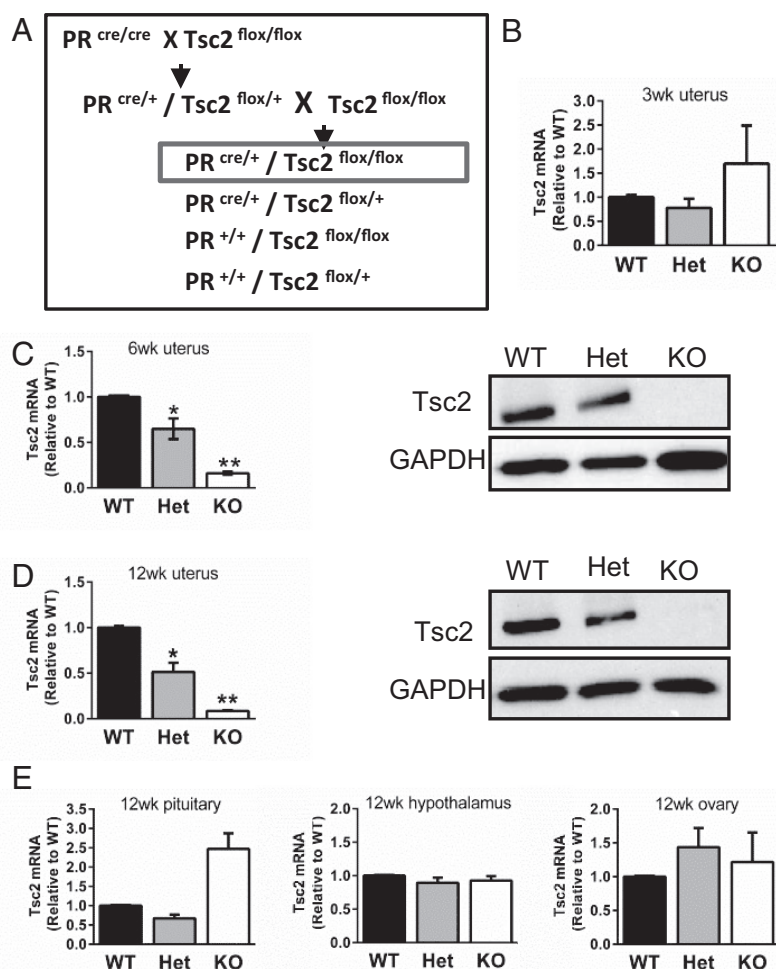


Figure 1. Generation of uterine-specific Tsc2 knockout mice. (A) Schematic of crossing strategy. Uterine Tsc2 mRNA expression in 3- (B), 6- (C), and 12- (D) week-old wild-type (WT), heterozygotic (Het), and uterine-specific Tsc2 knockout (KO) mice were determined using quantitative real-time PCR. Data were normalized to GAPDH and represented relative to WT uterus. Data are shown as mean $\pm$ SEM of at least 3 mice per genotype. *, $P < .05$; **, $P < .0001$ relative to WT by Student t test. Uterine Tsc2 protein by immunoblot is shown for 6- (C) and 12- (D) week-old mice. (E) Levels of Tsc2 mRNA in the pituitary, hypothalamus, and ovary.

compared with heterozygous and wild-type uteri (Figure 2C). Histological analysis demonstrated increased endometrial proliferation, with more endometrial glands and irregular luminal epithelium in uteri of uterine-specific Tsc2 knockout mice (Figure 2C). In addition, histological analysis and measurement of myometrial thickness, along with Ki-67 staining, showed significantly increased myometrial proliferation in uteri of 12-week-old uterine-specific Tsc2 knockout mice relative to wild-type and heterozygotic mice (Figure 2, C, E, and F). In older mice (24 wk of age) Tsc2 knockout uteri were appreciably larger, with continued endometrial overgrowth and internal bleeding (Figure 3A). Notably, for the purposes of this article, we did not focus on the endometrial proliferation, although our results are consistent with PR-Cre-induced phosphatase and tensin homolog (PTEN) knockout animals, where PTEN loss leads to enhanced Akt and

mTORC1 signaling, resulting in endometrial hyperplasia (32, 33). Focusing on the myometrium, we observed marked myometrial overgrowth, with tumors consistent with leiomyomas (Figure 3A). Although wild-type uteri contained a highly organized, distinct myometrial layer, the Tsc2 knockout myometrium was markedly thickened with rough borders and irregular, disoriented cells that stained for α -smooth muscle actin (SMA α) (Figure 3, B–G). In addition, Tsc2 knockout myometrium expressed high levels of phosphorylated S6 protein relative to wild-type myometrium, reflecting Tsc2 loss and mTORC1 activation (Figure 3, H and I). Notably, similar to leiomyomas, uteri from uterine-specific Tsc2 knockout mice expressed ER α and PR (Figure 3, J and K), suggesting that ER and/or PR signaling might play a role in the abnormal myometrial growth. In addition, although *MLANA* mRNA (encodes the melanocytic protein melanoma-antigen-recognized-by-T-cells—a protein known to be up-regulated in LAM) was barely detected in the uteri of 12-week-old wild-type mice, *MLANA* mRNA was markedly elevated in uteri of uterine-specific Tsc2 knockout mice (Figure 3L), confirming yet another similarity between the Tsc2 null uteri and LAM cells. Last, markedly elevated myometrial proliferation was corroborated by

Ki-67 staining at 24 weeks of age (Figure 3M).

Rapamycin prevents abnormal growth of Tsc2 knockout uteri

Rapamycin inhibits mTORC1 and is used as an immunosuppressant to prevent rejection after transplantation. Rapamycin effectively blocks tumor development in the global Tsc2^{+/-} mouse model (34) and reduces tumor size in Eker rats (35). Four-week-old (prepubertal) uterine-specific Tsc2 knockout, heterozygotic, and wild-type mice were treated with rapamycin for 8 weeks. Rapamycin treatment completely prevented the abnormal growth seen in 12-week-old uterine-specific Tsc2 knockout mice, with no differences in morphology, size, and weight relative to wild-type mice (Figure 4, A and B). Confirming that rapamycin effectively inhibited mTORC1, and S6

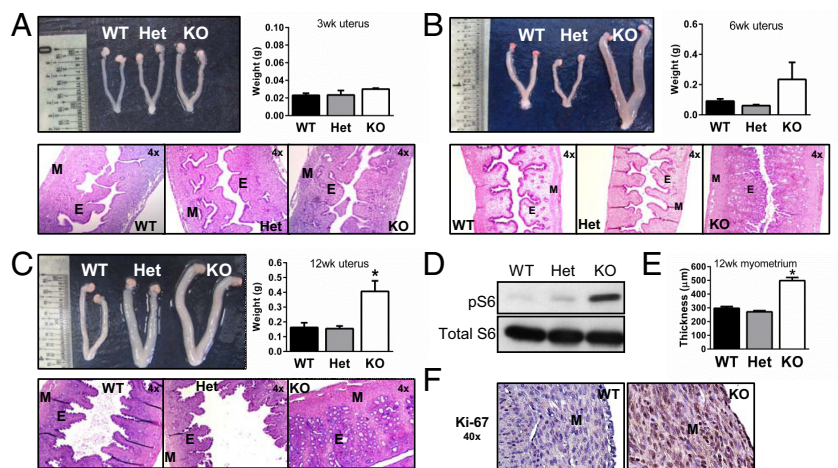


Figure 2. Uteri of uterine-specific Tsc2 KO mice show abnormal growth after puberty. (A) No detectable differences were seen in size (upper left panel), weight (upper right panel), or morphology (lower panel) between uteri of prepubertal 3-week-old WT, Het, and uterine-specific Tsc2 KO mice. At 6 (B) and 12 (C) weeks of age, Tsc2 KO uteri were larger (upper left panels) and heavier (upper right panels), and H&E stains showed increased endometrial (E) and myometrial (M) proliferation with respect to Het and WT uteri (lower panels). (D) Immunoblot demonstrating phosphorylated and total S6 in uteri of 12-week-old WT, Het, or KO mice. (E) Average myometrial thickness of 12-week-old WT, Het, or KO mice. (F) Uterine sections from 12-week-old WT and KO mice stained for Ki-67. Photos are representative of at least 3 mice per genotype. Results portray the mean $\pm$ SEM of at least 4 mice per genotype. *, $P < .05$ relative to WT by Student t test.

phosphorylation was low in Tsc2 knockout uteri (as opposed to constitutive S6 phosphorylation in untreated Tsc2 knockout uteri) (Figure 4C). Thus, as expected,

importance of steroid signaling in the abnormal uterine growth of uterine-specific Tsc2 knockout mice. Ovariectomies

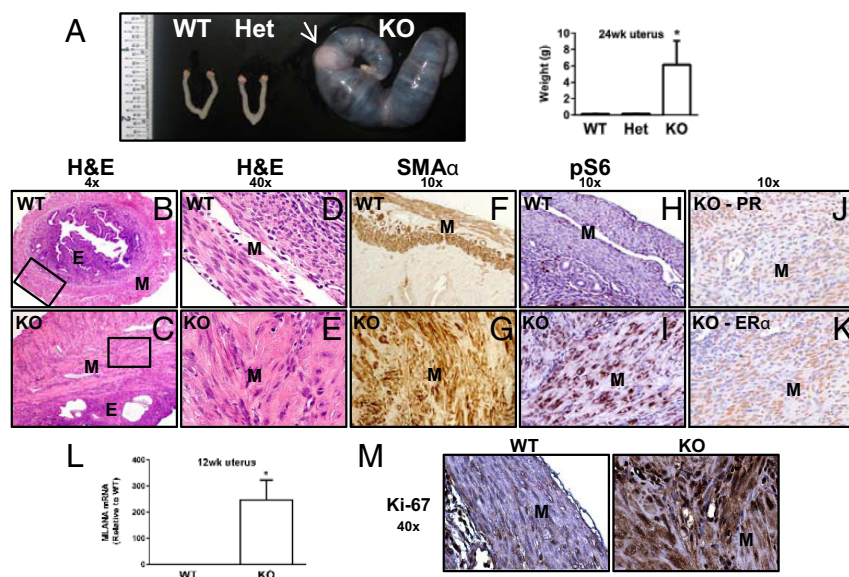


Figure 3. Uteri from 24-week-old uterine-specific Tsc2 KO mice contain leiomyomas and share features with LAM. (A) Uteri of 24-week-old WT, Het, and uterine-specific Tsc2 KO mice. Tsc2 KO uteri were larger, often blood-filled, and contained leiomyomas (arrow). (B–K) Uterine sections from 24-week-old WT and KO mice stained with H&E (B–E; M, myometrium, E, endometrium), anti-SMA α (F, G), antiphosphorylated S6 (H, I), anti-PR (J), and anti-ER α (K). (L) Uterine *MLANA* mRNA expression in 12-week-old WT and KO mice were determined using quantitative real-time PCR. Data were normalized to GAPDH and represented relative to WT uterus. Data are shown as mean $\pm$ SEM of at least 3 mice per genotype. (M) Uterine sections from 24-week-old WT and KO mice stained for Ki-67. Photos are representative of at least 3 mice per genotype. *, $P < .05$ relative to WT by Student t test.

mTORC1 activity appears critical for the myometrial overgrowth in uterine-specific Tsc2 knockout mice.

Ovariectomy prevents abnormal growth of Tsc2 knockout uteri and estrogen stimulates myometrial proliferation

Uterine leiomyomas and LAM usually develop during the reproductive years in women. In contrast, men with Tsc mutations rarely develop LAM (3). Clinical and biochemical studies report an important role for estrogen, and possibly progesterone, in the promotion of leiomyoma growth (36). Furthermore, like leiomyomas, lung LAM cells express ERs and PRs (2, 37) and appear to proliferate in response to estradiol (38). Because myometrial tumors from Tsc2 knockout uteri express PR and ER α (Figure 3, J and K), we examined the importance of steroid signaling in the abnormal uterine growth of uterine-specific Tsc2 knockout mice. Ovariectomies (OVX) were performed in 4-week-old uterine-specific Tsc2 knockout and wild-type mice. After 8 more weeks, uteri were examined. In the absence of ovarian steroids, uteri from uterine-specific Tsc2 knockout mice looked identical to those from wild-type mice, with no evidence of myometrial or endometrial proliferation by gross examination and weight (Figure 5A). Thus, ovarian steroids appear necessary for abnormal uterine growth. To determine which ovarian steroid regulated myometrial growth in uterine-specific Tsc2 knockout mice, pellets containing estradiol (5.5 μ g/d/mouse), progesterone (280 μ g/d/mouse), or both, were applied to OVX mice. Mice were treated for 8 weeks, after which they needed to be killed due to known estradiol-implant effects on the bladder that lead to urinary retention (39). After 8 weeks of steroid treatment, excess myometrial growth was restored to OVX uterine-specific

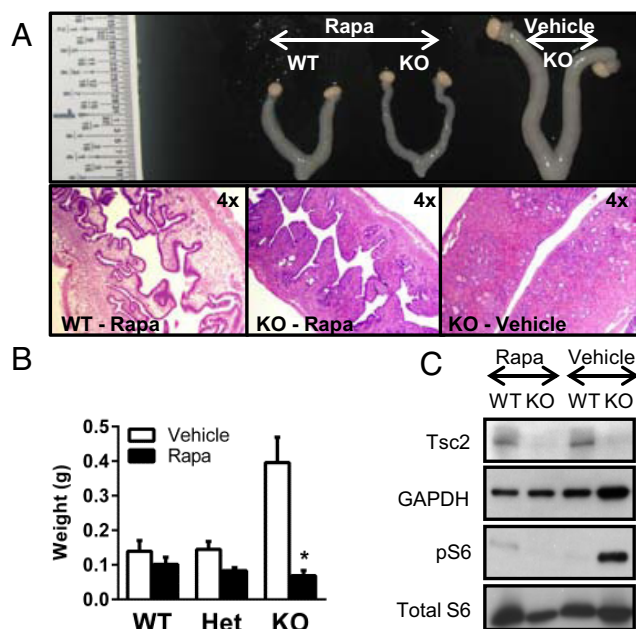


Figure 4. Rapamycin prevents abnormal growth of Tsc2 KO uteri. Four-week-old WT, Het, and uterine-specific Tsc2 KO mice were injected with rapamycin (Rapa, 5 mg/kg ip, 3 d/wk for 8 wk). (A) At 12 weeks of age, rapamycin prevented uterine overgrowth of KO mice as seen by size (upper panel), morphology (lower panel), and (B) weight. Photos are representative from 3 experiments with identical results. Results portray the mean $\pm$ SEM of 3 mice per group per treatment. *, $P < .05$ relative to untreated uterine-specific Tsc2 KO by Student t test. (C) To confirm inhibition of mTORC1, levels of uterine Tsc2, phosphorylated S6, total S6, and GAPDH were measured by immunoblot.

Tsc2 knockout mice by estradiol alone, as evidenced by uterine size and weight, as well as by thickened myometrial growth (Figure 5A). Uterine size and weight were also increased in wild-type OVX mice exposed to estradiol; however, these increases were primarily due to increased endometrial proliferation, with normal myometrial architecture (Figure 5A). In contrast, progesterone alone had no significant effect on uterine morphology or weight in either wild-type or uterine-specific Tsc2 knockout OVX mice, suggesting that progesterone is not a major regulator of myometrial growth in the absence of Tsc2 expression. Finally, treatment of OVX wild-type mice with estradiol plus progesterone decreased proliferation of epithelial and glandular tissue, reflected by the slightly decreased size and uterine weight relative to OVX wild-type treated with E2 alone (Figure 5A). This observation is consistent with progesterone's known opposing effects on estradiol-mediated endometrial proliferation (40). However, combined estradiol and progesterone treatment of OVX uterine-specific Tsc2 knockout mice increased uterine size and weight, with continued endometrial and myometrial growth (and often unilateral fluid buildup in one uterine horn, although this observation

was variable), suggesting that, in the setting of Tsc2 loss, progesterone does not as effectively oppose estrogen action in the uterus. Intriguingly, although Tsc2 expression remained low in the uteri of OVX uterine-specific Tsc2 knockout mice, S6 phosphorylation was no longer significant (Figure 5B), suggesting that mTORC1 activity might require steroid signaling, even in the absence of Tsc2. In fact, estradiol, but not progesterone, treatment of OVX uterine-specific Tsc2 knockout mice rescued mTORC1 activity, as evidenced by the statistically significant increase in total uterine S6 phosphorylation by Western blot (Figure 5B), and an increase in myometrial S6 phosphorylation by immunohistochemistry (Figure 5C). Similarly, estradiol plus progesterone enhanced myometrial S6 phosphorylation by immunohistochemistry (Figure 5C) and total uterine S6 phosphorylation by Western blot (Figure 5B), although the latter was not quite statistically significant in this series of 3 uteri.

Aromatase inhibition reduces myometrial proliferation in Tsc2 knockout uteri

Estrogens are generated from androgens by aromatase. Aromatase inhibitors, such as letrozole, are used in breast cancer patients to block estrogen production and subsequent ER signaling. Because estradiol regulated myometrial growth in our mouse model, we predicted that aromatase inhibition would attenuate growth in 12-week-old uterine-specific Tsc2 knockout mice. Starting at 4 weeks, uterine-specific Tsc2 knockout and wild-type mice were treated with letrozole for 8 weeks. As expected, uterine size and weight were significantly lower in letrozole-treated vs untreated uterine-specific Tsc2 knockout mice (Figure 6, A and B), confirming that estradiol is required for proliferation in the absence of Tsc2. Importantly, S6 phosphorylation in the uteri of letrozole-treated uterine-specific Tsc2 knockout was markedly reduced relative to intact uterine-specific Tsc2 knockout mice, with levels no different than wild-type uteri (Figure 6D), again suggesting that estradiol is required for mTORC1 activity, even in the absence of Tsc2. Finally, although estradiol levels were relatively low in all mice examined, serum LH levels were elevated in letrozole-treated wild-type and knockout mice (Figure 6C), confirming that chronic estradiol production was reduced by letrozole.

Metastatic myometrial lesions in the lungs of uterine-specific Tsc2 knockout mice

As mentioned, this mouse model was created to determine whether myometrial, or possibly LAM, lung lesions could be derived from the uterus. Interestingly, we found that most uterine-specific Tsc2 knockout females that

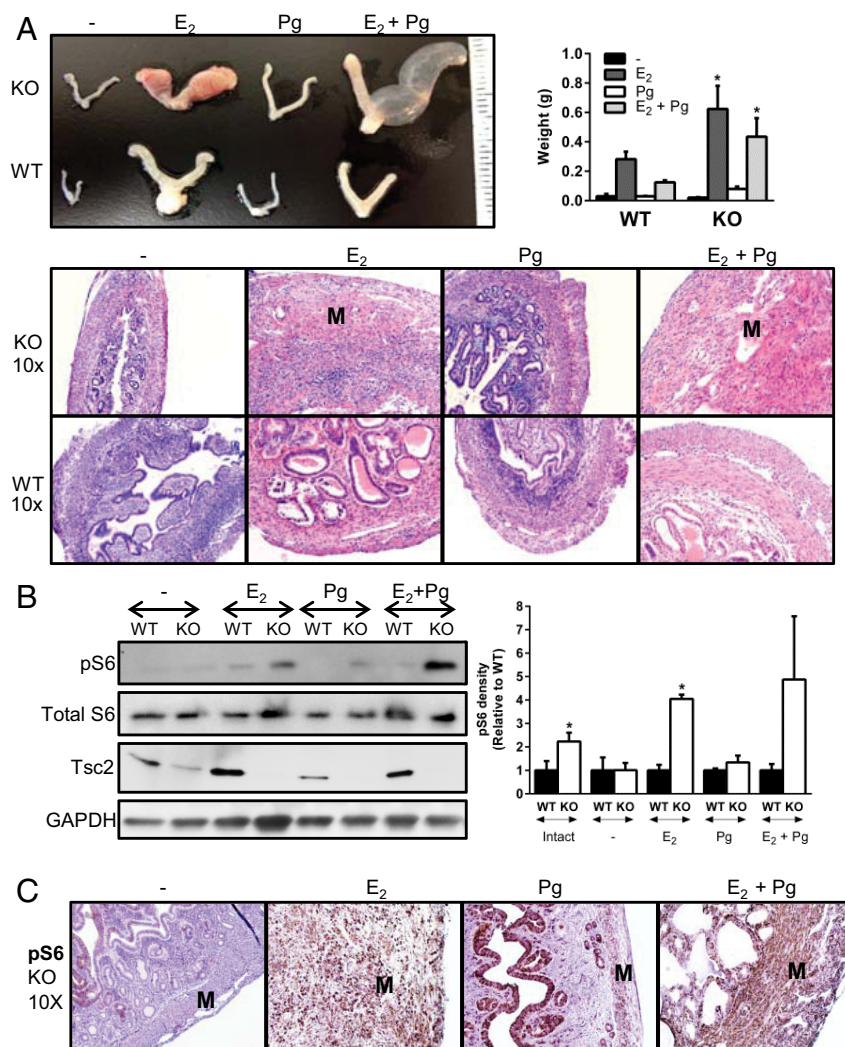


Figure 5. Ovariectomy prevents abnormal growth of Tsc2 KO uteri. Four-week-old WT and uterine-specific Tsc2 KO mice were OVX and treated with pellets of estradiol (E₂, 0.5 mg/pellet), progesterone (Pg, 25 mg/pellet), both, or neither for 8 weeks. (A) At 12 weeks of age, uterine gross morphology (upper left panel), weight (upper right panel), and microscopic morphology (lower panel) were analyzed. M, myometrium. Results portray the mean $\pm$ SEM of at least 3 mice per group per treatment. *, $P < .05$ relative to untreated OVX uterine-specific Tsc2 KO by Student t test. (B) Levels of Tsc2, GAPDH, and phosphorylated and total S6 were examined by immunoblot in OVX WT and KO uteri (left). Treatments are indicated. Quantification by densitometry of phosphorylated S6/total S6 is shown on the right. The y-axis is pS6/tS6 of KO relative to WT uteri for each condition. Intact represents mice that never received OVX. *, $P < .05$ relative to WT pairs by Student t test, $n \geq 3$ for each condition. (C) Immunohistochemistry for phosphorylated S6 was performed using sections from the indicated Tsc2 KO uteri.

reached 31 weeks and up (6 of 8) contained myometrial tumors in the lungs (Figure 7A). In contrast, no mice age 24 weeks or lower had any detectable lung tumors. Western blot analysis of uterine and lung tumors from a representative 34-week-old Tsc2 knockout mouse demonstrated extremely low Tsc2 protein expression (Figure 7B). Tsc2 expression was normal in unaffected lungs of these mice. The very faint Tsc2 protein expression in the KO lanes most likely reflects the heterogeneity of the tumors and the difficulty in specifically manually dissecting only tumor tissue. Moreover, the lung tumors expressed

PR, ER α , SMA α , and phosphorylated-S6 positive (all seen in the uterine myometrial lesions), but not the lung marker, Nkx2.1 (Figure 7C). Finally, when examining lungs in the knockout animals for early tumor formation, we find tumors originating from the lymphatics, as evidenced by LYVE1-positive staining of lymphatic endothelial cells surrounding the tumor (Supplemental Figure 2). These data strongly suggest that the lung tumors were metastatic from the uterus, perhaps entering the lungs via the lymphatics (as is seen in LAM), confirming that LAM-like myometrial lung tumors could indeed be derived from uterus.

Discussion

Although progress has been made in diagnosing and treating LAM, a cure remains elusive. One difficulty in finding a cure is that both the origin of LAM cells and the mechanism of the sexual dimorphism in LAM are still not well understood. Furthermore, because of the lack of amenable animal models, the relative importance of mTORC1 vs estrogen signaling in LAM is not well characterized. Here we started with the hypothesis that lung LAM cells, which resemble uterine leiomyoma cells, might be derived from uterine myometrial cells containing mutations in 1 of the 2 TSC alleles. We further proposed that the combination of estrogen and de-repressed mTORC1 signaling might be required for LAM proliferation. To test our hypothesis, we specifically knocked out Tsc2 in uterine cells. Nearly 100% of the uterine-specific Tsc2 knockout mice developed myometrial thickening by 12 weeks of age and uterine leiomyomas by 24 weeks of age. These uterine tumors share many features of leiomyoma and LAM, including PR, ER α , SMA α , and phosphorylated S6 protein expression. These uteri also express elevated *MLANA* mRNA levels, a melanocyte differentiation marker commonly seen in LAM cells. Finally, 75% of mice older than 31

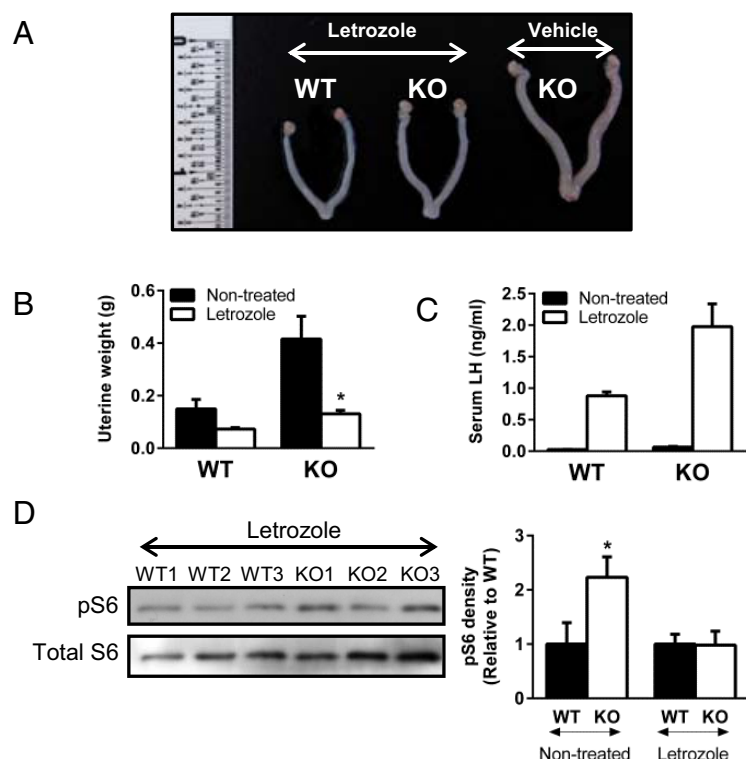


Figure 6. Letrozole prevents abnormal growth of Tsc2 KO uteri. Four-week-old WT and uterine-specific Tsc2 KO mice were injected with letrozole (10 μ g/mouse daily) sc for 8 weeks. Uterine gross morphology (A) and weights (B) in 12-week-old KO treated with letrozole are similar to those of WT. (C) LH levels in serum were analyzed by sandwich immunoradiometric assay. Results portray the mean $\pm$ SEM of 3 mice per treatment. *, $P < .05$ relative to untreated uterine-specific Tsc2 KO by Student t test. (D) Levels of phosphorylated and total S6 were examined by immunoblot, with 3 representative samples from letrozole-treated WT and KO mice shown on the left. Quantification by densitometry of phosphorylated S6/total S6 for WT and KO mice both treated and not treated with letrozole is shown on the right. The y-axis is pS6/tS6 of letrozole-treated vs untreated uteri for WT and KO mice. *, $P < .05$ relative to untreated WT by Student t test, $n \geq 3$ for each condition.

weeks of age developed lung myometrial lesions. Although the global lung pathology is not identical to LAM, the individual tumors share some pathologic features similar to LAM, including PR, ER α , SMA α , and phosphorylated-S6 protein expression. Thus, this mouse strain may serve as a useful model for both leiomyoma and LAM.

The first key physiologic feature shared between Tsc2 null mouse myometrial cells and LAM is the dependence on mTORC1 signaling for proliferation. As seen in LAM and other models (41, 42), rapamycin completely blocked myometrial overgrowth in our model (Figure 4), even in the presence of ovarian steroids. This observation demonstrates that mTORC1 signaling is necessary for the proliferation of Tsc2-null mouse myometrial cells. Similarly, LAM progression appears to require mTORC1 signaling, as rapamycin analogs stabilize lung function and reduce symptoms in LAM patients (41, 43, 44). Importantly, however, lung function again declines after discontinuation of therapy (45–47), suggesting that mTORC1

inhibition only slows growth but does not destroy LAM cells. Thus, the need for additional therapeutic targets in LAM remains.

Another potential therapeutic target, and the second feature shared between Tsc2 null myometrial cells and LAM cells, is the dependence on estrogen. We found that elimination of steroid signaling by ovariectomy abrogated myometrial overgrowth in uterine-specific Tsc2 knockout mice (Figure 5). This result demonstrates the critical role of steroid signaling for proliferation, even in the absence of Tsc2. Importantly, although elimination of ovarian steroidogenesis blocked myometrial proliferation in Tsc2 knockout uteri, growth was restored by the addition of estradiol but not progesterone (Figure 5A). This observation confirms that estradiol is the major ovarian steroid regulating myometrial cell proliferation and is consistent with previous work supporting the role of estrogen in the promotion of both leiomyoma and LAM. These earlier studies demonstrated that LAM and leiomyoma cells expressed ER α and progressed most rapidly in women during pregnancy and while taking oral contraceptives, when estrogen levels are highest (48–54). Furthermore, in vitro studies demonstrated estrogen-induced increased proliferation of primary human LAM cells as well as ELT3 Tsc2-null Eker rat leiomyoma cells (48, 55). In fact, estradiol not only regulated ELT3 cell proliferation, but also promoted metastatic spread of ELT3 cells in xenograft models (19). Notably, ER α promotes cell growth via both genomic transcriptional responses as well as non-genomic cross-talk between ER α and growth factor receptors (56). Thus, inhibition of genomic and non-genomic ER α signaling might have greater potential in battling LAM than blockade of any one individual pathway. Importantly, we found that ovariectomized or letrozole-treated uterine-specific Tsc2 knockout mice demonstrated markedly reduced uterine/myometrial mTORC1 signaling relative to untreated knockout mice, as reflected by decreased phosphorylation of S6 (Figure 5, B and C, Figure 6D). Furthermore, estradiol or estradiol/progesterone treatment of ovariectomized uterine-specific Tsc2 knockout mice rescued mTORC1 signaling in the myometrium. Together, these observations suggest that estradiol is required for complete activation of mTORC1/S6 signaling in myometrial cells, even in the absence of Tsc2 expression, underscoring the importance of estradiol for myometrial and possibly LAM proliferation. In fact, the

inhibition only slows growth but does not destroy LAM cells. Thus, the need for additional therapeutic targets in LAM remains.

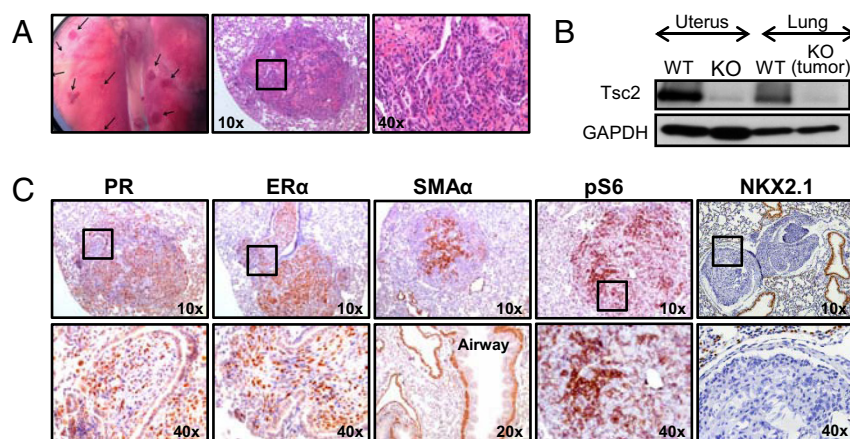


Figure 7. Myometrial tumors in the lungs of uterine-specific *Tsc2* KO mice. (A) Lung tumors (arrows) were found in 75% (6/8) of 31 week and older uterine-specific *Tsc2* KO mice. H&E staining showed leiomyoma-like appearance. (B) Immunoblots of uterine and a lung tumor from a representative mouse demonstrated lack of *Tsc2* protein. (C) Immunohistochemical staining demonstrated that the lung tumors were PR, ER α , SMA α , and phosphorylated S6 positive, but negative for the lung marker *Nkx2.1*.

need for estradiol signaling to keep mTORC1 signaling elevated in cells lacking *Tsc2* suggests that anti-estrogen treatment might be an effective means of suppressing proliferative signals in LAM.

An additional steroid that might contribute to the gender specificity of LAM is progesterone. The role of progesterone in leiomyomas and LAM is not well understood. Some studies have shown that progestins might promote leiomyoma growth and survival and therefore might be contraindicated in patients with leiomyomas or LAM (57, 58). Accordingly, although early studies suggested that progestins might improve symptoms in LAM patients (59, 60), a later study demonstrated no difference and possibly deterioration of LAM symptoms on treatment with progestin (21). In our model, progesterone did not promote myometrial proliferation on its own. Furthermore, the full opposing effect of progesterone on es-

tradiol-mediated effects was not present in *Tsc2* knockout uteri, as myometrial cells proliferated equally well in response to estradiol alone or estradiol plus progesterone (Figure 5). Thus, progesterone does not appear to be a major regulator of *Tsc2* null myometrial growth.

The final feature shared between our uterine-specific *Tsc2* knockout mouse and LAM is the presence of lung tumors. Genetic data suggest that LAM might originate from outside the lungs (8). Moreover, case studies have described recurrence of LAM within lungs after lung transplantation, again suggesting a metastatic origin of LAM (12, 61). Because leiomyoma cells share many

features with LAM cells, and because leiomyomas are found exclusively in women, the uterus is an intriguing potential source of LAM cells. Consistent with this possibility, a recent study found uterine leiomyomas in every LAM patient studied, with microscopic uterine LAM lesions in all but one patient (25). Furthermore, in the less aggressive lung disease, benign metastasizing leiomyoma, myometrial cells metastasize to the lungs (62, 63). We therefore propose that loss of *Tsc2* in myometrial cells might result in a more aggressive form of benign metastasizing leiomyoma that, in the presence of estradiol, leads to proliferating myometrial tumors and possibly LAM. In fact, in 75% of mice that survived to 31 weeks of age, we found myometrial lung tumors that were *Tsc2* and *Nkx2.1* negative, but expressed pS6, PR, ER α , and SMA α (Figure 7). Furthermore, some of these tumors can be seen emerging from the lymphatics (Supplemental Figure 2), which is thought to be a common mode of lung entry for LAM cells. All of these data are consistent with metastatic leiomyoma as well as LAM and suggest that uterine myometrial *Tsc2* null cells can indeed travel to the lungs. Although we do not see significant loss of *Tsc2* expression in any other tissue, nor do we see tumors in tissues other than the uterus and lungs (and the lung tumors are present less frequently and only later in life), it is still possible that the lung lesions described here were derived from other small pockets of PR-positive tissues, or even from PR-positive smooth muscle cells within the lungs. To address this possibility, studies are currently being performed where uteri (but not ovaries) are removed from uterine-specific *Tsc2* knockout mice before puberty and lungs are examined at 31 weeks.

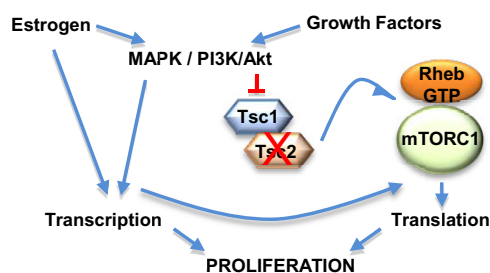


Figure 8. Schematic model for potential estrogen/mTORC1 crosstalk in LAM. Estrogen promotes proliferation both transcriptionally and through nongenomic MAPK/PI3K/Akt signaling. This nongenomic kinase signaling can both modulate ER-mediated transcription and regulate mTORC1 signaling through both *Tsc1/2*-dependent and possibly independent mechanisms. This complex of mTORC1 inhibitor proteins likely includes TBC1D7, as well as *Tsc1* and *Tsc2*. mTORC1 regulates proliferation through many pathways, including enhancement of translation.

Of note, alternative rodent models for LAM have provided important information. Perhaps the best known is the Eker rat, which contains a heterozygotic inactivating mutation in the *Tsc2* locus. Eker rats develop tumors in multiple locations within the body, including the uterine myometrium (64). In fact, this model was the first to demonstrate that *Tsc2* deletion in the myometrium can lead to leiomyoma formation. However, Eker rats did not develop myometrial lung lesions. Furthermore, because leiomyoma formation requires inactivation of the second *Tsc2* allele, only 65% of females develop leiomyomas, and the time to leiomyoma formation is over 1 year. In our uterine-specific *Tsc2* knockout mouse model, myometrial cell hyperproliferation occurs in nearly 100% of the mice by 12 weeks and leiomyoma formation by 24 weeks, potentially making this model more amenable for studying leiomyoma and LAM. In addition to the rats, both *Tsc1* and *Tsc2* global knockout mice have been made (65–68). Complete knockout mice are embryonically lethal, but heterozygotes develop tumors that, as in Eker rats, contain inactivation of the second allele. However, unlike the model presented here, none of these mice develop uterine leiomyomas or lung myometrial lesions. Using an alternative approach, labeled *Tsc2* null angiomylipoma cells injected into mice intratracheally developed histopathologic changes within the lung that were consistent with LAM as well as dissemination of *Tsc2* null cells throughout the lymphatics, thus creating a novel model for examining LAM progression (69). In addition, a recent mouse model has been introduced whereby *Tsc2* null mouse kidney cells were injected into the flanks of nude mice; flank tumors were removed and grown in vitro, and cultured cells were injected into the tail veins of nude mice (70). Injected nude mice then developed lung lesions consistent with pulmonary LAM. Like the intratracheal system, this powerful model holds great promise, but both involve using immunocompromised mice and do not address the potential uterine origin of the LAM cell.

In summary, mice with conditional knockout of *Tsc2* in the uterus provide a new model for both leiomyoma and possibly LAM. Myometrial tumors in the uteri and lungs share a number of key features with LAM, and both estrogen and mTORC1 signaling are necessary for growth (Figure 8). Furthermore, although not completely identical to overall LAM lung pathology (for example, the lungs in these mice did not develop the cysts seen in LAM patients), the presence of myometrial tumors that share many features of LAM within the lungs of uterine-specific *Tsc2* knockout mice suggests that LAM cells might metastasize from the uterus. This novel model may prove useful in understanding the pathophysiology of leiomyoma and LAM, identifying cellular events that allow

LAM cells to proliferate and travel to the lungs, and developing novel therapeutic strategies for the treatment of LAM.

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