Estrogen maintains myometrial tumors in a lymphangioleiomyomatosis model

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Abstract

Lymphangioleiomyomatosis (LAM) is a rare disease in women. Patients with LAM develop metastatic smooth-muscle cell adenomas within the lungs, resulting in reduced pulmonary function. LAM cells contain mutations in tuberous sclerosis genes (*TSC1* or *TSC2*), leading to up-regulation of mTORC1 activity and elevated proliferation. The origin of LAM cells remains unknown; however, inactivation of *Tsc2* gene in the mouse uterus resulted in myometrial tumors exhibiting LAM features, and approximately 50% of animals developed metastatic myometrial lung tumors. This suggests that LAM tumors might originate from the uterine myometrium, possibly explaining the overwhelming prevalence of LAM in female. Here, we demonstrate that mouse *Tsc2*-null myometrial tumors exhibit nearly all the features of LAM, including mTORC1/S6K activation, as well as expression of melanocytic markers and matrix metalloproteinases (MMPs). Estrogen ablation reduces S6K signaling and results in *Tsc2*-null myometrial tumor regression. Thus, even without *TSC2*, estradiol is required to maintain tumors and mTORC1/S6K signaling. Additionally, we find that MMP-2 and -9, as well as neutrophil elastase (NE), are overexpressed in *Tsc2*-null myometrial tumors in an estrogen-dependent fashion. *In vivo* fluorescent imaging using MMP- or NE-sensitive optical biomarkers confirms that protease activity is specific to myometrial tumors. Similar to LAM cells, uterine *Tsc2*-null myometrial cells also overexpress melanocytic markers in an estrogen-dependent fashion. Finally, we identify glycoprotein NMB (GPNMB) as a melanocytic marker up-regulated in *Tsc2*-null mouse uteri and human LAM samples. Our data highlight the potential importance of estradiol in LAM cells, suggesting that anti-estrogen therapy may be a treatment modality. Furthermore, proteases and GPNMB might be useful LAM biomarkers.

Key Words

- ▶ lymphangioleiomyomatosis
- ▶ uterus
- ▶ estrogen
- ▶ mTOR
- ▶ metalloproteinase
- ▶ GPNMB

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Introduction

Lymphangioleiomyomatosis (LAM) is a devastating rare lung disease exclusively seen in women (Kelly & Moss 2001). LAM patients develop small, diffuse smooth-muscle (SM) cell tumors within the lungs that have lost expression of functional tumor suppressor protein tuberous sclerosis 1 or 2 (*TSC1* or *TSC2*) due to

inactivating mutations (Smolarek *et al.* 1998, Carsillo *et al.* 2000), thus leading to constitutive mTOR complex 1 (mTORC1) activation, elevated proliferative signals by p70S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Kristof 2010, Zoncu *et al.* 2011), and ultimately increased cell growth

(Goncharova *et al.* 2002, Hayashi & Proud 2007). The mTORC1 inhibitor rapamycin (Sehgal 1998) effectively reduces tumor growth of *Tsc2*-null mouse embryo fibroblast allograft cells (Lee *et al.* 2005), increases cell death of *Tsc2*-null tumors in Eker rats (Kenerson *et al.* 2005), and prevents abnormal growth of *Tsc2*-null mouse uteri (Prizant *et al.* 2013). Furthermore, rapamycin stabilizes lung function in LAM patients (Taveira-DaSilva *et al.* 2011, Ando *et al.* 2013) and recently became the first FDA-approved drug for the treatment of LAM. However, rapamycin is not effective in and is not tolerated by everybody, and decline in lung function resumes when the drug should be stopped (McCormack *et al.* 2011). Therefore, alternative treatment options for LAM are needed.

In addition to the *TSC1* or *TSC2* mutations, LAM cells typically express melanocytic markers. One such marker, gp100 (PMEL), is often up-regulated in LAM cells (Kuhnen *et al.* 2001). PMEL is the target for the HMB-45 antibody; thus, HMB-45 staining by immunohistochemistry (IHC) is often used to diagnose LAM in pathology samples. Additional melanocytic markers are also present in LAM cells (Klarquist *et al.* 2009); however, their physiological significance remains unknown.

Another conundrum of LAM concerns the origin of the lung tumors. Some patients develop progressive lung failure resulting in lung transplantation; however, LAM tumors often recur in transplanted lungs (Bittmann *et al.* 2003, Karbowniczek *et al.* 2003), suggesting a metastatic nature of LAM. Accordingly, proteins associated with metastasis (e.g., matrix metalloproteases (MMPs)) are expressed in LAM tumors (Pacheco-Rodriguez & Moss 2010, Ruiz de Garibay *et al.* 2015). LAM cells express MMP-2 (Hayashi *et al.* 1997, Matsui *et al.* 2000, Chang *et al.* 2012), and MMP-2 activation increased invasiveness of LAM cells in culture (Glassberg *et al.* 2008). Furthermore, MMP-9 levels are elevated in the serum and urine of LAM patients (Odajima *et al.* 2009, Pimenta *et al.* 2011).

To unify the female sexual dimorphism, metastatic nature, and SM cell phenotype of LAM, we proposed that LAM cells might originate from uterine myometrial cells containing mutated *TSC* genes. LAM cells and uterine myometrial tumors (leiomyomas) share many features, including similar SM cell types, expression of estrogen and progesterone receptors (ERs and PRs) (Benassayag *et al.* 1999), and growth sensitivity to estrogens (Maruo *et al.* 2004). In fact, a study on the uteri of ten LAM patients found that nine contained microscopic LAM lesions (Hayashi *et al.* 2011).

To test our hypothesis, and to create a mouse model for LAM, we knocked out *Tsc2* expression in the uterus

(Prizant *et al.* 2013). One hundred percentage of uterine-specific *Tsc2*-null mice developed leiomyomas with characteristics of human LAM, including expression of ER α , PR, SM-actin (SMA), and the melanocytic marker MLANA. Although they do not develop cystic lung disease, approximately 50% of female mice developed myometrial lung tumors after 30 weeks, demonstrating that *Tsc2*-null myometrial tumors indeed can metastasize to the lungs (Prizant *et al.* 2013). Pre-pubertal elimination of sex steroid production prevented abnormal myometrial growth, and re-introduction of estrogen, but not progesterone, rescued the proliferative phenotype (Prizant *et al.* 2013), indicating the importance of estrogen in the growth of these LAM-like myometrial lesions. However, it is not known whether estrogen deprivation reverses existing *Tsc2*-null tumors, and further characterization of this LAM model is still required.

Here, we showed that anti-estrogen therapy reversed myometrial overgrowth in *Tsc2*-null mouse uteri. Anti-estrogen treatment also reduced S6 phosphorylation in *Tsc2*-null mouse uteri, suggesting that direct effects of estradiol on mTORC1 or S6K are required to maintain their activity and to regulate proliferation. RNAseq and other studies on *Tsc2*-null mouse uteri revealed increased MMP expression and activity in *Tsc2*-null leiomyomas, as well as increased expression of melanocytic markers. Similar to tumor progression and S6K activity, expression of these markers required estradiol, further emphasizing the importance of estrogen in *Tsc2*-null myometrial tumors, and possibly LAM. Finally, we showed that a new cell marker, glycoprotein NMB (GPNMB), is highly expressed in *Tsc2*-null mouse uteri, metastasizing lung tumors, and human LAM.

Materials and methods

Animal and human tissue preparation

Mouse studies were performed in accordance with the Care and Use of Laboratory Animals guidelines and approved by the University Committee on Animal Resources at the University of Rochester. Uterine-specific *Tsc2*-null mice were generated by crossing *Tsc2*-floxed mice with mice expressing *Cre* recombinase driven by the *Pr* (*Pgr*) promoter (Prizant *et al.* 2013). Animals were killed between 12 and 30 weeks of age. Uteri were removed, weighed, dissected, and fixed with 10% formalin. De-identified human tissues were from Thomas Colby (Mayo Clinic, AZ, USA) and were handled in accordance with the University Research Subjects Review Board. For histology, sections were stained with hematoxylin and eosin (H&E) or IHC.

In vivo treatments

Eighteen-week old uterine-specific *Tsc2*-null mice and littermate controls were oophorectomized or injected with rapamycin (Rapamune, LC Laboratories, Woburn, MA, USA) intraperitoneally (5 mg/kg, 3 days/week) or letrozole (Femera, Sigma-Aldrich) subcutaneously (10 µg/mouse/day) for 10–12 weeks. Rapamycin and letrozole were dissolved, as described in [Prizant et al. \(2013\)](#). Mice were killed at 30 weeks and uteri were analyzed. For long-term estradiol stimulation, four-week-old oophorectomized mice were treated with or without 90-day-release pellets of 17β-estradiol (0.5 mg/pellet; Innovative Research of America, Sarasota, FL, USA) for 8 weeks. For short-term estradiol stimulation, 12-week-old mice were injected with 1 µg 17β-estradiol (Sigma-Aldrich) and uteri were analyzed after 18 h. Myometrial thickness was measured as described in [Prizant et al. \(2013\)](#).

Cell cultures and reagents

ELT3 cells (Cheryl Walker, Texas A&M Health Science Center) were derived from a primary rat leiomyoma tumor, and 621-101 cells were derived from a primary AML tumor from LAM patient (Elizabeth Henske, Brigham and Women's Hospital). Cells were used within 3 weeks of thawing and maintained in DF8 medium ([Howe et al. 1995](#)). There is no genetic baseline with which these cells could be compared; however, there were early passages and phenotype was verified by expression of ER, PR, melanocytic markers, and estradiol-mediated up-regulation of PR. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were stimulated with 10 nM 17β-estradiol or 20 nM rapamycin. Cells were maintained for 24 h in DF8 medium containing 1% bovine serum albumin and 5% charcoal-stripped fetal bovine serum before experiments.

Immunoblotting and antibodies

Uteri and ELT3 cells were homogenized in RIPA (Santa Cruz, CA, USA). Western blots used 1:1000 rabbit polyclonal anti-phospho-S6 (Ser 235/236), anti-S6, anti-phospho-4EBP1 (Thr37/46), anti-4EBP1, anti-phospho-p44/42-ERK1/2 (T202/Y204), p44/42-ERK1/2, and 1:5000 anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; Cell Signaling) antibodies. IHC was performed, as described in [Prizant et al. \(2013\)](#). Sections were incubated with 1:1000 rabbit anti-SMA (Epitomics, Burlingame, CA,

USA), 1:400 anti-phospho-S6 (Ser 235/236), 1:100 anti-MMP-9, 1:300 anti-MITF, 1:500 anti-GPNMB (Sigma-Aldrich, for mouse), 1:100 anti-GPNMB (R&D Systems, for human) ([Hoashi et al. 2010](#), [Rose et al. 2010b](#)), or 1:50 anti-HMB-45 (Dako) primary antibody. For immunofluorescence, sections were incubated with 1:200 fluorescein-conjugated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Nuclei were labeled using 1 mg/mL Hoechst-33258 (Invitrogen). For immunofluorescence, cells were grown in poly-D-lysine pre-coated glass bottom dishes (MatTech Corporation, Ashland, MA, USA), fixed in 4% paraformaldehyde, and stained with 1:250 anti-MITF or 1:300 anti-GPNMB (Sigma-Aldrich) primary antibody.

RNA sequencing (RNAseq)

Total RNA was isolated using the RNeasy Plus Kit (Qiagen). RNAseq was performed by the UR Genomics Research Center. Illumina compatible library construction was performed using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Amplified libraries were hybridized to the Illumina single-end flow cell and amplified using the cBot (Illumina) at a concentration of 8 pM per lane. Single-end reads of 100 nt were generated per sample and aligned to the mouse genome.

Real-time quantitative PCR (qPCR)

RNA was isolated from wild-type and uterine-specific *Tsc2*-null mouse uteri, ELT3 cells, and 621-101 cells using E.Z.N.A kit (Omega, Norcross, GA, USA). mRNAs levels were normalized to GAPDH and validated by ΔΔCt method using inventoried TaqMan primers (Applied Biosystems) ([supplemental Table 1](#), see section on [supplementary data](#) given at the end of this article) ([Shen et al. 2010](#), [Lin et al. 2013](#)).

Fluorogenic enzyme activity imaging

MMP and neutrophil elastase (NE) activities were measured using enzyme-selective optical probes (MMPsense 680 and NE 680 fast; PerkinElmer). Thirty-week-old mice were injected with 100 µL (4 nmols) of probe via tail vein. *In vivo* imaging by the IVIS Spectrum system (PerkinElmer) was performed after 24 hours. Images were processed using Living Image 3.2 software (PerkinElmer). *Ex vivo* activity measurements were performed in excised uteri using fluorescent microscopy and intensity was analyzed using ImageJ (NIH).

Gelatin zymography

Gelatin zymography from uterine cell lysates was performed, as described in [Light & Hammes \(2015\)](#).

Statistical analysis

Group differences were analyzed using standard two-tailed Student's *t*-tests. Values are expressed as mean $\pm$ standard error of the mean (S.E.M.). $P \leq 0.05$ was considered statistically significant.

Results

Estrogen deprivation reverses myometrial overgrowth in uterine-specific *Tsc2*-null mice

As mentioned, we developed a mouse model for LAM in which detectable TSC2 was eliminated in the uterus and myometrial overgrowth and leiomyoma formation occurred within 12–18 weeks ([Prizant et al. 2013](#)). These tumors, which shared most characteristics of LAM, required estrogen, as oophorectomy or treatment with the aromatase inhibitor letrozole prevented their formation. To determine whether estrogen withdrawal would shrink pre-existing myometrial tumors, 18-week-old uterine-specific *Tsc2*-null and wild-type mice were treated for 10–12 weeks with letrozole, oophorectomy, or rapamycin. At 18 weeks, uterine-specific *Tsc2*-null mice developed abnormal uteri with myometrial overgrowth and leiomyomas ([Fig. 1A](#)). At 30 weeks, untreated uteri were significantly larger, contained bigger leiomyomas, and showed internal hemorrhage ([Fig. 1A](#)). mTORC1 inhibition with rapamycin for 10–12 weeks significantly reduced uterine size and weight ([Fig. 1A and B](#)). Remarkably, oophorectomy or letrozole treatment similarly reduced uterine size and weight to nearly normal ([Fig. 1A and B](#)), with almost normal myometrial thickness ([Fig. 1C and D](#)). No tumors were detected in the lungs of 30-week-old mice treated with estrogen deprivation or rapamycin. These results suggest that both mTORC1 and estrogen signaling are required to support myometrial tumors.

Estrogen promotes S6 phosphorylation in the absence of TSC2

As a first step toward determining why *Tsc2* knockout without estradiol is not sufficient to maintain myometrial growth, we examined a proliferative signal activated by mTORC1:S6K ([Vinals et al. 1999](#), [Espeillac et al. 2011](#)). As expected, in *Tsc2*-null uteri from 30-week-old female mice,

S6K is constitutively active, as indicated by phosphorylation of S6 ([Fig. 2A](#), control KO). This activity was localized to myometrial cells, as determined by IHC against phosphorylated S6 ([Fig. 2B, left](#)). Treatment with letrozole or oophorectomy from 18 to 30 weeks markedly reduced S6K activity ([Fig. 2A and B](#)), although not as complete as with mTORC1 inhibition by rapamycin. Interestingly, overnight stimulation with 17 β -estradiol in 12-week-old wild-type mice and uterine-specific *Tsc2*-null mice modestly but significantly increased S6, but not 4EPB1, phosphorylation ([Fig. 2C](#)). These results were confirmed *in vitro* in ELT3 rat *Tsc2*-null leiomyoma cells, where estradiol promoted S6 and ERK, but not 4EBP1, phosphorylation ([Fig. 2D](#)). These studies suggest that, in *Tsc2*-null myometrial cells and possibly in LAM cells, estrogen is required for mTORC1/S6K signaling and activates mTORC1 and/or S6K beyond that resulting from TSC2 inactivation.

Proteolytic enzyme activity is increased in *Tsc2*-null leiomyoma regions

To uncover additional genes/proteins regulated by TSC2 and estradiol, we performed RNAseq on uteri from 12-week-old, oophorectomized, wild-type mice and uterine-specific *Tsc2*-null mice that were either untreated or treated with 17 β -estradiol pellets for 8 weeks. We examined estrogen-dependent gene expression in wild-type and *Tsc2*-null mouse uteri ([Fig. S1A](#), see section on supplementary data given at the end of this article). We also compared gene expression among estradiol-treated wild-type and uterine-specific *Tsc2*-null mouse uteri to uncover genes regulated by TSC2 ([Fig. S1A, green](#)). We then identified genes that were mediated by both estradiol and TSC2 ([Fig. S1A and B](#)). We focused on two sets of up-regulated genes that are also enhanced in LAM cells: proteases and melanocytic markers.

With regard to proteases, mRNAs encoding MMP-2, -9, and -3, as well as NE, were up-regulated by both estradiol and loss of TSC2. MMPs regulate migration, invasion, and metastasis in other tumors ([Jimi et al. 1997](#), [Cockett et al. 1998](#), [Lin et al. 2004](#)), and NE mediates lung damage in injury, asthma, or emphysema ([Chughtai & O'Riordan 2004](#), [Moraes et al. 2006](#)). Furthermore, MMP-2 and -9 were identified in LAM nodules and in the blood of LAM patients ([Hayashi et al. 1997](#), [Odajima et al. 2009](#)). Thus, these proteases were intriguing candidates to be examined.

To confirm RNAseq results, mRNA was collected from uteri of 18- and 30-week-old wild-type mice and uterine-specific *Tsc2*-null mice and gene expression of MMPs was examined by quantitative PCR. As expected, *Mmp-2*, -9, and -3 mRNA levels were elevated in

Figure 1

Estrogen deprivation reverses myometrial overgrowth in uterine-specific *Tsc2*-null mice. Eighteen-week (wk)-old wild-type (WT) mice and uterine-specific *Tsc2*-null (KO) mice were either untreated (Ctl; $n = 5$ at 18 weeks, $n = 7$ at 30 weeks per genotype), oophorectomized (OVX, $n = 3$ WT, $n = 5$ KO), or treated with letrozole (Let; $10 \mu\text{g}/\text{mouse}/\text{day}$ s.c., $n = 3$ WT, $n = 4$ KO) or rapamycin (Rapa; $5 \text{ mg}/\text{kg}$ i.p., 3 days/week, $n = 3$ per genotype) for 10–12 weeks. (A) At 30 weeks of age, all treatments reversed uterine overgrowth in KO mice (representative uteri showed), as seen by the uterus size (scale bar, 1 cm) and (B) weight. (C) Corresponding representative H&E stains show myometrial (M) morphology of untreated and treated mice and (D) myometrial thickness was measured. Images are representative of at least three mice per genotype per treatment. Results represent the mean $\pm$ s.e.m. * $P \leq 0.05$ relative to 18 weeks Ctl by Student's *t*-test. A full color version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0505>.

Tsc2-null mouse uteri compared with wild-type mouse uteri (Fig. 3A). Zymography from uterine lysates from these same mice confirmed up-regulation of pro-enzyme and active forms of MMP-2 and -9 proteins (Fig. 3B).

MMP function in *Tsc2*-null myometrial tumors was examined using an optical biomarker consisting of two fluorophores linked to a peptide targeted for MMP-2, -3, -9. Upon peptide cleavage, the fluorophores separate and emit fluorescent signal. Uteri from 28-week-old wild-type mice displayed minimal activity, whereas uteri from uterine-specific *Tsc2*-null mice demonstrated significant signal *in vivo* and *ex vivo* (Fig. 3C and D). Signal was highest both qualitatively (Fig. 3D, left) and quantitatively (Fig. 3D, right) at regions of myometrial overgrowth, confirming that MMP-2 and -9 (and possibly -3) activity associates with *Tsc2*-null myometrial tumors.

Similarly, NE enzyme expression by IHC was higher in 30-week-old *Tsc2*-null myometrium compared with wild type (Fig. 3E). Furthermore, using a NE-sensitive biomarker, NE activity was highest in areas of myometrial overgrowth, with minimal activity in wild-type uteri (Fig. 3F).

MMP expression and activity in *Tsc2*-null cells is induced by estradiol

We next examined whether estradiol regulates MMP expression and activity. Uteri from 30-week-old uterine-specific *Tsc2*-null mice that had been deprived of estrogen for 12 weeks (oophorectomy or letrozole treatment at 18 weeks) were compared with control 30-week-old *Tsc2*-null mice. *Mmp-2*, -9, and -3 mRNA levels were markedly lower in the absence of estrogen (Fig. 4A).

Myometrial MMP-9 protein expression by IHC was similarly reduced in the absence of estrogen (Fig. 4B). Finally, zymography showed reduced MMP-9 pro- and active enzyme, as well as reduced active but not pro-MMP-2, following oophorectomy or letrozole treatment (Fig. 4C; left qualitative, right quantitative). Interestingly, while rapamycin reduced the expression of *Mmp-2*, *-9*, and *-3* mRNAs (Fig. 4A), it had minimal effect on MMP-2 and *-9* pro-enzyme expression and only partially decreased MMP-2 active enzyme expression (Fig. 4C). These data suggest that mTORC1 may partially induce MMP expression and activity; however, estrogen is required for the markedly elevated MMP expression and activity in *Tsc2*-null mouse uteri, and possibly LAM cells.

Although estrogen is required for MMP expression, can it directly stimulate MMP expression? As estradiol increases MMP-2 expression and activity in ELT3 cells (Li *et al.* 2013), we focused on MMP-9 in the same cells. Estradiol responsiveness was confirmed by up-regulation of *Oxtr* and *Pgr* mRNAs in response to overnight stimulation with 17 β -estradiol (Fig. 5A). *Mmp-9* mRNA expression was also significantly up-regulated by estradiol (Fig. 5A), confirming that estradiol promotes MMP-9 expression

beyond that seen with TSC2 loss. Interestingly, while *in vivo* overnight stimulation with 17 β -estradiol in 12-week-old wild-type mice and *Tsc2*-null mice induced uterine *Oxtr* mRNA expression, it had no effect on *Mmp-9* mRNA levels (Fig. 5B). In contrast, long-term (from 4 to 12 weeks of age) exposure to 17 β -estradiol increased *Mmp-9* mRNA (Fig. 5C, right) and protein (Fig. 5D) in oophorectomized *Tsc2*-null, but not wild-type, uteri. *Mmp-2* mRNA and protein expression was not induced by estradiol in either genotype (Fig. 5C and D), suggesting that, *in vivo*, MMP-9 expression is more sensitive to estrogen. Similar to MMP-9, overnight estradiol *in vivo* had no effect on *Elane* (NE) mRNA levels (Fig. 5E); however, 8 week estradiol exposure markedly increased *Elane* mRNA (Fig. 5F) and protein expression by IHC (Fig. 5G) in oophorectomized *Tsc2*-null uteri (and wild-type uteri).

Expression of melanocytic markers requires both mTORC1 and estradiol

We next turned to melanocytic markers, which are expressed in LAM cells and identified in our RNAseq data. These include MLANA, DCT, and PMEL, all of which are

Figure 2

Estradiol promotes S6 phosphorylation in the absence of TSC2. (A) Levels of phosphorylated S6 (pS6) and total S6 (TS6) were examined by western blot in mouse uteri of 30-week (wks)-old untreated (Ctl) and treated (OVX, oophorectomized; Let, letrozole 10 μ g/mouse/day s.c.; Rapa, rapamycin 5 mg/kg i.p.; 3 days/week, for 10–12 weeks) wild-type (WT) and *Tsc2*-null (KO) mice (left panel). Quantification by densitometry of pS6/TS6 in KO mouse uteri is shown in the right panel ($n = 4$ Ctl, $n = 3$ each OVX, Let, and Rapa). (B) IHC of myometrial (M) pS6 was performed on uterine sections from untreated and OVX 30-week-old KO mice (images are representative of $n = 3$ per treatment). (C) Twelve-week-old WT and KO mice were stimulated overnight (~18 h) with 17 β -estradiol (E_2 , 1 μ g/mouse; $n = 3$ per genotype per treatment). Uterine levels of pS6, TS6, p4EBP1, and T4EBP1 were examined by western blot. Quantification by densitometry of pS6/TS6 and p4EBP1/T4EBP1 in WT and KO mouse uteri are shown. (D) ELT3 cells were cultured in 5% charcoal-stripped DF8 media for 24 h before overnight stimulation with 10 nM E_2 . Cell lysate levels of pS6, TS6, pERK, TERK, p4EBP1, T4EBP1, and GAPDH were examined by western blot (image is a representative of $n = 3$ independent experiments). Results represent the mean $\pm$ S.E.M. * $P \leq 0.05$ relative to Ctl or Ctl-WT; # $P \leq 0.05$ relative to Ctl-KO; NS, Not significant by Student's *t*-test. A full color version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0505>.

Figure 3

Proteolytic enzyme activity is increased in *Tsc2*-null leiomyoma regions. (A) Uterine *Mmp* mRNA expression in 18- and 30-week (wk)-old wild-type (WT; $n = 3$ and 5 respectively) and uterine-specific *Tsc2* null (KO; $n = 5$ each) mice was determined using quantitative PCR. Data were normalized to *Gapdh* mRNA and represented relative to WT 18 week. (B) Corresponding uterine protein levels of pro- and active MMP-2 and -9 enzymes were determined by gelatin zymography. Twenty-eight-week-old WT and KO mice were injected with an MMP-2, -3, -9-sensitive optical probe (images are representative of $n = 3$ per genotype) and MMP activity was measured (C) *in vivo* in the whole body using IVIS or (D) *ex vivo* in excised uteri using fluorescent microscopy (left panel). MMP activity was quantified by pixel intensity measurements using ImageJ (right panel). (E) NE enzyme expression was determined by IHC in the myometrium of 30-week-old WT and KO animals (images are representative of $n = 3$ per genotype). (F) NE activity was measured *ex vivo* in excised uteri using NE-sensitive optical probe (left panel; $n = 3$ per genotype). Quantification by pixel intensity of NE activity was measured using ImageJ (right panel). Results represent mean $\pm$ S.E.M. * $P \leq 0.05$ relative to WT by Student's *t*-test.

induced by MITF (Zhe & Schuger 2004). In addition, we detected overexpression of another melanocytic marker, GPNMB (Loftus *et al.* 2009), a transmembrane protein overexpressed in several cancers, including breast (Rose *et al.* 2010b) and melanoma (Tse *et al.* 2006), that correlates with metastasis (Maric *et al.* 2013). To confirm the RNAseq data, *Mitf*, *Pmel*, *Gpnmb*, *Mlana*, and *Dct* mRNA were measured by qPCR in wild-type and *Tsc2*-null uteri at 12, 18, and 30 weeks of age. In 12-week-old mice, mRNAs, excluding *Pmel*, were significantly higher in *Tsc2*-null uteri relative to wild-type uteri (Fig. 6A). At 18 and 30 weeks of age, uterine mRNA levels of all markers were significantly elevated in uterine-specific *Tsc2*-null mice, with *Pmel*, *Mlana*, and *Gpnmb* mRNAs increasing in an age-dependent fashion (Fig. 6A) that correlated with uterine weight (Fig. 6B). Increase in mRNA expression may be due to changes in cell size or number rather than an increase in relative transcription; however, as data were normalized to GAPDH, these possibilities seem less likely. As reported, some >30-week-old uterine-specific

Tsc2-null mice developed metastasizing myometrial lung lesions (Prizant *et al.* 2013). We found that these lung lesions were positive for the melanocytic markers MITF and GPNMB, whereas the surrounding lung lesions was negative (Fig. 6C), confirming that metastatic lung tumors in uterine-specific *Tsc2*-null mice share almost all known markers of human LAM (Prizant *et al.* 2013).

To determine the effect of estrogen and mTORC1 inhibition on the expression of melanocytic markers, we examined the uteri of 30-week-old uterine-specific *Tsc2*-null mice after 10–12 weeks of estrogen deprivation or rapamycin treatment in comparison to untreated 30-week-old *Tsc2*-null mice. As with MMP expression, mRNA levels of all markers were significantly down-regulated following oophorectomy or treatment with letrozole or rapamycin (Fig. 6D). The exception was *Pmel* expression, which did not reach significance in rapamycin-treated mice. To confirm that mRNA levels correlated with expressed protein levels, we performed immunofluorescence staining on uterine samples from 30-week-old mice. *Tsc2*-null

uteri expressed higher MITF and GPNMB protein levels compared with wild-type myometrium (Fig. 6E), and both were significantly decreased following oophorectomy at 18 weeks (Fig. 6E, right). Thus, as with proliferation and protease expression, melanocytic markers in *Tsc2*-null myometrial tumors, and possibly in LAM, require both mTORC1 and estrogen signaling.

Estrogen is necessary but not sufficient for MITF and GPNMB expression in *Tsc2*-null cells

As estrogen is necessary for the expression of melanocytic markers, we next tested whether estrogen directly stimulates the expression of melanocytic markers. *Tsc2*-null ELT3 cells expressed MITF and GPNMB (Fig. 7A) by immunofluorescence. GPNMB localized primarily to the peri-nuclear and cytoplasm regions but also to protruding membranes, as seen in other cancer cells (Rose *et al.* 2010b, Torres *et al.* 2015). Estradiol stimulation in ELT3 cells did not alter mRNA levels of either *Mitf* or *Gpnmb*; however, mTORC1 inhibition with rapamycin significantly reduced *Gpnmb* mRNA without affecting *Mitf* (Fig. 7B). Similarly, in 621-101 cells, GPNMB localized to the cytoplasm and the cell surface (Fig. 7C), estradiol did not promote *GPMP* or *MITF* mRNA expression, and rapamycin significantly reduced GPNMB but not *MITF* mRNA levels (Fig. 7D). *In vivo*, overnight and 8 week estradiol stimulation did not increase *Mitf* or *Gpnmb*

mRNA expression in oophorectomized, 12-week-old, wild-type mice and uterine-specific *Tsc2*-null mice (Fig. 7E and F). Thus, estrogen is necessary but not sufficient for MITF and GPNMB expression.

LAM cells in the uterus, lungs, and lymph node from LAM patients express GPNMB

We next determined whether GPNMB is expressed in human LAM tumors. In the uterus of a LAM patient, LAM-like nodules were characterized by bundles of spindle-shaped myometrial cells with eosinophilic-to-clear cytoplasm and round-to-oval nuclei, separated by narrow slit-like luminal spaces from normal myometrium (Fig. 8A). Staining with SMA distinguished normal myometrium composed of arranged circular SM bundles from the LAM-like nodule composed of more scattered SM-like cells. As expected, LAM-like nodules, but not surrounding uterus, were positive for phosphorylated S6. Furthermore, GPNMB co-localized with phosphorylated S6 in these nodules (Fig. 8A). Similarly, LAM-like tumor cells in a lymph node from a LAM patient were positive for SMA, phosphorylated S6, MITF, and GPNMB (Fig. 8B). Finally, LAM lung cells from five different patients also stained positive for GPNMB (Fig. 8C). While HMB-45 staining in a LAM lung section showed scattered positive epithelioid-like LAM cells, GPNMB showed more uniform staining

Figure 4

MMP expression and activity in *Tsc2*-null cells require estradiol. (A) Uterine *Mmp* mRNA expression in 30-week (wk)-old untreated (Ctl, $n = 5$) and treated (OVX, oophorectomized, $n = 5$; Let, letrozole 10 $\mu\text{g}/\text{mouse}/\text{day}$ s.c., $n = 4$; Rapa, rapamycin 5 mg/kg i.p., 3 days/week, $n = 3$; all for 10–12 weeks) uterine-specific *Tsc2*-null (KO) mice was determined using quantitative PCR. Data were normalized to *Gapdh* mRNA and represented relative to Ctl. (B) IHC of myometrial (M) MMP-9 was performed on uterine sections from untreated and OVX 30-week-old KO mice (images are representative of $n = 3$ per treatment). (C) Uterine protein levels of pro- and active MMP-2 and -9 enzymes were determined by gelatin zymography (left panel; $n = 4$ Ctl, $n = 3$ per treatment). Quantification by densitometry of indicated MMP relative to GAPDH (determined by western blot) is shown on the right panel. Results represent mean $\pm$ S.E.M. * $P \leq 0.05$ relative to Ctl by Student's *t*-test. A full color version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0505>.

Figure 5

Long-term, but not short-term, estrogen stimulation up-regulates protease expression and activity in *Tsc2*-null mouse uteri. ELT3 cells were cultured in 5% charcoal-stripped DF8 media for 24 h before overnight (o/n) stimulation (~18 h) with 10 nM 17 β -estradiol (E₂). (A) *Oxt*, *Pgr*, and *Mmp-9* mRNA expressions were determined using quantitative PCR. Data were normalized to *Gapdh* mRNA ($n = 6$ independent experiments). Indicated mRNA expression in the uterus of 12-week (wk)-old wild-type (WT) mice and uterine-specific *Tsc2*-null (KO) mice that were (B) untreated or stimulated overnight with E₂ (1 μ g/mouse; $n = 3$ per genotype per treatment) or (C) oophorectomized (OVX) and either left untreated (estrogen absent) (Ctl, $n = 4$ per genotype) or treated with E₂ pellets (0.5 mg/pellet) for 8 week ($n = 3$ WT, $n = 4$ KO) was determined using quantitative PCR. (D) Uterine levels of pro- and active MMP-2 and -9 enzymes were determined by gelatin zymography. (E) *Elane* (NE) mRNA expression in the uterus of 12-week-old WT and KO mice that were untreated (Ctl) or stimulated overnight with 1 μ g E₂ ($n = 3$ per genotype per treatment) or (F) OVX and either left untreated (Ctl, $n = 4$ per genotype) or treated with estradiol pellets (0.5 mg/pellet) for 8 week ($n = 3$ WT, $n = 4$ KO) was determined using quantitative PCR. Data were normalized to *Gapdh* mRNA. (G) IHC of myometrial (M) NE was performed on uterine sections from 12-week-old OVX WT and KO mice with and without estradiol pellets (0.5 mg/pellet) for 8 week (images are representative of $n = 3$ per genotype per treatment). Results represent mean $\pm$ S.E.M. * $P \leq 0.05$ relative to control, WT-OVX, or WT-Ctl, as indicated, by Student's *t*-test. A full color version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0505>.

within the LAM nodule and in the walls of the lung cysts (Fig. S2). Thus, GPNMB may serve as a new marker for LAM.

Discussion

The female sexual dimorphism of LAM in combination with the time of presentation (reproductive age women) and the detection of ERs and PRs in LAM cells (Berger *et al.* 1990, Kinoshita *et al.* 1995) suggest that the development and progression of LAM involve estrogen signaling. Indeed, LAM seems to worsen in patients taking exogenous estrogens (Shen *et al.* 1987, Yano 2002), as well as during pregnancy (Brunelli *et al.* 1996, Urban *et al.* 1999). In fact, nearly every study that tested estrogen effects on the proliferation, migration, or metastasis of LAM-like cells, using various cellular and animal models of LAM (Yu *et al.* 2004, Glassberg *et al.* 2008, Yu *et al.* 2009, Gu *et al.* 2013, Prizant *et al.* 2013, Li *et al.* 2014), has

found that estrogen was a critical driving force in these processes. Yet, there is no conclusive indication that anti-estrogen treatments are effective in LAM, as, apart from a few small studies, no controlled prospective trials have been performed.

Here, we examined estrogen-dependent processes in a uterine-specific *Tsc2*-null mouse model in which 100% of knockout mice develop myometrial proliferation and leiomyomas that share almost every characteristics of LAM, including up-regulation of mTORC1 activity and expression of SMA, PR, ER, MMPs, and multiple melanocytic markers (Figs 3 and 6) (Prizant *et al.* 2013). We previously showed that estrogen withdrawal before puberty prevented myometrial proliferation and tumor formation. Here, we demonstrate that estrogen withdrawal after tumor formation, either by oophorectomy or letrozole treatment, completely reversed myometrial overgrowth. Thus, loss of TSC2 is not sufficient to maintain tumors: an estrogen signal is required as well, perhaps to promote

myometrial cell growth and/or possibly to prevent myometrial cell death. This concept is reminiscent of steroid-dependent breast and prostate tumors, where, at least initially, hormone withdrawal causes tumors to shrink and sometimes almost completely disappear.

Interestingly, in addition to its profound effects on tumor burden, estrogen withdrawal led to a significant reduction in S6 phosphorylation within *Tsc2*-null mouse uteri (Fig. 2), suggesting that, even in the absence of TSC2, estrogen is required for full mTORC1 or S6K activity. In fact, overnight estradiol stimulation of rat ELT3 leiomyoma cells increased S6 as well as ERK phosphorylation (Fig. 2D), implying that estrogen directly activates the

mTORC1/S6K pathway, even beyond that resulting from loss of TSC suppression. This result contrasts with studies showing that estradiol did not further enhance S6 phosphorylation in ELT3 and 621-101 cells (Li *et al.* 2014, Sun *et al.* 2014); however, these differences may be due to varying stimulation and culture conditions. Notably, *in vivo* stimulation with one overnight estradiol injection similarly induced S6 phosphorylation in *Tsc2*-null mouse uteri (Fig. 2C), further indicating that estradiol, through unknown mechanisms, activates S6K in the setting of *Tsc2* knockout. *In vitro*, phosphorylation of 4EBP1 never changed in response to estradiol (Fig. 2C and D), suggesting that estradiol might bypass mTORC1

Figure 7

Estrogen is necessary but not sufficient for MITF and GPNMB expression in *Tsc2*-null cells. (A) Protein expression by immunofluorescence (arrows point to cell surface localization) and (B) mRNA expression of MITF and GPNMB by qPCR were detected in ELT3 cells treated as indicated (vehicle; Ctl; E₂; 10 nM, Rapa, rapamycin; 20 nM overnight). Data were normalized to *Gapdh* mRNA ($n = 3$ independent experiments). (C) Protein expression by immunofluorescence (arrows point to cell surface localization) and (D) mRNA expression by qPCR of MITF and GPNMB were detected in 621-101 cells treated as indicated. Data were normalized to *GAPDH* mRNA ($n = 3$ independent experiments). (E) Uterine *Mitf* and *Gpnmb* mRNA expressions in the uterus of 12-week-old wild-type (WT) and uterine-specific *Tsc2*-null (KO) mice that were untreated (Ctl) or stimulated overnight with 1 μ g 17 β -estradiol (E₂; $n = 3$ per genotype per treatment) or (F) oophorectomized (OVX) and either left untreated (Ctl, $n = 4$ per genotype) or treated with estradiol pellets (0.5 mg/pellet) for 8 weeks ($n = 3$ WT, $n = 4$ KO) were measured by qPCR. Data were normalized to *Gapdh* mRNA. Results represent mean $\pm$ S.E.M. * $P \leq 0.05$ relative to WT-Ctl, WT-OVX, or control, as indicated, by Student's *t*-test. A full color version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0505>.

and directly activate S6K, perhaps through the ERK/RSK/S6 pathway (Shim *et al.* 2013, Choi *et al.* 2014, Lesma *et al.* 2015). This indicates that we cannot conclusively determine whether estradiol is directly activating S6K or stimulating mTORC1 above its constitutive activation in the absence of TSC-mediated suppression.

In addition to regulating *Tsc2*-null myometrial cell proliferation and death, estradiol also markedly increased expression and activity of matrix proteolytic enzymes, including MMP-2, -9, and NE. Proteolytic enzymes are known to regulate migration, invasion, and metastasis in other tumors (Jimi *et al.* 1997, Cockett *et al.* 1998, Lin *et al.* 2004) due to their ability to remodel and degrade

extracellular matrices (Woessner 1991). ELT3 and human lung LAM cells displayed up-regulated MMP-2 protein and activity as well as increased invasiveness by estradiol (Glassberg *et al.* 2008), suggesting that up-regulated MMP expression and activity in LAM cells (Hayashi *et al.* 1997, Matsui *et al.* 2000, Chang *et al.* 2012) may contribute to cell migration and lung destruction. Similarly, NE is up-regulated and may play a role in diseases that promote lung damage (Chughtai & O'Riordan 2004, Moraes *et al.* 2006). In fact, NE-mediated degradation of the anti-tumorigenic factor thrombospondin-1 in the mouse lungs promotes lung metastasis, possibly explaining why the lungs are a common metastatic site in LAM and other

cancers (El Rayes *et al.* 2015). In mice with normal ovarian estrogen production, both expression and activity of MMP-2 and -9 were higher in *Tsc2*-null uteri compared with wild-type uteri (Fig. 3). Using an MMP-sensitive optical probe, through whole-body imaging, we detected high MMP activity specifically in the *Tsc2*-null mouse uterus, and in excised uteri, the highest activity was in leiomyoma regions (Fig. 3). Similarly, using an NE-sensitive probe, we found that NE is highly active in LAM-like myometrial tumors compared with normal myometrium (Fig. 3G). Notably, although MMP-9 and NE expressions were induced by estradiol *in vivo*, only MMP-9 was detected in ELT3 and 621-101 cells (Fig. 5 and not shown), suggesting that MMPs might originate directly from myometrial (or

possibly LAM) cells, whereas NE might originate from myeloid cells in surrounding stroma of tumors. Although clinical studies suggest that MMP inhibition is ineffective in patients with LAM lung disease (Chang *et al.* 2014), it is still possible that MMP and NE activity can be used as biomarkers for LAM. MRI strategies are in process to use similar protease-sensitive optical biomarkers to track tumor enzyme activities in humans (Haris *et al.* 2014, Yoo *et al.* 2014). Although more work is needed, future development of optical probes recognized by MMP-2/9 or NE may eventually be useful non-invasive diagnostic tools to track tumor burden in LAM patients.

Another set of genes known to be overexpressed in LAM cells that were identified in our RNAseq data are

Figure 8

LAM or LAM-like cells in the uterus, lungs, and lymph node from LAM patients express GPNMB. H&E and GPNMB, pS6, SMA, and MITF immunofluorescence staining (as indicated, green) were performed on human tissues from patients with (A) uterine LAM ($n = 1$), (B) lymph node (LN) with LAM cell infiltration ($n = 2$), and (C) lung LAM ($n = 5$). *Vessel.

melanocytic markers. Although melanocytic markers are known to be up-regulated in LAM cells (Tanaka *et al.* 1995, Zhe & Schuger 2004), the significance and mechanism(s) of this up-regulation is not known. Here, we identified a melanocyte-associated marker that is regulated by MITF called GPNMB (Tomihari *et al.* 2009). In a variety of cancers, including breast cancer (Rose *et al.* 2010b) and melanoma (Tse *et al.* 2006), pro-invasive and pro-metastatic phenotypes were positively correlated with GPNMB expression. Our data suggest that both estrogen and mTORC1 signaling are necessary for up-regulation of *Mitf* and *Gpnmb* in *Tsc2*-null mouse uteri, as estrogen withdrawal, as well as rapamycin treatment, significantly reduced expression levels of these markers (Fig. 6). Although rapamycin significantly reduced GPNMB mRNA levels in ELT3 and 621-101 cell lines, estradiol stimulation, *in vitro* and *in vivo*, had no effect on GPNMB expression in our *Tsc2*-null mouse uteri or in cultured ELT3 and 621-101 cells (Fig. 7). These results suggest that the decrease in 30-week-old uterine melanocytic marker expression following estrogen withdrawal (Fig. 6D and E) was likely due to the down-regulation in mTORC1/S6K activity that is observed in estrogen-deprived uteri (Fig. 2A and B) rather than a direct effect by estrogen. Thus, estrogen is necessary but not sufficient for GPNMB expression in *Tsc2*-null cells, and loss of TSC2 is the primary promoter of melanocytic marker expression in *Tsc2*-null mouse uteri and possibly LAM cells.

Finally, we confirmed that GPNMB, which was overexpressed in LAM-like *Tsc2*-null myometrial and metastasizing lung tumors (Fig. 6), was also expressed in human LAM tumors. While healthy tissue of the lungs, lymph-node, and uterus that contained LAM or LAM-like lesions was negative for GPNMB expression, LAM or LAM-like tumor cells were positive for GPNMB expression (Fig. 8). Future studies should determine whether GPNMB plays a role in LAM cell migration and invasion, as suggested in other cancers such as breast, melanoma, and prostate (Qian *et al.* 2008, Rose *et al.* 2010a, Fiorentini *et al.* 2014). In addition, GPNMB localized to the cytoplasm as well as the cell surface of LAM-like cells ELT3 and 621-101 (Fig. 7A and C). Although GPNMB localization was reported to be cytoplasmic in normal cells, such as macrophages and melanocytes (Ripoll *et al.* 2007, Tomihari *et al.* 2009), it is similarly enriched on the cell surface of cancer cells such as breast and melanoma (Rose *et al.* 2010b, Torres *et al.* 2015). Membrane-localized GPNMB might therefore be used as a therapeutic target, as a GPNMB-specific antibody conjugated to a cytotoxic compound, CDX-011, was recently developed as a novel drug. Upon CDX-011

internalization into GPNMB-expressing cancer cells, cell cycle arrest followed by apoptosis was reported, leading to xenograft tumor regression (Tse *et al.* 2006, Rose *et al.* 2010b). Our findings that LAM-like cells in mice, as well as true LAM cells in humans, express high levels of GPNMB, suggest that GPNMB may serve as a biomarker for LAM, and opens a new target for CDX-011.

In summary, our uterine-specific *Tsc2*-null mouse model shares nearly all characteristics of LAM cells in women, as both myometrial cells and metastasizing lung cells in these mice are sensitive to estrogen, TSC2-negative, contain increased mTORC1 activity, and are positive for SMA, ER, PR, MMPs, and melanocytic markers. This mouse model allowed us to discover new markers of LAM (e.g., GPNMB) and to uncover mechanisms regulating key characteristics of LAM. Specifically, estrogen is required to maintain the LAM phenotype as well as mTORC1/S6K activation and tumor growth, emphasizing the importance of systematically studying anti-estrogen treatment in LAM.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0505>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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