Urokinase-type plasminogen activator (uPA) is critical for progression of tuberous sclerosis complex 2 (TSC2)-deficient tumors

Lymphangioleiomyomatosis (LAM) is a fatal lung disease associated with germline or somatic inactivating mutations in tuberous sclerosis complex genes (TSC1 or TSC2). LAM is characterized by neoplastic growth of smooth muscle-a-actin-positive cells that destroy lung parenchyma and by the formation of benign renal neoplasms called angiolipomas. The mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin slows progression of these diseases but is not curative and associated with notable toxicity at clinically effective doses, highlighting the need for better understanding LAM’s molecular etiology. We report here that LAM lesions are enriched in brain (Rheb)-GTP (1, 9–13). Activation of mTOR, in turn, induces cell growth, motility, and invasion and development of “benign” tumors in LAM and TSC-LAM (14, 15).

Although many but not all patients with LAM respond, at least initially, to the mTOR inhibitor rapamycin (sirolimus), the development of “benign” tumors in LAM and TSC-LAM (14, 15). Inactivating mutations in TSC1 and/or TSC2 genes, encoding the proteins hamartin and tuberin, respectively, lead to constitutive activation of mTOR kinase through the Ras homolog enriched in brain (Rheb)-GTP (1, 9–13). Activation of mTOR, in turn, induces cell growth, motility, and invasion and development of “benign” tumors in LAM and TSC-LAM (14, 15). Although many but not all patients with LAM respond, at least initially, to the mTOR inhibitor rapamycin (sirolimus), the disease state occurs in the absence of germline mutations as a result of two inactivating mutations and LOH in somatic LAM cells (5–8).

Lymphangioleiomyomatosis (LAM)4 is a fatal progressive lung disease that primarily affects women of childbearing age characterized by neoplastic growth of smooth muscle (SM)-like cells that destroy lung parenchyma and cause recurrent lung collapse and loss of pulmonary function (1). LAM may develop through a “two-hit” mechanism of loss of heterozygosity (LOH) (2) similar to other tumor syndromes associated with loss of tumor suppressor function (3, 4). LAM occurs in ~30% of adult women with the genetic disorder tuberous sclerosis complex (TSC) (TSC-associated LAM), which is characterized by germ-line mutations of the tuberous sclerosis complex 1/2 (TSC1 or TSC2) genes together with a distinct mutation in a second allele at the same locus in a somatic cell, resulting in LOH for the normal allele. In sporadic LAM, neoplastic transformation occurs in the absence of germline mutations as a result of two hit TSC mutations and LOH in somatic LAM cells (5–8).

4 The abbreviations used are: LAM, lymphangioleiomyomatosis; SM, smooth muscle; mTOR, mammalian target of rapamycin; uPA, urokinase-type plasminogen activator; TSC, tuberous sclerosis complex; MEF, mouse embryonic fibroblast; qRT, quantitative RT; WB, Western blotting; MMP, matrix metalloproteinase; LV, lentiviral; LOH, loss of heterozygosity; uPAR, uPA receptor; AMPK, AMP-activated kinase.

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ease progresses in others despite treatment (16) thus necessi-
tating lung transplantation (17). Although rapamycin analogs
(rapalogs) show beneficial effects, the recurrence of symptoms,
a decline in pulmonary function and regrowth of angiomyolipomas
after cessation of treatment, suggests a need for determining
novel molecular targets for treatment either alone or as adju-
vant therapy with rapalogs (18–20). The reasons for the varia-
tion in response to rapamycin, the need for persistent treat-
ment, and the reason for delayed progression are uncertain (17,
19, 20) may originate from the inability of rapalogs to inhibit all
mTORC1 substrates along with induction of the feedback
loops, resulting in re-activation of the receptor tyrosine kinases,
Akt and ERK1,2 (10, 21–24).

LAM is a multisystem disorder that affects the lungs, pleural
space, kidney, liver, lymphatic system, and uterus. The origin of
the LAM cells is unknown, but renal angiomyolipomas and
uterine lesions have been proposed as potential primary sites
(25). Renal angiomyolipomas develop in nearly 80–90% of
patients with TSC and 50% of patients with sporadic LAM.
Renal angiomyolipomas and LAM cells from individual patients
with sporadic LAM share the same mutation in TSC2, suggest-
ing a common cellular origin and suggesting that LAM cells
disseminate from one site to another (7). LAM can also recur
within transplanted lung (26, 27). The fact that recurrent lung
tumors carry the same TSC2 mutation as the host’s LAM cells
suggests that these tumors are capable of metastasizing from
the other organs to donor lung (7, 28, 29). However, the path-
ways leading to dissemination of LAM cells have not been well
delineated (1).

The urokinase-type plasminogen activator (uPA) is a serine
protease that has been implicated in tumor growth, adhesion,
migration, tissue invasion, and angiogenesis (30–32). Expres-
sion of uPA is very low in quiescent non-dividing cells but
increases dramatically in most malignant tumors (31). uPA
converts plasminogen into the active serine protease plasmin
(33, 34), which in turn activates multiple matrix metallopro-
teinases MMPs (MMP-2, -3, and -9) (35–37), VEGF-A (38),
VEGF-C and VEGF-D (39), and other growth factors impli-
cated in the proliferation of LAM cells (40–43) and in many
other types of tumor cells. uPA binds cells with high affinity
through a glycosylphosphatidylinositol-linked receptor (uPAR/
CD87) that is mobile in the plasma membrane and permits
proteolytic activity to localize to the leading edge of migrating
cells (44, 45). Although uPAR lacks transmembrane and cyto-
plasmic domains, it transduces intracellular signals through
interactions in cis with several transmembrane receptors (46–
48). The proteolytic activity of uPA is regulated by specific
inhibitors, which belong to a serine protease inhibitors (SERO-
PIN) family (Plasminogen Activator Inhibitors PAI-1, PAI-2,
and PN-1) (49). Immunohistochemical analysis suggests that
LAM nodules underexpress PAI-1 (50), which, together with
overexpression of uPA (50), may contribute to the processes of
tissue destruction in the lung. We have previously reported that
uPA also rapidly translocates to cell nuclei where it up-regu-
lates transcription of genes encoding VEGFR1 and VEGFR2
(FLT-1 and KDR, respectively) (51) and down-regulates expres-
sion of the tumor suppressor p53 (52) via non-proteolytic
mechanisms. However, little is known whether uPA-dependent
signaling pathways contribute to neoplastic growth in LAM.

Although LAM lesions are often designated as benign
tumors, up-regulation of uPA expression may not only enhance
local growth with destruction of surrounding parenchyma but
may also promote vascular and lymphatic invasion and confer
metastasizing capacity, similar to its role in the progression of
many common cancers (53, 54). In view of this, we investigated
the role of uPA in the pathogenesis of LAM. In this study, we
demonstrate the following: 1) uPA is up-regulated within
LAM lung and renal angiomyolipomas; 2) growth of TSC2-
null tumors is significantly impaired in uPA-knock-out mice
(uPA−/− mice); 3) inhibiting expression of uPA in TSC2-null
lung tumor cells reduces their tumorigenic capacity in mice; 4) treat-
ment of TSC2-null tumor-bearing mice with the uPA inhibitor
amiloride significantly impairs tumor growth in the lung; 5) 
up-regulation of uPA is a direct consequence of loss of TSC
function; 6) mTOR inhibitors further up-regulate expression of
uPA in cells with compromised TSC function; and 7) rapa-
mycin-induced up-regulation of uPA is prevented by glucocor-
ticoids and inhibition of FOXO1/FOXO3 transcription fac-
tors. Together, these data suggest that uPA may serve as a
potential therapeutic target to prevent neoplastic growth and
dissemination of LAM cells.

Results
Expression of uPA is increased in LAM lesions of patients with
LAM and angiomyolipomas

To explore the role of uPA in LAM, we compared the expres-
sion of uPA in lung sections containing LAM lesions and renal
sections containing angiomyolipomas to normal lung and renal
samples. The expression of SM α-actin was used as a positive
control to localize LAM lesions (55). Expression of uPA in nor-
mal lung was low as expected (Fig. 1A) and as reported by others
(50). In contrast, uPA expression was markedly increased in SM
α-actin–positive LAM lesions (Fig. 1B), in accordance with the
previously reported data (50). In normal kidney, uPA-positive
staining was localized to tubular epithelial cells and vasculature,
and anti-SM α-actin antibody was only detected in vessels (Fig.
1C), but marked staining for uPA was seen throughout the
SM α-actin–positive angiomyolipomas (Fig. 1D). These results
affirm that uPA is up-regulated in LAM lesions and in angi-
omyolipomas (Fig. 1).

Loss of TSC induces overexpression of uPA

Loss of TSC function affects the expression of multiple genes
(56). Therefore, we next asked whether up-regulation of uPA in
LAM and angiomyolipoma lesions is associated with loss of
TSC function. To examine this question, we compared the
expression of uPA in Tsc1−/− versus Tsc1+/+ mouse embry-
onic fibroblasts (MEFs) and in Tsc2−/−/p53−/− versus Tsc2−/−/p53−/−
MEFs. The Western blotting data shown in Fig. 2, A and
B, demonstrate that knockdown of either Tsc1 or Tsc2 leads to
marked up-regulation of uPA expression. Up-regulation of uPA
mRNA in Tsc1−/− and Tsc2−/−/p53−/− versus Tsc1+/+ and
Tsc2−/−/p53−/− MEFs, respectively, was confirmed by qRT-
PCR (shown in Table 1). TSC2 silencing in wild-type MEFs

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using lentiviral shRNA resulted in up-regulation of uPA expression (Fig. 2C).

**mTOR inhibition increased uPA expression in TSC-deficient cells**

Inactivation of TSC function in LAM results in hyper-activation of the mTOR pathway (13). Therefore, we asked whether mTOR hyperactivation due to TSC loss of function is responsible for up-regulation of uPA expression in TSC-compromised cells, and whether addition of the mTOR complex 1 (mTORC1) inhibitor rapamycin to Tsc1−/− or Tsc2−/−/pS3−/− MEFs lowers the expression of uPA to levels expressed by Tsc1+/+ or Tsc2+/+ or pS3+/− MEFs, respectively. Unexpectedly, rapamycin not only failed to suppress uPA expression in Tsc1−/− and Tsc2−/−/pS3−/− MEFs, but it actually further increased uPA expression in these cells. In contrast, rapamycin did not affect uPA expression in Tsc1+/+ or Tsc2+/+ or pS3+/− MEFs (Fig. 2D), Rapamycin and Torin 1, which inhibit both mTORC1 and mTOR complex 2 (mTORC2), also up-regulated uPA expression in the mouse TSC2-null tumor cells (Fig. 3A, 4A and 5A) (57–59), in the human TSC2-null AML621-101 cells (Fig. 3B) (60), and in the TSC2-null rat ELT3 cells (Fig. 3C) (61). These data demonstrate that TSC-dependent up-regulation of uPA is not reversed by inhibition of mTORC1. We examined the length of time necessary for 100 nM rapamycin to induce expression in TSC2-null mouse tumor cells. The results shown in Fig. 3D show that up-regulation of uPA at the level of protein required more than 6 h but less than 18 h.

**Signaling pathways that up-regulate uPA expression by rapamycin in TSC-deficient cells**

uPA expression is regulated by glucocorticoids and the FOXO3 transcription factor in a glucocorticoid receptor-dependent manner (62). Based on these findings, we next investigated the signaling pathways that are involved in up-regulation of uPA in response to rapamycin in TSC-deficient cells. To do so, we incubated TSC2-null tumor cells with rapamycin in the presence or absence of the HMG-CoA reductase inhibitor simvastatin, which inhibits mTORC2-mediated activation of RhoA and induces apoptosis of TSC2-null tumor cells (63); the MEK inhibitor PD98059, which prevents activation of ERK1,2 (64); the FOXO1 inhibitor FOXO1i (AS1842856) (65), which also inhibits FOXO3 at higher concentrations; and dexamethasone, which activates the glucocorticoid receptor and inactivates FOXO3 through serum/glucocorticoid-regulated kinase-1 (SGK-1) activation (62). Neither simvastatin nor PD98059 reduced basal levels of expression nor prevented uPA up-regulation in response to rapamycin, whereas inhibition of FOXO family of the transcription factors or addition of dexamethasone abrogated up-regulation of uPA (Fig. 4, A, C and D). Inhibition of SGK-1 by GSK650394 resulted in a decrease in FOXO3 phosphorylation at Ser-315 and an increase of both basal and rapamycin-induced levels of uPA (Fig. 4C), confirming that SGK-1 contributes to inhibition of uPA expression (62).
in a FOXO3-dependent manner. As AMP-activated kinase (AMPK) phosphorylates and activates FOXO3 (66) and is activated upon loss of TSC2 (67, 68), we tested the effect of the AMPK inhibitor dorsomorphin (compound C) on uPA expression in TSC2-null cells and found that it partially inhibits up-regulation of uPA expression by rapamycin (Fig. 4 B). Importantly, both pre-exposure of mouse TSC2-null tumor cells to dexamethasone before addition of rapamycin and adding dexamethasone 24 h after addition of rapamycin blocked up-regulation of uPA by the mTORC1 inhibitor by rapamycin further up-regulates uPA in TSC-deficient MEFs. uPA, pS6 (phospho-S-235/236), total S6, GAPDH, TSC1, or TSC2 were detected in lysates of cells incubated for 24 h in the absence or presence of the indicated concentrations of rapamycin. D, Tsc1–/– and Tsc1+/+/MEFs; E, Tsc2–/–/p53–/– and Tsc2+/+ /p53–/– MEFs. The vertical black lines indicate that the molecular weight standards were run together with the other samples but were non-contiguous on the gel. The fold-increase in expression of uPA is shown above the panel illustrating the uPA WB.

| Sample 1          | Sample 2          | Fold increase in sample 1 vs. sample 2 |
|-------------------|-------------------|---------------------------------------|
| TSC2+/+ (p53−/−) MEF | TSC2+/+ (p53+/+) MEF | 28.5                                  |
| TSC1+/− MEF       | TSC1+/+ MEF       | 5.8                                   |
| TSC2-null tumor cells | TSC2-null tumor cells + rapamycin | 2.8                                   |

**Table 1** Comparative expression of *plau* mRNA in mouse cells

tumor cells using the LV-mediated shRNA targeting. The results shown in Fig. 4D demonstrate that silencing of FOXO3 suppressed expression of uPA. These results complement our data obtained using the chemical inhibitor of FOXOs confirming that FOXO3 is required for uPA overexpression in TSC2-deficient cells. Collectively, these data indicate the glucocorticoid receptor pathway, AMPK, and FOXO3 participate in TSC-dependent expression of uPA and in their response to rapamycin.

**Rapamycin enhances the motility and invasiveness of TSC2-null tumor cells**

In view of the finding that rapamycin up-regulates expression of uPA in TSC2-null cells (Figs. 2, D and E, and 3, A–E), we asked whether rapamycin affects their migration and invasive capacity. TSC2-null cells pre-incubated with rapamycin migrated through a Matrigel-coated porous membrane more effectively than did untreated cells (Fig. 5). We also asked whether inhibition of uPA activity or expression would prevent this rapamycin-mediated increase of TSC2-null tumor cell migration. As shown in Fig. 5, pre-treatment of cells with either dexamethasone or FOXOi or addition of the uPA inhibitor...
UK122 abolished the rapamycin-induced increase in cell migration (Fig. 5A) and invasion (Fig. 5B). These data suggest that rapamycin increases the invasive capacity of TSC-deficient cells in a uPA-dependent manner and that this effect is mitigated by dexamethasone and by FOXOi. Growth of TSC2-null tumors is reduced in uPA-knock-out (uPA−/−) mice

Homozygous TSC2−/− mice are not viable (58). Therefore, TSC2-null tumor cells (see “Experimental procedures”) were derived from kidney tumors that develop in heterozygous Tsc2−/− mice that spontaneously acquired an inactivating mutation in the second allele of the Tsc2 gene due to LOH (57, 58). We injected equal numbers of the TSC2-null tumor cells into the jugular veins of uPA−/− mice and control WT littermates. Twenty days post-injection, animals were sacrificed and lungs were subjected to immunohistological analysis. As seen in Fig. 6A, visual examination of the lungs from WT mice injected with TSC2-null cells showed multiple lesions throughout the lungs; in contrast, in uPA−/− mice, a few small TSC2-null lesions were observed. Staining with hematoxylin-eosin (H&E) revealed clear differences in the number and size of the lesions. Statistical analysis of H&E staining, performed by measuring the areas of lesions relative to the entire lung, revealed that the growth of the TSC2-null cell-derived tumors was significantly decreased in uPA−/− mice (p < 0.05) as seen in Fig. 6, B and C. These data indicate that host-derived uPA also contributes to tumor growth.

Inhibition of uPA expression in TSC2-null tumor cells decreases proliferation and migration and promotes apoptosis in vitro

To further examine the role of uPA in the growth and motility of TSC2-null cells, we next used LV transfer of mouse uPA mRNA-targeting shRNA (TSC2-null/uPA-sh) or control shRNA (TSC2-null/con-sh) to inhibit expression of uPA (Fig. 7A). Control lentiviral transduction did not attenuate the expression of uPA in tumor cells. TSC2-null/uPA-sh cells exhibited impaired migration through Matrigel-coated porous membranes toward serum-supplemented media compared with TSC2-null/con-sh cells (Fig. 7B), suggesting that uPA in required for serum-induced TSC2-null cell migration. TSC2-null/uPA-sh cells exhibited decreased cell growth relative to TSC2-null cells transduced with control lentivirus (Fig. 7C), and the cells were more sensitive to apoptosis induced by simvastatin (Fig. 7D) (59).
Rapamycin increases the migration and invasion of TSC2-null cells in a uPA-dependent manner. TSC2-null tumor cells were starved in DMEM, 0.1% BSA for 24 h and incubated with either dexamethasone (Dex, 1 μM) or AS184856 (FOXOi, 1 μM) for 30 min and then rapamycin (20 nM) or vehicle (DMSO) was added for an additional 18 h. The cells were detached with trypsin/EDTA, washed in starvation medium, and resuspended in the same medium alone or in the presence of the uPA inhibitor UK122. Cell migration and invasion were assessed as described under “Experimental procedures.” Cells that had undergone migration were photographed and counted using the EVOS FL Auto Imaging System microscope software Auto count mode. Each condition was set up in three wells, and three images were taken at different sites within each transwell. The bar graphs on left and right show fold-change in the number of cells per microscopic field that migrated (A) or invaded (B) the Matrigel, respectively, in response to serum (mean ± S.E.). Few cells migrated in starvation medium (2–3 per microscopic field), and therefore these results do not appear on the graph. *, p < 0.001.

Figure 5. Rapamycin increases the migration and invasion of TSC2-null cells in a uPA-dependent manner. TSC2-null tumor cells were starved in DMEM, 0.1% BSA for 24 h and incubated with either dexamethasone (Dex, 1 μM) or AS184856 (FOXOi, 1 μM) for 30 min and then rapamycin (20 nM) or vehicle (DMSO) was added for an additional 18 h. The cells were detached with trypsin/EDTA, washed in starvation medium, and resuspended in the same medium alone or in the presence of the uPA inhibitor UK122. Cell migration and invasion were assessed as described under “Experimental procedures.” Cells that had undergone migration were photographed and counted using the EVOS FL Auto Imaging System microscope software Auto count mode. Each condition was set up in three wells, and three images were taken at different sites within each transwell. The bar graphs on left and right show fold-change in the number of cells per microscopic field that migrated (A) or invaded (B) the Matrigel, respectively, in response to serum (mean ± S.E.). Few cells migrated in starvation medium (2–3 per microscopic field), and therefore these results do not appear on the graph. *, p < 0.001.

Figure 4. FOXO1/3- and glucocorticoid receptor-mediated signaling drive rapamycin-induced uPA overexpression in TSC-deficient cells. TSC2-null tumor cells were starved in DMEM, 0.1% BSA for 24 h, pre-incubated with simvastatin (10 μM), PD98059 (50 μM), AS184856 (FOXOi, 1 μM) (A); dorsomorphin (20 μM) (B); GSK650394 (GSKi, 50 μM) or dexamethasone (Dex, 1 μM) (C) for 30 min and then rapamycin (20 nM) or vehicle (DMSO) was added for 18 h in presence of these inhibitors. D, TSC2-null tumor cells were starved in DMEM, 0.1% BSA for 24 h and exposed to rapamycin (Rapa, 20 nM) or vehicle for 24 h, and the media were replaced with fresh DMEM, 0.1% BSA supplemented with either vehicle (ethanol) or dexamethasone (Dex, 1 μM) for additional 24 h. Cells were lysed in RIPA buffer and uPA, pS6 (as an efficacy control for rapamycin (78)), pERK1,2, pFOXO3(Ser-315) (as an efficacy control for SGKi (68)), pFOXO1(Thr-24)/pFOXO3(Thr-32), phospho-RAPTOR(Ser-792) (as an efficacy control for AMPK inhibitor dorsomorphin (66)), and total S6, ERK1,2 RAPTOR, RICTOR (as an efficacy control for FOXO1/3 inhibitor (121)), and FOXO3 were detected in lysates by Western blotting as in Fig. 3. The fold-increase in expression of uPA is shown above the panel illustrating the uPA WB. E, qRT-PCR analysis of Plau mRNA in TSC2-null cells treated with rapamycin in the absence or presence of AS184856 (FOXOi, 1 μM), dorsomorphin (AMPKi, 20 μM), or dexamethasone (Dex, 1 μM) as in A–C. Results are expressed as the relative arbitrary units calculated relative to β-actin. Experiments were performed in three biological replicates. *, p < 0.05. F, down-regulation of FOXO3 expression results in inhibition of basal expression of uPA in TSC2-null cells. Cells were transfected with lentivirus encoding either control shRNA (consh) or mouse FOXO3-targeting shRNA (FOXO3sh) and selected using 2 μg/ml puromycin. Lysates of the cells were analyzed by WB as in A.
Inhibition of uPA expression in TSC2-null tumor cells reduces tumorigenic potential in vivo

To assess the effect of uPA on the tumorigenic potential of TSC2-null cells in vivo, equal numbers of TSC2-null/uPA-sh or TSC2-null/con-sh cells were injected intravenously into WT C57/BL6 mice. TSC2-null/con-sh cells formed multiple lesions in the lungs. B, representative images of H&E-stained lungs collected on day 20 after injection of tumor cells from a WT mouse (top) and an uPA−/− mouse (bottom). Images taken using EVOS® FL Auto Cell Imaging System with a ×4 objective were stitched using Scan and Stitch function of EVOS® FL software (left). An individual representative image taken at ×4 magnification is presented on the right. Scale bar, 1000 μm. C, quantification of nodule area. The y axis shows the values calculated as the ratio between the area of the nodules and the entire lung area measured in square pixels, the mean value ± S.D. Sections, obtained at five levels for each lung, were analyzed. Lungs of three animals were analyzed in each group. *, p < 0.01.

Pharmacological inhibition of uPA with amiloride inhibits TSC2-null tumor growth

To determine whether uPA catalytic activity contributes to the tumorigenic potential of TSC2-null cells in vivo, we injected WT C57/BL6 mice with TSC-2-null cells followed 1 day later by daily intraperitoneal injections of amiloride (10 mg/kg) to inhibit uPA catalytic activity or with control vehicle alone. The dose of amiloride was selected based on previously published studies in mice and rats (69). Mice injected with the TSC2-null cells and vehicle developed multiple lung lesions as seen in Fig. 8C, left panel. Administration of amiloride significantly reduced the size and number of tumors (Fig. 8C, right panel). Analysis of H&E staining revealed a statistically significant reduction in the occurrence and size of TSC2-null lesions in the lungs of the mice treated with amiloride (p < 0.02) (Fig. 8D). These data demonstrate that the catalytic activity of uPA contributes to tumorigenic potential of TSC2-null tumor cells in vivo.

Discussion

Increased expression of uPA is a strong risk factor for the development of metastasis by many types of malignant tumors (70), but its role in LAM has not been established. Our data suggest that urokinase may play an important role in the progression of LAM as well. Neoplastic LAM lesions in the lung and angiomyolipomas in the kidney express high levels of uPA (Fig. 1), which is consistent with a previous report (50) showing overexpression of uPA in human LAM nodules. We now show that inactivation of TSC induces overexpression of uPA in
TSC2-null LAM cells. We found that Tsc1<sup>-/-</sup> and Tsc2<sup>-/-</sup> MEFs all express higher levels of uPA than their WT counterparts (Fig. 2, A and B), and silencing of TSC2 in WT MEFs resulted in an increase of uPA expression (Fig. 2C).

In view of our finding that uPA is up-regulated in TSC-deficient MEFs, we investigated how mTORC1 and mTORC2 inhibitors affected uPA expression in Tsc2<sup>-/-</sup> and Tsc1<sup>-/-</sup> MEFs and other cell types with deregulated TSC2 function. However, rapamycin, while inhibiting mTOR and inducing growth arrest of TSC-deficient cells, enhanced up-regulated expression of uPA in human TSC2-null human angiomyolipoma cells (60), mouse TSC2-null tumor cells, Tsc2<sup>-/-</sup> MEFs (71), and TSC2-null rat ELT3 cells (Figs. 2E, 3, A–D) (72), but the effect was less prominent in Tsc1<sup>-/-</sup> MEFs (Fig. 2D) (71), and no induction was seen in cells with intact TSC (Fig. 2D and E). Patients with TSC1 mutations are generally less severely affected than those with TSC2 mutations matched for age (73, 74). It will therefore be of interest to determine in future studies whether this phenotypic difference stems from the preferential binding of TSC2 to several targets like FOXO1 relative to TSC1 (75) or whether these two complexes differ in downstream pathways that result in up-regulation of uPA.

We investigated the mechanism by which inhibition of mTORC1 increases uPA expression in TSC-null cells. Similar to uPA, several other genes implicated in the progression of LAM, including various MMPs, TIMPs, phospholipase A<sub>2</sub>, PARP1, and members of Wnt signaling cascade are up-regulated upon the loss of TSC function, but none are negatively regulated by rapamycin (56, 76). We found that an HMG-CoA reductase inhibitor that blocks mTORC2-mediated RhoA activation and causes TSC2-null cells to undergo apoptosis (63) did not inhibit uPA expression in Tsc2-null mouse tumor cells (Fig. 4A), nor did it prevent rapamycin-induced up-regulation of uPA.

Rapamycin, an allosteric inhibitor of mTOR, does not inhibit mTORC1 signaling completely (22, 77–79), which is consistent with its cytostatic effect and its inability to induce the death of LAM cells (80). Based on this evidence, we examined the possibility that enhanced uPA expression in TSC-deficient cells was mediated by the capacity of rapamycin to block negative feedback loops emanating from the S6K1 to PI3K signaling pathway (81), which activates proliferative and pro-survival effectors such as Akt (21, 81–84) and up-regulates specific receptor tyrosine kinases (85, 86) and mitogen-activated protein kinase (87). However, neither inhibition of PI3K, ERK1,2, nor AKT prevented rapamycin from increasing expression of uPA in Tsc2-null mouse tumor cells (Fig. 4A), nor did it prevent rapamycin-induced up-regulation of uPA.

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We did find that activation of glucocorticoid receptor-mediated signal transduction by dexamethasone or pharmacological inhibition of FOXO1/3 lowered both basal levels of uPA and abolished the rapamycin-induced increase in uPA expression in TSC2-compromised cells (Fig. 4, A, C, and D). These data are consistent with observations made in breast cancer cells (62) implicating FOXO3 in activation of uPA expression, showing that FOXO3 binding and activation of the uPA promoter is prevented by glucocorticoid-induced activation of SGK-1 along with concomitant down-regulation of uPA expression (62). AMPK phosphorylates and activates FOXO3 (88) and is hyperactivated in TSC2-null cells and tumors (68). Rapamycin also increases AMPK activity in TSC2-null cells (89). We found that inhibition of AMPK partially inhibited the rapamycin-induced increase in uPA expression in TSC2-null cells (Fig. 4B), which suggests that AMPK contributes to up-regulation of uPA expression in TSC2-negative cells through activation of FOXO3. The data showing that up-regulation of uPA in response to rapamycin in TSC2-negative cells was paralleled by the increase of FOXO3 protein (Fig. 4, A—C and F) are consistent with previously reported observations that FOXO3 expression is up-regulated upon mTORC1 inactivation (90). Of importance, dexamethasone, FOXO1/3 inhibitor, and an inhibitor of uPA catalytic activity by UK122 prevented the acceleration of TSC2-null cell migration and invasion by rapamycin (Fig. 5). However, additional studies are needed both to ascertain how FOXOs are regulated by mTORC1 inhibition in TSC-negative cells and to delineate the mechanism by which FOXOs up-regulate uPA expression in TSC-compromised cells following inhibition of mTORC1.

Our findings suggest that overexpression of uPA by TSC-deficient cells exposed to rapamycin might exacerbate the destruction of surrounding lung or kidney parenchyma and foster spread of LAM to other organs when therapy must be interrupted due to

Figure 8. Molecular or pharmacological targeting of uPA with shRNA and/or amiloride significantly inhibits growth of TSC2-null lung lesions. Equal numbers of TSC2-null/uPA sh and TSC2-null/con sh cells were injected in WT mice as in Fig. 6A. A, representative images are shown of H&E-stained sections of the lungs after injection of control-sh or uPA-sh tumor cells. Images taken using EVOS® FL auto cell imaging system with a ×4 objective were stitched using Scan and Stitch function of EVOS® FL software (left). An individual representative image taken at ×4 magnification is presented on right. Scale bar, 1000 μm. B, quantification of nodule area. The y axis shows the values calculated as the ratio between the area of the nodules and the entire lung area measured in square pixels and normalized, the mean value ± S.D. Sections obtained at five levels from each lung were analyzed for each of nine animals in the group injected with control sh LV-cells and four animals injected with uPA sh LV-infected TSC2-null tumor cells. *, p < 0.01. C and D, inhibition of TSC2-null tumor growth by amiloride. TSC2-null cells were injected in WT mice as in Fig. 6A. Mice received 10 mg/kg amiloride or vehicle alone daily as described under “Experimental procedures.” C, representative images of H&E-stained sections of the lungs of the vehicle- and amiloride-treated mice are shown. Images were taken using Olympus SZX-16 stereomicroscope equipped with Axiocam HRC camera (Carl Zeiss) and were recorded using Carl Zeiss Axio Vision 3.1 software. D, quantification of the areas occupied by nodules in six vehicle-treated and four amiloride-treated animals was performed as in Fig. 6B. Lungs of four of eight amiloride-treated animals did not contain detectable tumor nodules, an outcome that was never observed in vehicle-treated animals. The y axis shows the values calculated as the ratio between the area of the nodules and the entire lung area measured in square pixels and normalized, the mean value ± S.D. Sections, obtained at five levels for each lung, were analyzed. *, p < 0.02.
serious adverse events commonly encountered in practice (91, 92) and why rapamycin cannot be withdrawn without a rebound in growth of angiolipomas (18) and decline in lung function (20). Additional studies will be needed to assess whether rapamycin induces expression of uPA in TSC-deficient tumors in animal models and in tissues from patients with LAM and TSC-LAM and whether uPA contributes to disease progression or unresponsiveness to rapamycin in the clinical setting.

Our studies indicate that uPA is critical for progression of TSC2-null tumors. Growth of TSC2-null tumors is impaired in the lungs of uPA-knock-out mice (Fig. 6), suggesting stromal cell-derived uPA contributes to the growth of TSC2-null tumors. It has been shown that uPA and its uPA receptor contribute to the interaction between tumor cells and infiltrating stromal cells during tumor growth and dissemination (93–95). The relative contribution of tumor-derived versus host-derived uPA on tumor development was not established by our study, and additional studies will be needed to determine whether stromal cells contribute to the high levels of uPA expression in human LAM lesions.

Our studies show that silencing of uPA mRNA in LAM-like TSC2-null tumor cells using lentiviral shRNA significantly reduces tumorigenesis in vivo (Fig. 8, A and B). This outcome can be attributed in part to lower invasive, mitogenic, and survival capacities of TSC2-null/uPA-sh cells (Fig. 7, B–D). The results provide independent confirmation that uPA contributes directly to the development of TSC-null tumors rather than suggesting an off-target effect of uPA gene deletion in uPA−/− mice. Growth of TSC2-null/uPA-sh cell-derived tumors might be further reduced in uPA−/− mice if stroma-derived uPA also contributes to the progression of TSC2-null tumors.

Amiloride is a moderately potent inhibitor of uPA ($K_i = 7 \times 10^{-4} \text{m}$) that does not inhibit tissue-type plasminogen activator or other serine proteases, such as kallikrein, thrombin, or plasmin (96), and is used clinically as an oral potassium-sparing diuretic that inhibits the sodium–hydrogen exchanger 1 (NHE1). NHE1 is partially responsible for the low extracellular pH of tumors and has been identified as a potential target for anti-cancer therapeutics (97). Our studies demonstrate that amiloride suppresses the growth of tumor nodules in the lungs of WT mice injected with TSC2-null cells (Fig. 8, C and D). Additional studies will be necessary to determine whether the NHE1-inhibiting effects of amiloride contribute to the reduction in the growth of TSC2-null tumor cells in addition to its capacity to block uPA catalytic activity.

Together, these findings indicate that the uPA system contributes to the growth and dissemination of benign LAM-like tumors, similar to its role in the spread of malignant cancer cells (32, 98). The elastic fibers lining remodeled alveoli in the lungs of patients with LAM are scant and are often disrupted (99). It has been hypothesized that LAM tumor cells destroy host lung tissue and form cystic cavities by degrading the extracellular matrix through overproduction and activation of matrix-degrading proteases (MMPs) that are not properly counterbalanced by their endogenous inhibitors, TIMPs (100). Increased levels of MMP-1, MMP-2, MMP-9, MMP-11, and MMP-19 are found in lungs from patients with LAM (101). Using the experimental mouse model of LAM, we demonstrated previously that growth of TSC2-null lesions in murine lung is associated with an increase in MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12 (59), all with known elastase activity (102), which results in significant loss of elastin fibers in alveoli of mice bearing TSC2-null lesions (59). It is well known that uPA, which activates plasminogen to plasmin, is proximate to the proteolytic cascade that triggers the mutual activation of MMPs (103, 104). Of importance, FOXO3a, which activates uPA expression (62), also induces transcription of MMP-9 and -13 in breast cancer cells (105). We have also previously shown that uPA up-regulates MMP-9 expression via mechanisms that involve intracellular signal transduction (106). Therefore, our findings suggest that overexpression of uPA in LAM nodules might be responsible, at least in part, for overproduction and hyper-activation of MMPs involved in destruction of lung parenchyma.

Cell signaling, triggered by uPA binding to its cognate receptor uPAR, might also contribute to increased invasion, growth, and survival of uPA-overexpressing cells (107–109). uPA might also enhance the growth of renal angiolipomas by stimulating the expression of VEGF receptors by up-regulating gene expression directly, as we have shown previously (51). In support of the concept that uPA is involved in progression of LAM-like tumors, inhibition of uPA expression in TSC2-null tumor cells reduced their invasive and mitogenic potentials (Fig. 7, B and C) and increased their susceptibility to the pro-apoptotic agent simvastatin (Fig. 7D) (59, 63). Reduction in the invasive capacity of TSC2-null/uPA-sh cells might be directly linked to the ability of uPA to initiate proteolysis of extracellular matrix components. On the other hand, decrease in the growth rate, migration, and sensitization of TSC2-null/uPA-sh cells to simvastatin might involve intracellular signaling pathways activated by uPA (110, 111) through uPAR (108) or other cell membrane receptors (112–116) or through translocation of uPA to cell nuclei where it stimulates expression of VEGF receptors as part of a genetic program that enhances cell survival and tumor angiogenesis (51, 52).

In summary, our findings show that uPA is up-regulated in LAM nodules and TSC-null angiomyolipomas and mediates progression of TSC2-negative tumors in vivo, and the uPA inhibitor amiloride suppresses tumor growth in vivo. This raises the possibility that inhibition of uPA activity, for example with amiloride or WX-671 (117), or suppression of uPA synthesis using periodic pulse administration of dexamethasone or other glucocorticoids (as is used now to treat diverse hematopoietic cell-derived tumors and autoimmune conditions) might be effective in patients who are not responsive to or do not tolerate rapamycin and might permit dose reduction or even withdrawal of treatment in responding patients in some settings in this otherwise progressive and potentially fatal disease.

Experimental procedures

**Immunohistochemical analysis**

Human LAM and normal lung samples were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). Normal kidney and renal angiomyolipoma samples were obtained from the Cooperative Human Tissue Network (Eastern Division CHTN, Philadelphia, PA). Paraffin-embedded sections of LAM tissue and angiomyolipomas were depar-

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affinized in xylene and rehydrated in graded ethanol solutions. Blocking of endogenous peroxidase and permeabilization was performed in a 2.2% solution of H2O2 in methanol. Permeabilized sections were blocked with 10% horse serum diluted in 1% BSA, 1× automation buffer solution (blocking buffer) for 20 min at room temperature. Sections were incubated overnight at 4 °C with primary mouse monoclonal anti-human uPA antibody (American Diagnostica, catalog no. 3689) diluted in blocking buffer. The cells were washed and incubated with biotinylated horse anti-mouse secondary antibody diluted in blocking buffer for 30 min at 37 °C, followed by incubation with streptavidin-HRP Vectastain Elite ABC HRP kit (catalog no. PK-6100, Vector Laboratories, Burlingame, CA). The tyramide signal amplification reaction was performed using the Alexa Fluor 488 tyramide signal amplification kit (catalog no. T20912, Invitrogen-Thermo Fisher Scientific) following the manufacturer’s instructions. Tissue sections were then incubated with Cy3-conjugated mouse monoclonal anti-SM α-actin, followed by incubation with streptavidin-HRP Vectastain Elite ABC HRP kit (catalog no. PK-6100, Vector Laboratories, Burlingame, CA). The nuclei were counterstained with DAPI (catalog no. 015-000-003; Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated total mouse IgG (catalog no. bs-0296P-Cy3, Bioss Antibodies, Inc, Woburn, MA) were used as negative controls.

**Cell culture**

Tsc2−/−/p53−/− MEFS, Tsc2+/−/p53−/− MEFS, Tsc1−/− MEFS, and Tsc1+/− MEFS were generously provided by Dr. David Kwiatkowski (Brigham and Women’s Hospital, Boston, MA) (71); human TSC2-negative AML621-101 cells were generously provided by Dr. Elisabeth Henske (Brigham and Women’s Hospital) (60); rat TSC2-null ELT3 cells were the generous gift of Dr. Cheryl Walker (Texas A&M University) (71); human TSC2-negative AML621-101 cells were generously provided by Dr. Elisabeth Henske (Brigham and Women’s Hospital) (60); rat TSC2-null ELT3 cells were the generous gift of Dr. Cheryl Walker (Texas A&M University) (61, 118). Cells were maintained in DMEM supplemented with 10% FCS until studied.

**Derivation of TSC2-null tumor cells**

TSC2-null cells were derived from kidney tumors that develop in heterozygous Tsc2+/− mice that spontaneously acquired an inactivating mutation in the second allele of the Tsc2 gene (57, 58). The cells were injected into the flanks of immunodeficient mice where they formed subcutaneous tumors (59). Cells were then isolated from these subcutaneous nodules. These tumor-derived cells form tumors in the lungs of the immunodeficient mice after intravenous injection (59) or subcutaneous tumors if injected into the flanks of the immunocompetent mice. Tumor cells dissociated from these subcutaneous nodules are capable of forming tumors in the lungs of the immunocompetent mice after intravenous injection.5

5 K. Maisel, M. J. Merrilees, L. Lian, K. Obraziatsova, R. Rue, E. N. Atochina-Vasserman, N. Zuo, L. F. Angel, A. J. Gow, I. Kang, T. N. Wight, M. A. Swartz, E. Eruslanov, and V. P. Krymskaya, submitted for publication.

**Silencing of uPA and FOXO3 in mouse TSC2-null tumor cells**

These TSC2-null cells were infected with lentivirus encoding either control scrambled shRNA (TSC2-null/con-sh cells) or mouse uPA-targeting shRNA (TSC2-null/uPA-sh cells) or mouse FOXO3-targeting shRNA (Santa Cruz Biotechnology, catalog nos. sc-108080, sc-36779-V, and sc-37888-V, respectively). Transfected cells were selected with puromycin (1–2 μg/ml). Expression of uPA and FOXO3 was monitored by Western blotting using anti-mouse uPA rabbit polyclonal antibodies (Meridian Life Sciences, catalog no. K63679R) and anti-FOXO3 rabbit monoclonal antibodies (Cell Signaling Technology, catalog no. 2497).

**Silencing of Tsc2 in mouse embryonic fibroblasts**

TSC1+/+ MEFS (wild type, WT) were infected with lentivirus encoding either control scrambled shRNA or mouse TSC2-targeting shRNA (Santa Cruz Biotechnology, catalog nos. sc-108080 and sc-36763-V, respectively). Transfected cells were selected with puromycin (4 μg/ml). Expression of tuberin was monitored by Western blotting using a rabbit anti-tuberin polyclonal antibody (Cell Signaling Technology, catalog no. 893).

**Migration and invasion assays**

Mouse TSC2-null tumor cells infected with control or mouse uPA-targeting shRNA LVs were serum-deprived for 24 h in DMEM, 0.1% BSA for TSC2-null tumor cells. Starved cells were detached by trypsin as above, washed, and resuspended in DMEM, 0.1% BSA or complete DMEM, 10% FBS medium for TSC2-null cells. FluoroBlokTM transwell inserts (Corning, catalog no. 3511520, Tewksbury, MA) were left uncoated or were coated with the growth factor-reduced Matrigel (Costar, catalog no. 356230), diluted 1:50 with DMEM, 0.1% BSA, which was allowed to solidify for 30 min in a humidified incubator. Transwells were inserted into their holders; 0.5 ml of the cell suspensions (5 × 10^3 cells/ml) were added, and cells were allowed to migrate for 18 h per the manufacturer’s instructions. No increase in total cell number was observed during this time (data not shown). Migrating cells were visualized by pre-loading with calcein AM and Hoechst 33342 dyes and then fixed in 4% paraformaldehyde/PBS. Cells were photographed using with an EVOS® FL Auto Cell Imaging System (Thermo Fisher Scientific, Waltham, MA) using a ×4 objective. Cell numbers in each microscopic field were quantified using EVOS® software Auto Count feature.

**Growth and apoptosis luminescence assays**

To measure cell growth, equal numbers of mouse TSC2-null tumor cells infected with control or mouse uPA-targeting shRNA LVs were plated in clear bottom black 96-well plates (Corning, catalog no. 3603, Corning, PA) (500 cells per well) in three separate plates. Immediately, 48 and 96 h later, cells were quantified using the CellTiter-Glo® Luminescent Cell Viability Assay (catalog no. G7570, Promega, Madison, WI). To measure apoptosis, equal numbers of mouse TSC2-null tumor cells infected with control or mouse uPA-targeting shRNA LVs were
plated in clear bottom black 96-well plates (5,000 cells per well). Twenty four hours later, the cells were incubated with simvas-
tatin (catalog no. 567022, Calbiochem) for 24 h. Apoptosis was measured using the Caspase-Glo® 3/7 assay (catalog no. G8091, Promega), and luminescence was quantified using a Synergy 2 multimode microplate reader and Gene5 software (BioTeK, Winooski, VT)

**Cell treatments, lysis, and Western blot analysis**

Cells were plated at 50% confluence in 60-mm dishes in DMEM, 10% FBS (complete) medium, starved in 0.1% BSA/ DMEM (serum-free) for 24 h. Cells were incubated with rapa-
mycin (catalog no. 8781, Sigma) at the indicated concentrations for 24 h. In separate experiments, starved cells were pre-incu-
bated with various inhibitors (simvastatin (10 μM, catalog no. 567022), PD98059 (50 μM, catalog no. 513001), LY294002 (10 μM, catalog no. 440202), 1-(2-(hydroxymethyl)-chiro-inositol-
2-[(R)-2-O-methyl-3-O-octadecylcarbonate] (10 μM, catalog no. 124005), AS184586 (1 μM catalog no. 506081), dorsomor-
phin (compound C) (20 μM, catalog no. 171260) (all from Cal-
biochem), or dexamethasone (1 μM, catalog no. API-04, GBio-
sciences, St. Louis, MO) or SGK-1 inhibitor (50 μM, catalog no. 3572, Tocris-Bio-Technne, Minneapolis, MI) and were then incubated with rapamycin (20 nM) for 24 h. In some experiments TSC2-null cells were starved for 24 h, incu-
bated with 20 nM rapamycin for 24 h, washed, and then incu-
bated with 1 nM dexamethasone (or vehicle control) for an additional 24 h. The cells were then lysed in 1× RIPA buffer diluted 10 times from 10× RIPA buffer (catalog no. 98065, Cell Signal-
ing Technologies, Beverly, MA) with added 1× proteinase inhibitor mixture diluted from a 100× concentrate (catalog no. 8340, Sigma) and 1× phosphatase inhibitors mixture twice diluted from a 100× concentrate (catalog no. P5726, Sigma). The protein concentrations in cell lysates were measured using the Bradford Protein Assay (Bio-Rad, catalog no. 5000006).

**Reverse transcription and quantitative PCR**

Total RNA was isolated using RNaseasy mini kit (Qiagen, catalog no. 74174) from Tsc1−/− and Tcs1+/+ MEFs, Tsc2−/− (p53−/−) and Tsc2+/+ (p53−/−) MEFs, and mouse Tsc2-null tumor cells incubated with rapamycin in the absence or presence of AS184586, dexamethasone, or dorsomorphin or con-
trol media. RNA samples were converted to cDNA using Ther-
moScript reverse transcriptase kit (Thermo Fisher Scientific, 11146-016). Quantitative PCR was performed in triplicate using the StepOne real time PCR system (Applied Biosystems), SYBR Green Fast master mix (Applied Biosystems, catalog no. 4385612), and the primers specific to Plau gene (listed below). Differential expression was calculated as fold-increase using the ΔΔCt method (119) with normalization to β-actin. The primers used were as follows: uPA forward, 5′-GAGCAGCTC-
ATCTTGACGGAATAAC-3′, and reverse, 5′-GCCAGTGATC-
TCACAGTCTGAACC-3′; and β-actin forward, 5′-CATCCG-
TAAAGACCTCTATGCCAAC-3′, and reverse, 5′-ATGGAG-
CCACCGATCCACA-3′.

**Animals**

uPA−/− mice on C57BL/6 background and WT C57BL/6 mice were obtained under a Material Transfer Agreement between the Russian Cardiology Research and Production Center (Moscow, Russia) and the FIRI Institute for Molecular Oncology (Milan, Italy). The colony was maintained at the Pushchino nursery (Pushchino, Russia). In the first set of experiments, TSC2-null, TSC2-null/con-sh, or TSC2-null/uPA-sh cells were plated in cell culture dishes in DMEM supplemented with 10% FCS as described (59). After 48 h in culture, the cells were detached by trypsin/EDTA, washed, resuspended in DMEM, filtered, and counted; 5 × 10⁶ cells were injected into the jugular vein of 8-week-old male uPA−/− and/or WT mice. The weights of the animals were monitored throughout the experiment. In a second series of experiments, mice were given daily intraperitoneal injections of the uPA inhibitor amiloride HCl (10 mg/kg in 100 μl, Sigma, catalog no. A7410) (69, 120) starting 1 day after the injection of tumor cells. In both sets of experiments, animals injected with TSC2-null cells were eutha-
nized on day 20 or if an animal showed signs of respiratory distress or significant weight loss.

Lungs were inflated at 25-cm water pressure with formalin. The tracheas were tied off, and the lungs were excised, placed in formalin, paraffin-embedded, and sectioned into 5-μm-thick slices followed by staining with H&E and immunohistochemi-
ical analysis. Each experimental group included a minimum of five animals per condition. Tissue samples were analyzed by two independent investigators at the Cardiology Research Cen-
ter and by one investigator at the University of Pennsylvania. The area occupied by LAM-like tumor area relative to total lung area was determined using Meta Imaging Series software (Molecular Devices.).

**Statistical analysis**

Differences between groups were compared using the one-
way analysis of variance statistical test and non-parametric Mann-Whitney U test. Statistical analyses were performed using the EZAnalyst add-in to Microsoft Excel software and Statistica 10.0 software (StatSoft, Tulsa, OK). Significance was set at a p value of less than 0.05.

**Study approval**

All experimental procedures were performed according to the "Rules for Carrying Out Experiments Using Laboratory
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Animals” of the Russian Cardiology Research and Production Center, Moscow, Russia.

Author contributions—V. S., Ye. V. P., V. P. K., and D. B. C. contributed to the conception of the research. V. S., K. V. D., K. R. H., Z. I. T., E. A.-V., M. T., A. V., S. V. Z., S. P. L., F. G. Z., and K. O. performed the experiments and analyzed the data. V. S., V. P. K., and D. B. C. wrote the manuscript.

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