Development of a Lymphangioleiomyomatosis Model by Endonasal Administration of Human TSC2−/− Smooth Muscle Cells in Mice

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Lymphangioleiomyomatosis (LAM) is an interstitial lung disease characterized by invasion and proliferation of abnormal smooth muscle (ASM) cells in lung parenchyma and axial lymphatics. LAM cells bear mutations in tuberous sclerosis (TSC) genes. TSC2−/− ASM cells, derived from a human renal angiomyolipoma, require epidermal growth factor (EGF) for proliferation. Blockade of EGF receptors (EGFR) causes cell death. TSC2−/− ASM cells, previously labeled with PKH26-GL dye, were endonasally administered to 5-week-old immunodeficient female nude mice, and 4 or 26 weeks later anti-EGFR antibody or rapamycin was administered twice a week for 4 consecutive weeks. TSC2−/− ASM cells infiltrated lymph nodes and alveolar lung walls, causing progressive destruction of parenchyma. Parenchymal destruction was efficiently reversed by anti-EGFR treatment and partially by rapamycin treatment. Following TSC2−/− ASM cell administration, lymphangiogenesis increased in lungs as indicated by more diffuse LYVE1 expression and high murine VEGF levels. Anti-EGFR antibody and rapamycin blocked the increase in lymphatic vessels. This study shows that TSC2−/− ASM cells can migrate and invade lungs and lymph nodes, and anti-EGFR antibody is more effective than rapamycin in promoting lung repair and reducing lymphangiogenesis. The development of a model to study metastasis by TSC cells will also help to explain how they invade different tissues and metastasize to the lung. (Am J Pathol 2012, 181:947–960; http://dx.doi.org/10.1016/j.ajpath.2012.05.017)

Lymphangioleiomyomatosis (LAM) is a rare lung disease that leads to progressive respiratory failure and that affects women of childbearing age almost exclusively. LAM is characterized by widespread proliferation of abnormal smooth muscle–like (ASM) cells in the lung, which leads to destruction of lung parenchyma, fluid-filled cystic structures in the axial lymphatics, and abdominal tumors (ie, angiomyolipomas).1–3 LAM ASM cells are part of characteristic LAM nodules responsible for cystic destruction and angiomyolipomas in kidney. LAM can be sporadic or associated with tuberous sclerosis complex (TSC). TSC is an autosomal dominant syndrome characterized by hamartoma-like tumor growths in organs such as brain, kidney, skin, retina, and heart.4 TSC is caused by mutations in TSC1 or TSC2, which encode hamartin and tuberin, respectively. These two proteins function together to inhibit mammalian target of rapamycin (mTOR)–mediated signaling to S6K.5 In the absence of hamartin or tuberin, mTOR is highly activated, leading to abnormal translation and cellular proliferation. Identical TSC2 mutations and/or loss of heterozygosity (LOH) in cells of sporadic LAM patients that have no identifiable germline TSC2 mutation suggest a probable common genetic association.6–8 Moreover, consistent with the metastatic theory, cells bearing LOH for TSC genes have been isolated from blood of LAM patients.9 Whether the ASM cells arise in the airways or the vasculature is unclear. ASM cells could also be the result of a distant metastasis from angiomyolipomas or the uterus.10–12

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We isolated pure human TSC2−/− smooth muscle (ASM) cells derived from a renal angiomyolipoma. The cells were rich in smooth muscle α-actin, showed HMB45 antibody labeling, EGF-dependent proliferation, and hyperphosphorylation of S6 kinase and its substrate S6. The lack of tuberin underlies EGF dependency, as transfection of TSC2 into TSC2−/− ASM cells removed the EGF requirement for proliferation. EGF dependency is caused by the loss of TSC2 gene function. In another human TSC2 cell line with LOH caused by promoter methylation, the lack of tuberin leads to EGF dependence. No EGF supplementation was necessary once these cells were treated with demethylating agents and tuberin was expressed. Moreover, antibodies to EGF receptor (anti-EGFR) cause cell death in both TSC2-deficient cell types. To evaluate the invasiveness of the TSC2-deficient cells and the therapeutic potential of an anti-EGFR antibody, we developed an in vivo model of LAM by endonasal administration of TSC2−/− ASM cells to nude mice. Lymph nodes and alveolar lung walls were quickly infiltrated and lesions formed. Anti-EGFR antibody labeling, EGF-dependent proliferation, and hyperphosphorylation of S6 kinase and its substrate were reported by Lesma et al. The CT/G human aorta vascular smooth muscle cells (VSMCs; ATCC, Manassas, VA) were grown in DMEM containing 10% fetal bovine serum.

Cell Culture Labeling

TSC2−/− ASM cells, VSMCs, and TSC2−/− ASM cells transfected with TSC2 were labeled with PKH26-GL using Red Fluorescent Cell Linker kits (Sigma-Aldrich). PKH-26 has been used in long-term follow-up studies. Cells were incubated with PKH26-GL in diluent C at 37°C for 4 minutes, and the reaction was stopped by adding an equal volume of FBS. The cell suspension was centrifuged, washed with DMEM, washed with PBS, and resuspended in 25 μL of physiological solution before being administered to the mice.

Generation of Tuberin-Expressing Cell Lines

Transfection was performed as previously described by Astrinidis et al. Briefly, GP2-293 cells were co-transfected with 2 μg of the retroviral vector pMSCVneo (a gift from Dr. A. Astrinidis and E.P. Henske, Fox Chase Cancer Center, Philadelphia, PA), 1 μg pVSV-G (BD Biosciences–Clontech) encoding the viral glycoprotein, and 6 μg of FuGene6 (Roche, Indianapolis, IN). Replication-deficient retroviruses were collected from the culture after 72 hours and applied to subconfluent TSC2−/− ASM cells in the presence of 8 μg/mL of polybrene (Sigma-Aldrich). Cells were transduced with empty pMSCVneo vector as a control, or a pMSCVneo construct containing the coding region of the human TSC2 gene. Stable clones were selected for 2 weeks in the presence of 100 μg/mL of G418 (Sigma-Aldrich).

Endonasal Cell Administration

After light anesthesia by intramuscular injection of 4% chloral hydrate, 2 × 105 TSC2−/− ASM cells, TSC2−/− ASM stably transfected with TSC2 or VSMCs, all labeled with PKH26-GL, were endonasally administered to each immunodeficient female nude mouse using a microtip and allowing the mice to breathe the drop (25 μL) of physiological solution (0.9% NaCl) containing the cells. Mice were randomly assigned to control and experimental groups.

Pharmacological Treatments

At 4 or 26 weeks posttreatment, mice were injected intraperitoneally (i.p.) with anti-EGFR antibody twice weekly at a starting dose of 400 mg/m² followed by a subsequent dose of 250 mg/m² (Merck, Darmstadt, Germany), or 4 mg/kg rapamycin (Rapamune-Sirolimus; Wyeth Europe, Maidenhead, UK) twice weekly for 4 weeks. At 8 or 30 weeks after cell administration, the mice were sacrificed and blood, lungs, and lymph nodes removed.

To study the effects of cessation of treatment, mice were administered anti-EGFR antibody or rapamycin for 4 weeks,
from week 26 to week 30, as described above, and then were taken off the drugs for 8 weeks before being sacrificed.

**Locomotor Climbing Activity**

Mice were exercised and run to exhaustion for 5 minutes in a wire mesh grid that was 5 × 50 cm in a support with a 70° inclination angle. Barriers were placed along the perimeter to prevent the mice from climbing around to the back of the apparatus. Run time was recorded after 5 minutes of training. The maximal time allowed was 60 seconds.

**Tissue Preparation**

The mice were sacrificed by exsanguination under 4% chloral hydrate anesthesia. Next the chest cavity was opened and the diaphragm incised. Lungs were then inflated (25 cm H₂O) with 10% paraformaldehyde (Sigma-Aldrich) for 10 minutes, after which the trachea was tied off and the lungs excised. The lymph nodes were then removed.

All specimens were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin or optimal cutting temperature (OCT) (Bio-Optica, Milan, Italy) compound. After fixation, serial sagittal sections of lungs were cut parallel to a plane perpendicular to the diaphragmatic pleura passing through the apex of the lobe. These ran from the costal surface toward the hilum. Lymph nodes were trimmed and randomly oriented in preparation for sectioning. Slices were made in random directions.

The paraffin samples were sectioned, deparaffinized, and rehydrated in xylene and graded concentrations of ethanol to distilled water. The sections were stained with hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) for morphological evaluation. To obtain cryostat sections, after fixation in paraformaldehyde, specimens were immersed in 25% and 30% sucrose (Sigma-Aldrich) solution overnight for cryoprotection, embedded in OCT compound, and frozen at −80°C. Frozen sections were cut; air dried, rehydrated with TBS and stained using the immunohistochemical protocol described below.

**Histology and Immunohistochemistry**

Immunohistochemical analyses were performed using standard avidin-biotin–alkaline phosphatase complex (ABC-AP Vector Laboratories, Burlingame, CA) or avidin-biotin immunoperoxidase (ABC Ultrasensitive Peroxidase Staining kit; Pierce) staining procedures using paraffin sections. For staining with monoclonal anti-human-HLA-A, B, C, and estrogen and progesterone receptors, frozen sections were used.

Sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and heated at 95°C for 20 minutes for antigen retrieval. Nonspecific binding was blocked with 3% BSA/TBS for 1 hour. Antibodies were incubated overnight at 4°C: human-HLA-A, B, C (1:50; BD Biosciences, Le Pont-De-Claix Cedex, France), phospho-S6 (Ser235/236) (1:50; Cell Signaling Technology, Beverly, MA), CD68 (1:300, Thermo Scientific, Freemont CA), and CD45 (1:300, DAKO). Samples were incubated with the appropriate secondary biotin-conjugated antibody for 1 hour at room temperature. ABC-AP or ABC was applied for 30 minutes, and detection was performed with Vector Red or 3,3′-diaminobenzidine tetrahydrochloride (DAB Substrate kit, Pierce). For double immunostaining, the first color reaction was visualized with Vector Red and the second antibody pair was visualized with the ABC reagent and DAB. Incubations were conducted at room temperature. Finally, sections were counterstained with Mayer’s hematoxylin. The phospho-S6 labeling was quantified by counting the number of phospho-S6-positive cells and the total number of cells (identified by nuclei) and expressed as a percentage. For each lung, at least five randomly selected fields per slide were evaluated.

For immunofluorescence, after fixation and blocking for 1.5 hours with 3% BSA, sections were incubated with LYVE-1 (5 μg/mL, R&D System, Minneapolis, MN) or HMB45 antibody (1:70 DAKO, Glonstrup, Denmark) for 1.5 hours at room temperature, and then with an Alexa Fluor 488-conjugated secondary antibody (1:200; Molecular Probes, Eugene, OR). Nuclei were stained with 2 μg/mL DAPI (Sigma-Aldrich). Appropriate controls for immunofluorescence were performed by substituting the primary antibody with normal goat serum or isotype control (mouse IgG1K). Sections were analyzed using a laser scanning confocal microscope (LEICA DMIRE2, Wetzlar, Germany). Relative optical density of protein expression was measured using Leica confocal software (Quantification, Tool Leica Confocal Software-TCS SP2, Leica Confocal Microscopy, Mannheim, Germany) on the confocal images and analyzing at least five randomly selected fields for each group.

For cell immunofluorescence, TSC2−/− ASM were cultured on glass slides, permeabilized with Cytoskeleton (Cytoskeleton, Denver, CO) and air dried. Samples were incubated with primary antibodies against estrogen receptors (1:250; Santa Cruz) or progesterone receptors (1:250; Santa Cruz), overnight at 4°C. Slides were incubated with FITC-conjugated anti-rabbit antibody (1:200; Molecular Probes). Nuclei were detected by DAPI staining. Sections were analyzed using a confocal microscope.

**Counting TSC2−/− ASM Cells in Lungs**

The number of PKH-26-GL–positive TSC2−/− ASM cells in lung parenchyma was determined by two independent observers who counted 10 randomly selected fields for each treatment group. TSC2−/− ASM cell number was based on the following formula: ASM cells/mm² = (TSC2−/− ASM cells/mm³) × (10³×[5.53 μm² + 5 μm]), where 5.53 μm represents the mean TSC2−/− ASM cell diameter and 5 μm is the section thickness.
**TUNEL Assay**

Nuclei were detected by DAPI staining. The number of apoptotic cells in lung sections was evaluated using a DeadEnd Fluorometric TUNEL kit (Promega, Madison, WI).

**Analysis of TSC2−/− ASM Cell Proliferation in Lungs**

Cell proliferation was detected by staining with Ki-67 antibody and hematoxylin counterstaining. The Ki-67 labeling index for the parenchyma was determined by counting the number of Ki-67–positive nuclei per total number of nuclei and was expressed as a percentage. For each lung, at least five randomly selected fields per slide were evaluated.

**Real-Time Reverse Transcription–PCR**

Total RNA was isolated from fresh tissue using the TRIzol reagent (Invitrogen, Paisley, UK). Reverse transcription of 1 µg of total RNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Paisley, UK) was used to obtain cDNA. For Taqman analysis, primers for PCNA (Mm 00448100_g1) and 18S (Eukariotic 18s rRNA endogenous Control) were obtained from the Applied Biosystems Assays on Demand system (Applied Biosystems, Foster, CA). Primer production and validation was carried out by Applied Biosystems.

Real-time PCR analysis of gene expression was performed using an MJ Opticon PCR analyzer (MJ Research, Waltham, MA). The PCR protocol was one cycle of 50°C for 2 minutes, one cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The data generated were analyzed using autoCt and autobaseline as outlined by the supplier (Applied Biosystems User Bulletin #2), and Ct values were exported to Excel. A comparative Ct method of analysis was used, whereby endogenous 18S control was used to normalize expression levels of the target gene by correcting for differences in starting material.

**Morphometric Analysis**

The density of air-exchanging parenchyma, Aa (ae/lu), was determined by point counting based on computer-assisted image analysis. The number of points falling on air-exchanging parenchyma (peripheral lung parenchyma excluding airspace) in random lung fields was divided by the number of points falling on the entire field (tissue and airspace).

**ELISA**

Murine VEGF (R&DSystem) and murine EGF (Peprotech, Rocky Hill, NJ) levels in serum and lung homogenates were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Frozen lungs were homogenized in homogenization buffer (50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EGTA, 5 mmol/L EDTA, 2%[v/v] Nonidet P-40, 10 µg/mL leupeptin, 50 ng/mL phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine-HCl, and 1 mmol/L aprotinin). The levels of murine VEGF and EGF were then measured according to the manufacturer’s instructions.

**Dissociation and Flow-Cytometric Analysis of Cells from Lungs and Lymph Nodes of Nude Mice**

Lungs and lymph nodes dissected from control and TSC2−/− ASM cell–injected mice were excised, washed in PBS, cut into small fragments, and suspended in a pre-warmed cell dissociation solution containing protease, collagenase and DNase activities (ACCUMAX, Milipore, Vimodrone, Italy). The tissue fragments were then incubated for 1.5 hours at 37°C with occasional agitation. Following complete disaggregation, cells were transferred to sterile tubes and centrifuged at 1200 rpm to pellet the cells and remove the ACCUMAX solution. Cells were then fixed in fixation buffer (BD Bioscience) for 1 hour at 4°C. Fixed samples were pelleted and permeabilized with PermWash buffer I (BD Bioscience) for 1 hour at room temperature. The samples were incubated with anti-phospho-S6 (Ser235/236) (1:50; Cell Signaling Technology) for 1.5 hours at room temperature. After washing with PermWash buffer, samples were incubated with Alexa Fluor 488-conjugated secondary antibody (1:200; Molecular Probes) for 1.5 hours at room temperature in the dark. Data were collected using a Cytomics FC500 flow cytometer (Beckman Coulter, Milan, Italy). Fluorescence signals were collected using amplifiers that reported on a logarithmic scale (FL1 channel for phospho-S6 signal, FL2 channel for PKH-26 signal); forward and side scatter signals were recorded on a linear scale. Data analyses were performed using CXP 2.2 software (Beckman Coulter).

**Data Analysis**

Data are expressed as mean ± SEM and were statistically analyzed using Student’s t-test. Statistical significance is indicated for P values of *P < 0.05; **P < 0.01; ***P < 0.001; †P < 0.05; ††P < 0.01; and †††P < 0.001 in the figures. Error bars throughout the figures indicate SEM.

**Results**

**Human TSC2−/− ASM Cells Survive and Migrate to Lungs and Lymph Nodes**

Nude mice have been extensively used as an experimental model to study the tumorigenicity of tumor cell lines. They may also constitute an ideal model for gaining insights into the mechanisms of in vivo expansion and migration of TSC2 smooth muscle cells and the effectiveness of drugs against such cells. The ability of PKH26-labeled TSC2−/− ASM cells to survive and migrate in vivo was monitored after endonasal administration of the cells to 5-week-old female nu/nu Hsd:athymic nude mice.
TSC2\(^{-/-}\) ASM cell were detected in the lungs, but not in the lymph nodes, 24 hours after endonasal administration, which suggests that TSC2\(^{-/-}\) ASM cells spread through pulmonary airspaces (Figure 1A). The labeled cells were observed in cervical, mediastinic, and retroperitoneal lymph nodes at 7 and 8 days; and 30 weeks, after administration (Figure 1A). TSC2\(^{-/-}\) ASM cells aggregated in clusters in lymph nodes at the early stages of infiltration; but later, the cells were more dispersed (Figure 1A). By contrast, labeled TSC2\(^{-/-}\) ASM cells were evenly distributed in the lungs, particularly at longer times (Figure 1A, bottom). Antibodies against human HLA-A, B, C also confirmed the presence of human TSC2\(^{-/-}\) ASM cells in the lungs (Figure 1B). The inhaled TSC2\(^{-/-}\) ASM

**Figure 1.** After endonasal administration, TSC2\(^{-/-}\) ASM cells accumulated in lymph nodes and lungs. TSC2\(^{-/-}\) ASM cells (2.5 \(\times\) 10\(^5\) cells) were labeled with PKH26-GL (red) and endonasally administered to 5-week-old nude mice. **A:** PKH26-labeled cells were not detectable in lymph nodes 24 hours after cell administration, but were detected in lungs (left panels, low magnification, right panels, high magnification). Scale bars: 75 \(\mu\)m (left); 30 \(\mu\)m (right). By 7 days after cell administration, PKH26-labeled cells were detected, by confocal microscopy, in mediastinic and retroperitoneal lymph nodes (scale bars: 300 \(\mu\)m for low magnification and 100 \(\mu\)m for high magnification). At longer times, 8 or 30 weeks post-administration of TSC2\(^{-/-}\) ASM cells, PKH26-labeled TSC2\(^{-/-}\) ASM cells were still present in the lymph nodes and lungs of nude mice. Scale bars = 75 \(\mu\)m and 30 \(\mu\)m, respectively. Sections were stained with DAPI to show nuclei (blue) (**B**) TSC2\(^{-/-}\) ASM cells could be detected in nude mice lungs by anti–human-HLA staining (arrows) 30 weeks after administration. Scale bars: 100 \(\mu\)m (upper panels, low magnification); 50 \(\mu\)m (lower panels, high magnification).
Anti-EGFR Antibody Treatment Reduces the Numbers of TSC2−/− ASM Cells in Lungs and Lymph Nodes

To quantitatively assess the number of TSC2−/− ASM cells in lungs, PKH26 positive cells were counted 30 weeks after administration (Figure 2, A and B). The average number of positive cells in lung parenchyma was 2.1 ± 0.6/mm² or 197.1 ± 58.27/mm³ (Figure 2B). We previously reported that the anti-EGFR antibody blocks the in vitro growth of TSC2−/− ASM cells, and causes their death more efficiently and specifically than rapamycin.13,15 To assess the in vivo action of the two drugs, anti-EGFR antibody and rapamycin were administered i.p. 26 weeks after cell inhalation. Drug treatments were given twice weekly for 4 weeks. The average number of PKH26-positive TSC2−/− ASM cells was significantly reduced to 0.34 ± 0.13/mm² or 32.17 ± 12.74/mm³ by anti-EGFR antibody. However, rapamycin was only marginally effective and reduced the TSC2 cell number to 1.41 ± 0.57/mm² or 134.18 ± 53.94/mm³ (Figure 2, A and B). Thus, anti-EGFR antibody was significantly more effective in reducing the number of TSC2−/− ASM cells harbored by lung parenchyma. To assess the number of TSC2−/− ASM cells, flow cytometry was applied to cells isolated from lungs. By 30 weeks after cell administration, 0.2% of lung cells were PKH26 positive, whereas no PKH26 labeling was detectable after drug treatment. In lymph nodes, no cells with a PKH26 signal were detectable by this method.

The presence of TSC2−/− ASM cells in lungs and lymph nodes 30 weeks after inhalation was also indicated by the presence of cells that reacted positively with the HMB45 antibody (Figure 2, A and C), which recognizes gp100 on TSC and LAM cells.21 All of the HMB45 antibody-positive cells were also labeled by PKH26, whereas some PKH26 positive cells (~20%) were not recognized by the HMB45 antibody, similar to in vitro observations.15 Following anti-EGFR antibody treatment, the extent of HMB45 labeling was markedly reduced, whereas rapamycin was much less effective (Figure 2, A–E). The effects of combined treatment of anti-EGFR antibody and rapamycin are now under investigation, and preliminary results indicate that the combination is more effective in reducing the number of TSC2−/− ASM cells.

TUNEL analysis of the lungs showed a very small number of TUNEL-positive apoptotic cells that was similar in all experimental groups (Figure 2F). Thus, under the conditions and drug concentrations used in the current study, neither anti-EGFR antibody nor rapamycin caused apoptosis in lungs.
Evaluation of S6 Phosphorylation and Cell Proliferation After Endonasal Administration of TSC2−/− ASM Cells and Effect of Anti-EGFR Antibody and Rapamycin on Lungs

Higher phosphorylation of S6 is typical of TSC cells, as S6 activation occurs downstream of the mTOR/S6K1 signaling pathway. The number of phospho-S6–positive cells was much higher in lungs at 8 and 30 weeks after TSC2−/− ASM cell administration (Figure 3, A and C, and B and D, respectively). Both rapamycin and anti-EGFR antibody treatments reduced the number of phospho-S6–positive cells in lungs. Quantification of phospho-S6–positive cells in lungs by FACS analysis confirmed these data (Table 1). Rapamycin exposure rapidly affects the extent of S6 activation in lungs. Human COX IV staining (brown) shows human TSC2−/− ASM cells. Arrows indicate the co-localization of COX IV and phospho-S6 expression. Scale bars = 50 μm. The percentage of phospho-S6–positive cells was assayed by counting phospho-S6–positive cells versus the total number of nuclei (C and D) in lungs, 8 or 30 weeks after cell administration, respectively. E: By 30 weeks after TSC2−/− ASM cell administration, the extent of cellular proliferation in the lungs was increased, as determined by Ki-67 labeling. Sections were stained with hematoxylin and Ki-67, and the percentage of Ki-67 positive cells versus the total number of nuclei was determined (B). Both drug treatments affected cell proliferation in the lungs. TSC2−/− ASM cells were identified using anti-human–COX IV (red). Arrows indicate co-localization of COX IV and Ki-67 expression. Scale bars = 50 μm. G: Multiplex Taqman analysis of PCNA expression relative to 18S rRNA in lungs indicated an increase in PCNA mRNA following rapamycin treatment. Bars represent the mean ± SEM. **P < 0.01, ***P < 0.001 percentage of phospho-S6 or Ki-67–positive cells in TSC2−/− ASM cell–administered mice versus percentage of phospho-S6–or Ki-67–positive cells in control mice; †P < 0.05, ††P < 0.01, and †††P < 0.001, percentage of phospho-S6–or Ki-67–positive cells in drug-treated mice versus TSC2−/− ASM cell–administered mice.

Figure 3. The number of cells with S6 phosphorylation at Ser235/236 (red staining) was increased in lungs 8 or 30 weeks after TSC2−/− ASM cell administration (A–D). Anti-EGFR antibody (Anti-EGFR Ab) and rapamycin reduced the extent of S6 activation in lungs. Human COX IV staining (brown) shows human TSC2−/− ASM cells. Arrows indicate the co-localization of COX IV and phospho-S6 expression. Scale bars = 50 μm. The percentage of phospho-S6–positive cells was assessed by counting phospho-S6–positive cells versus the total number of nuclei (C and D) in lungs, 8 or 30 weeks after cell administration, respectively. E: By 30 weeks after TSC2−/− ASM cell administration, the extent of cellular proliferation in the lungs was increased, as determined by Ki-67 labeling. Sections were stained with hematoxylin and Ki-67, and the percentage of Ki-67 positive cells versus the total number of cells was determined (F). Both drug treatments affected cell proliferation in the lungs. TSC2−/− ASM cells were identified using anti-human–COX IV (red). Arrows indicate co-localization of COX IV and Ki-67 expression. Scale bars = 50 μm. G: Multiplex Taqman analysis of PCNA expression relative to 18S rRNA in lungs indicated an increase in PCNA mRNA following rapamycin treatment. Bars represent the mean ± SEM. **P < 0.01, ***P < 0.001 percentage of phospho-S6 or Ki-67–positive cells in TSC2−/− ASM cell–administered mice versus percentage of phospho-S6–or Ki-67–positive cells in control mice; †P < 0.05, ††P < 0.01, and †††P < 0.001, percentage of phospho-S6–or Ki-67–positive cells in drug-treated mice versus TSC2−/− ASM cell–administered mice.

Table 1. Phospho-S6 Expression in Lungs of Nude Mice

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<th>Control</th>
<th>TSC2−/− ASM cells</th>
<th>Anti-EGFR Ab</th>
<th>Rapa</th>
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<td>P-S6-positive cells (%)</td>
<td>4.3 ± 0.9452</td>
<td>18.2 ± 1.1504*</td>
<td>14.9 ± 1.1015†</td>
<td>5.4 ± 0.5605‡</td>
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Flow-cytometric analysis of phospho-S6 expression in cells isolated from murine lung specimens. Phospho-S6 is significantly more abundant in lungs of mice injected with TSC2−/− ASM cells. Drug treatments significantly reduced such expression.

*P < 0.001 versus control.

†P = 0.05 versus TSC2−/− ASM cells.

‡P < 0.001 versus TSC2−/− ASM cells.

vitro; phosphorylation is almost abolished within 48 hours, but the TSC2 cells are fully viable. Thus the reduced number of phospho-S6–positive cells in rapamycin-treated mice may not necessarily imply a significant reduction in the TSC2 cell number, as shown by the quantitative evaluation of labeled TSC2−/− ASM cells (Figure 2).

The proliferative potential of TSC2−/− ASM cells in vivo was assayed by measuring the expression of the proliferation markers Ki-67 and PCNA. During interphase, the Ki-67 antigen can be detected exclusively within the cell nucleus; whereas, in mitosis, most of the protein is relocated to the surface of the chromosomes. Ki-67 is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent in resting cells (G0). The percentage of Ki-67 positive cells was as high as 40% in lungs of mice 30 weeks after TSC2−/− ASM cell admin-
Antibody or Rapamycin on Lymph Nodes

(TSC2 antibody (Anti-EGFR Ab) and rapamycin reduced the extent of S6 activation in lymph nodes. Anti-human–COX IV (brown) was used to identify human cells (Figure 4, A–D). A real-time RT-PCR assay showed that PCNA mRNA levels were increased significantly after TSC2−/− ASM cell administration. Although anti-EGFR antibody treatment reduced PCNA mRNA levels to those of the controls, rapamycin treatment did not (Figure 4G).

**TSC2−/− ASM Cells Cause Lung Degeneration**

On gross examination, no distinguishable differences were found among the lungs belonging to the different experimental groups. After infiltration of TSC2−/− ASM cells, we observed enlarged airspaces throughout the lungs that were similar to those observed during emphysema, and enlargement of alveolar spaces compared with the control (Figure 5). The morphological changes were much more pronounced at 30 weeks than at 8 weeks. When animals were treated for 4 weeks with anti-EGFR antibody, lung destruction was almost completely reversed at both 8 and 30 weeks, whereas rapamycin treatment caused thickening of lung parenchyma (Figure 5, A and C). Quantitative evaluation of the density of air-exchanging parenchyma [\( A_{\text{ae}}/lu \)] confirmed the positive effect of anti-EGFR antibody treatment and the thickening action of rapamycin on lung parenchyma (Figure 5, B and D). Compared with control lungs, histological sections, 30 weeks after TSC2−/− ASM cell administration, revealed a severe disruption in the architecture of the mice lungs characterized by enlarged airspaces and

**Evaluation of S6 Phosphorylation and Proliferation After Endonasal Administration of TSC2−/− ASM Cells and Effect of Anti-EGFR Antibody or Rapamycin on Lymph Nodes**

As observed in lungs, the number of phospho-S6-positive cells was much higher in lymph nodes following TSC2−/− ASM cell administration than in the controls (Figure 4, A–D). Both rapamycin and anti-EGFR antibody treatments reduced the number of phospho-S6-positive cells in lymph nodes. In contrast to the lungs, after cell administration, Ki-67 labeling in the lymph nodes was not significantly different from control values (Figure 4, E and F). A real-time RT-PCR assay showed that PCNA mRNA levels were increased significantly after TSC2−/− ASM cell administration. Although anti-EGFR antibody treatment reduced PCNA mRNA levels to those of the controls, rapamycin treatment did not (Figure 4G).
observed an almost absent and comparable cellular inflammation evaluated by cell positivity to macrophage and leukocyte markers such as CD68 and CD45 (data not shown). Endonasal administration of TSC2−/− ASM cells in male mice caused lung lesions comparable to those observed in female mice (see Supplemental Figure S2 at http://ajp.amjpathol.org).

To verify the effect of the drugs on lung parenchyma, mice were treated with anti-EGFR antibody and rapamycin for 4 weeks, from week 26 to 30 after cell administration, maintained without further drug treatment for the following 8 weeks, and then the tissues were examined. The lung parenchyma features were comparable to those observed at the end of drug treatments, and the densities of air-exchanging parenchyma were similar (Figure 6, A

**Figure 5.** Administration of TSC2−/− ASM cells caused progressive lung degeneration (arrows) that was counteracted by anti-EGFR antibody (Anti-EGFR Ab) treatment. Hematoxylin and eosin staining was performed in lungs 8 weeks (A) or 30 weeks (C) after cell administration and drug treatments. TSC2−/− ASM cell administration caused enlarged airspaces that were counteracted by anti-EGFR Ab treatment. After rapamycin treatment lung parenchyma partially collapsed and thickened with a high number of red blood cells compared with controls. The Aα (ae/lu) (fraction of air-exchanging parenchyma relative to lung parenchyma) is shown as the mean of ± SEM of (n) animals per group, expressed as a percentage (B and D). After TSC2−/− ASM cell administration, some septi were thickened with larger pneumocytes (E). Anti-EGFR Ab promoted a recovery from the changes caused by TSC2−/− ASM cell administration. After rapamycin treatment, lungs were collapsed with a thickened parenchymal layer richer in red blood cells (E). *P < 0.05 and **P < 0.001, Aα (ae/lu) in TSC2−/− ASM cell-administered mice versus Aα (ae/lu) in control; ††P < 0.01 and †††P < 0.001, Aα (ae/lu) in drug-treated mice versus Aα (ae/lu) in TSC2−/− ASM cell-administered mice. Scale bars: 200 μm (A and C), 20 μm (E).

the absence of normal alveoli (Figure 5E). The large airspaces were bounded by septa that in some points appeared thickened compared with normal alveolar septa. The epithelial cells of alveoli walls presented an increase in the cytoplasm volume that appeared cloudy. Treatment with anti-EGFR antibody reversed these alterations, giving a picture similar to the control, whereas lungs of mice treated with rapamycin showed strong parenchymal distortion associated with enhanced thickness (Figure 5E). In addition, lungs looked partially collapsed in mice treated with rapamycin. In all experimental groups, we...
and B). The average number of PKH26-positive TSC2<sup>−/−</sup> ASM cells was practically unchanged in the lungs of mice treated with anti-EGFR antibody. By contrast, 8 weeks after cessation of rapamycin treatment, the number of PKH26-positive cells was higher, suggesting that cell proliferation had recommenced (Figure 6, C and D). Eight weeks after cessation of drug administration, the PCNA mRNA level was higher in TSC2<sup>−/−</sup> ASM cell–treated lungs, whereas it was comparable to the levels of the controls in the drug-treated groups (Figure 6E).

**TSC2<sup>−/−</sup> ASM Cell Administration Causes a Reduction of Exercise Capability**

LAM commonly presents with progressive breathlessness and a limiting exercise capacity that differs from patient to patient. To evaluate the effect of the morphological changes caused by cell administration, the locomotor climbing activity of the mice was evaluated over a 5-minute period. After cell administration, the mice exhibited a reduced capacity to sustain exercise compared with control mice (control: 6.5 ± 2.38; mice after cell administration: 15 ± 6.37, *P < 0.05 versus control).

**EGFR Distribution Does Not Change After TSC2<sup>−/−</sup> ASM Cell Administration and Drug Treatments**

EGFR was homogenously distributed in the lungs of nude mice (see Supplemental Figure S3A at http://ajp.amjpathol.org). The distribution pattern did not change after TSC2<sup>−/−</sup> ASM cell injection and anti-EGFR antibody or rapamycin administration. In the lymph nodes, cells expressing EGFR were very rare, and this pattern remained similar after drug treatments (see Supplemental Figure S3B at http://ajp.amjpathol.org).

**TSC2<sup>−/−</sup> ASM Cells Activate Lymphangiogenesis**

The lymphatic vessel hyaluronan receptor LYVE 1 is a well-known lymphatic capillary marker. In the lungs, the optical density of LYVE 1 labeling was much greater than in the controls at 30 weeks after TSC2<sup>−/−</sup> ASM administration, as shown by qualitative (Figure 7A) and quantitative (Figure 7C) assessments. These findings suggest that TSC2<sup>−/−</sup> ASM cells, once in the lungs, promote the proliferation of LYVE 1–positive lymphatic vessels. Both anti-EGFR antibody and rapamycin treatments counteracted the increase of LYVE 1 expression (Figure 7, A and C). Comparable changes were observed in lymph nodes (Figure 7, B and D).

Growth factors, such as VEGF-C and VEGF-D, are directly involved in lymphatic vessel growth, and VEGF-D levels are suggested to be higher in LAM patients. In homogenate of lungs with 30 weeks of TSC2<sup>−/−</sup> ASM cell administration, the levels of murine VEGF were significantly higher than in the controls (Figure 8A). Both anti-EGFR antibody and rapamycin treatments abrogate this increase in the VEGF level. The 30-week exposure to TSC2<sup>−/−</sup> ASM cells resulted in lower systemic serum levels of VEGF, which remained low even after either drug treatment (Figure 8B). The EGF level was very low in the serum of all experimental mice groups with no appreciable differences, and was not detectable in lungs (data not shown). Human VEGF and EGF were not detectable.

**Estrogen and Progesterone Receptors Are Expressed in TSC2<sup>−/−</sup> ASM Cells**

Because LAM is a disease that almost exclusively affects women, we examined the expression of estrogen and progesterone receptor. In all, 78% of TSC2<sup>−/−</sup> ASM cells expressed estrogen receptors (Figure 9A, left panel) the levels of which were maintained after drug administration (Figure 9, A and B, right panels). In addition, TSC2<sup>−/−</sup> ASM cells expressed progesterone receptors (Figure 9B, left panel).

**Discussion**

This study described the development of an animal model of LAM. The in vivo application of TSC2-deficient smooth muscle cells allowed a detailed examination of their migratory ability, which could further our understanding of how these cells move within tissue and from tissue to tissue. This model is also important as an experimental tool for developing and defining novel pharmacological strategies aimed at curing LAM. At present, there is only one model of LAM, which depends on the development of lung metastasis from Tsc2-null ELT3 cells in female and male mice following estrogen treatment. The model was successful in demonstrating the crucial role of the MEK pathway in the estrogen-dependent metastasis of Tsc2-deficient ELT3 cells. Different animal models of TSC and LAM have been generated. The Eker rat is a spontaneous autosomal dominant hereditary model of the Tsc2 mutation that predisposes the rat to renal adenoma, carcinoma, and uterine leiomyomas. Tsc2<sup>−/−</sup> mice also develop kidney tumors, but, in contrast to the Eker rat, they develop hemangiomas as well.

LAM cells are believed to have the capability to migrate or metastasize in the patient; although, LAM cells appear to be histologically benign. In our study, human TSC2<sup>−/−</sup> ASM cells isolated from an angiomyolipoma in a TSC2 patient penetrated into the lungs and lymphatic system, after endonatal administration to nude mice, where they survived and proliferated. Enhanced lymphatic infiltration with progressive enlargement of the airspaces, emphysema-like tissue formation, and thickness of the few left primary septa ensued in female and male mice administered TSC2<sup>−/−</sup> ASM cells and appeared uniformly distributed through the lung. It appeared that thickening of airspace walls after TSC2<sup>−/−</sup> ASM cell administration was due to alveolar cells with enlarged cytoplasm. The high predominance of LAM in female and the aggravation of the disease during pregnancy or after the administration of estrogens strongly suggest a key role of estrogen in LAM pathogenesis. However, direct TSC2<sup>−/−</sup> ASM cell administration into the lungs via the respiratory
system allowed cellular accumulation. The lung damage was comparable in male and female mice, suggesting that, at least in this murine model, the destructive action of TSC2−/− ASM cells in lungs may not require hormonal support. After TSC2−/− ASM cell administration, we observed reactivity to HMB45 and enhanced labeling to phospho-S6 antibodies throughout the lymph nodes and lungs. This result shows that TSC2−/− ASM cells can invade alveolar walls and migrate through the lymphatic system while maintaining their typical biochemical features. The proliferation of TSC2−/− ASM cells was higher in lungs than in lymph nodes, as has been observed for some types of cancer cells.31 Because, by definition, replication at the site of metastasis, together with detachment, entrance and survival in the lymphatic or hematological circulatory systems, are cellular capabilities needed for recruitment to a metastatic site, these results suggest that TSC2 cells possess metastatic potential.31 Lymph node metastasis is a characteristic feature of carcinoma, and cancer cells arriving in the lymph nodes often proliferate and colonize the lymph nodes. By contrast, TSC2−/− ASM cells appear not to proliferate in the lymph nodes.

The more diffuse labeling of LYVE1 in lungs and lymph nodes following TSC2−/− ASM cell administration strongly supports the hypothesis that TSC2 cells promote lymphatic vessel growth in the tissue in which they are located. There are strong functional links between LAM cells and lymphatic vessels. LAM cell clusters enveloped by lymphatic endothelial cells have been identified in LAM lesions.32 In the case of inappropriate lymphangiogenesis, LAM lesions exhibit expression of lymphatic endothelial markers such as VEGFR-3, podoplanin, and prox-1.33 VEGF-D, a lymphangiogenic factor that binds VEGFR-3, is elevated in serum of LAM patients,25 and VEGF-D serum concentrations are suggested to correlate with clinical phenotype severity and are considered a measure of lymphatic involvement in LAM patients.34,35 Under the experimental conditions used in this study, TSC2 cells promoted enlarged airspaces through the lung parenchyma with increased lymphatic vessel ingrowth, which was correlated with the production of high.
...discontinuation of the drug. In patients with LAM, 12 cystadenomas, but marked tumor regrowth occurred after effective in suppressing the growth of Tsc mouse kidney substantial decrease in the number of TSC2 and HMB45 during rapamycin administration that does not result in a we found that there was a reduction in cell proliferation, it does not affect cell survival. In this study, that although rapamycin can effectively reduce TSC cell proliferation, it does not affect cell survival. In this study, that although rapamycin can effectively reduce TSC cell proliferation, it does not affect cell survival.

Rapamycin acts by arresting the progression of the cell cycle between the G1 phase to S phase through interactions with proteins such as FK-binding protein and mTOR, which in turn results in the blockade of numerous signal pathways, including key cytokine signaling pathways that regulate the cell cycle. Rapamycin was effective in promoting the regression of TSC angiomyolipomas, which tended to increase again in volume once the therapy was stopped. Similarly, rapamycin was highly effective in suppressing the growth of Tsc mouse kidney cystadenomas, but marked tumor regrowth occurred after discontinuation of the drug. In patients with LAM, 12 months of rapamycin treatment stabilized lung function, reduced VEGF-D levels, and improved functional performance; but, after discontinuation of the therapy, the decline in lung function returned to the levels observed in untreated patients. Consistent with these data, present and previous studies by our group, have shown that although rapamycin can effectively reduce TSC cell proliferation, it does not affect cell survival. In this study, we found that there was a reduction in cell proliferation during rapamycin administration that does not result in a substantial decrease in the number of TSC2 and HMB45 positive cells. Thus, rapamycin seems to be effective in blocking proliferation, but not in causing complete TSC2−/− ASM cell death. This is particularly relevant when this effect is compared with that of anti-EGFR anti-

**Figure 8.** Murine VEGF levels in lung homogenates and serum were evaluated by ELISA. A: VEGF levels were significantly increased in lung homogenates 30 weeks after TSC2−/− ASM cell administration. Anti-EGFR antibody (Anti-EGFR Ab) or rapamycin treatment reduced VEGF concentrations. B: TSC2−/− ASM cell administration caused a reduction in murine VEGF serum levels that was further reduced by anti-EGFR antibody (Anti-EGFR Ab) or rapamycin treatment. *P < 0.05, **P < 0.1, and ***P < 0.001. TSC2−/− ASM cell–administered mice versus control mice; ††P < 0.01 and †††P < 0.001 drug-treated mice versus TSC2−/− ASM cell–administered mice.

**Figure 9.** Estrogen receptors (A, left panel) and progesterone receptors (B, left panel) are expressed in 78% and 100% of TSC2−/− ASM cells, respectively. The expression of these receptors did not change after cell administration (A and B, right panels). Anti-human-HLA shows TSC2−/− ASM cells (red). Estrogen and progesterone receptors are expressed in lungs (brown). Arrows indicate the co-localization of HLA and estrogen or progesterone receptor expression. Scale bars: 150 μm (left panels); 50 μm (right panels).

VEGF levels by the host. No human VEGFA, B, or C that might be secreted by TSC2 cells was detected. Thus, TSC2 cells modified the lung environment by increasing tissue accumulation of murine VEGF, which was either produced locally or originated from the blood stream, as suggested by the decrease in serum VEGF levels. These observations suggest a dual mechanism underlying lung destruction in LAM: the infiltration and accumulation of TSC2 cells causes local destruction and modifies the lung environment by increasing local levels of VEGF; this, in turn, promotes massive invasion by lymphatic vessels.

The lung damage caused by TSC2−/− ASM cells is likely responsible for the reduced ability of these animals to perform physical tests, as shown by the reduced ability to climb.

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the number of infiltrated TSC2 cells, the lung parenchyma regained a normal morphology counteracting the epithelial alveolar thickness caused by TSC2-/- ASM administration. In animals treated with rapamycin, lungs were partially collapsed with thicker septal walls rich in enlarged alveolar cells. Rapamycin has been reported to cause thickening of the parenchymal walls and diffuse alveolar hemorrhaging, which has previously been described as a side effect of the drug.40,41 The occurrence of hemoptysis in patients treated with rapamycin can be solved by stopping the therapy.43 However, the increase in alveolar septal cellularity may involve lymphocyte and neutrophil accumulation, which causes alveolitis and pneumonia in patients.40,41 Pulmonary toxicity triggered by rapamycin may involve an immune-mediated effect that manifests itself as lymphocytic pneumonitis without hemorrhage or alveolar hemorrhage without lymphocytic alveolitis. However, further investigation is required to evaluate whether inflammation may influence the thickness of murine lungs after rapamycin treatment.

In conclusion, this study describes the development of an in vivo model of LAM using TSC-/- ASM cells. The model provides a tool to study LAM in preclinical models. The metastatic process had clearly been suggested by the recurrence of the disease in LAM patients who had undergone single lung transplantation.43 Anchoring of TSC cells in the lung may be accompanied by their transit through the lymphatic and cardiovascular system.11 Consistent with in vitro results, anti-EGFR antibody is more effective and specific than rapamycin in counteracting TSC2-/- ASM cell proliferation and reversing the structural lung alterations without eliciting side effects on lung tissue. Thus these data support the therapeutic value of anti-EGFR antibody for the control of ASM cell growth in LAM and TSC patients.

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