CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Evidence Supporting a Lymphatic Endothelium Origin for Angiomyolipoma, a TSC2[−] Tumor Related to Lymphangioliomyomatosis

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Angiomyolipoma (AML) is a tumor closely related to lymphangioliomyomatosis (LAM). Both entities are characterized by the proliferation of smooth muscle actin and melanocytic glycoprotein 100 (recognized by antibody HMB-45)—positive spindle-shaped and epithelioid cells. AML and LAM are etiologically linked to mutations in the *tsc2* and *tsc1* genes in the case of LAM. These genes encode the proteins tuberous sclerosis complex (TSC)-1 and TSC2, which are directly involved in suppressing the mechanistic target of rapamycin cell growth signaling pathway. Although significant progress has been made in characterizing and pharmacologically slowing the progression of AML and LAM with rapamycin, our understanding of their pathogenesis lacks an identified cell of origin. We used an AML-derived cell line to determine whether TSC2 restitution brings about the cell type from which AML arises. We found that AML cells express lymphatic endothelial cell markers consistent with lymphatic endothelial cell precursors *in vivo* and *in vitro*. Moreover, on TSC2 correction, AML cells mature into adult lymphatic endothelial cells and have functional attributes characteristic of this cell lineage, suggesting a lymphatic endothelial cell of origin for AML. These effects are dependent on TSC2-mediated mechanistic target of rapamycin inactivation. Finally, we demonstrate the *in vitro* effectiveness of norcantharidin, a lymphangiogenesis inhibitor, as a potential co-adjuvant therapy in the treatment of AML. (*Am J Pathol* 2016, 186: 1825–1836; <http://dx.doi.org/10.1016/j.ajpath.2016.03.009>)

Angiomyolipoma (AML) and lymphangioliomyomatosis (LAM) are members of the perivascular epithelioid cell tumor (PEComa) family, which is characterized by the proliferation of spindle-shaped and epithelioid cells expressing smooth muscle actin and melanocytic glycoprotein 100 (classically recognized by the antibody HMB-45).¹ In AML, the PEComa cells are admixed with different proportions of mature fat and thick-walled blood vessels. AMLs affect mainly young patients² and are typically found in the kidney but have also been described in the liver and less commonly in the ovary, fallopian tube, spermatic cord, palate, and colon.³ Although most AMLs are benign, they tend to spread to local lymph nodes⁴ and may grow such that kidney function is impaired or the blood vessels within the tumor may dilate and rupture, leading to often life-threatening retroperitoneal hemorrhage.⁵ As with most PEComas, AML is etiologically linked to mutations in the

tsc2 gene encoding the protein tuberous sclerosis complex (TSC)-2 (tuberin).⁶ Both TSC-associated LAM and sporadic LAM are primarily associated with *tsc2* gene mutations,^{7,8} although in rare cases LAM is caused by *tsc1* mutations.^{9,10} TSC2 dimerizes with TSC1 (hamartin) to inhibit the mechanistic target of rapamycin complex 1 (mTORC1) by directly inhibiting the activity of its upstream effector, the small GTPase ρ enriched in brain (Rheb) via the GTPase-activating protein domain of TSC2.¹¹

LAM, which primarily involves the lung bilaterally, is a low-grade malignant tumor characterized by the proliferation of PEComa cell nodules and the presence of cysts that often affects women of childbearing age. The LAM nodules, constituted by cells phenotypically indistinguishable from

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AML cells, enlarge, multiply, and cause cystic destruction of the lung, leading to respiratory insufficiency. By examining histologic sections of multiple LAM cases, we previously found that, along with the well-established myogenic and melanocytic differentiation, LAM cells possess a third lineage of differentiation because they express lymphatic endothelial cell (LEC) markers.¹² Most AML and LAM cases occur sporadically, but a few develop in patients with TSC. When sporadic AML and LAM coexist in the same patient, which is not uncommon,¹³ identical TSC2 mutations are seen in both processes.⁷ Therefore, AML and LAM are considered to be different manifestation of the same disease. Furthermore, it has been postulated that LAM may originate from AML cells that metastasize to the lungs.^{14,15} Against this possibility, however, stands the fact that the two conditions are more often seen independently than in combination and that AML affects men and women, whereas LAM rarely affects men. Therefore, currently it is hypothesized that TSC2-deficient cells from a third site may metastasize to both the lung and the kidney in the sporadic form of LAM.¹⁶

Despite intense speculation,^{14,15,17} the precursor cell from which AML and LAM originate remains unknown. A neural crest cell (NCC) origin has been mostly favored^{14,15,17} because of the coexistence of melanocytic and smooth cell markers in the AML and LAM cells, two cell lineages known to arise, partially in the case of smooth muscle cells, from NCCs.^{18,19} However, melanosomes, melanin, and HMB-45 positivity can be found in a variety of non-melanocytic cells known to be of non-NCC origin.^{20,21}

Although significant progress has been made in characterizing and pharmacologically slowing the progression of AML and LAM through the use of the mTORC1 inhibitor rapamycin,^{22–25} our understanding of the pathogenesis of these two conditions remains incomplete in part because of the lack of an identified cell precursor, which could provide the opportunity for directly targeting such a cell for therapeutic purposes. We sought to elucidate the source of these neoplastic cells, departing from the knowledge that a lack of active TSC2 underlies all AML cases studied so far²⁶ and that the currently available genetic data indicate that AML and LAM arise solely from such a deficiency. Therefore, we reasoned that TSC reconstitution could revert the AML cell phenotype to that of its precursor. To test such a possibility, we used a *tsc2* mutated immortalized AML cell line (621-101 cells) derived from a patient with sporadic LAM, which does not express TSC2.^{27,28} We referred to this cell line as TSC2[−] and compared it with the same cell line stably transfected with TSC2,^{24,29,30} 621-103 cells here referred as TSC2⁺, to determine whether and what type of cell differentiation is induced by TSC2 restitution.

Although our studies did not support the notion that AML cells are derived from NCCs, they found that, as well as LAM, AML cells express LEC markers and that TSC2 correction points to a LEC derivation. Consistent with these findings, norcantharidin (NCTD), a lymphangiogenesis

inhibitor, halted the proliferation of both TSC2⁺ and TSC2[−] AML cells. Finally, we found an additive effect between rapamycin and NCTD, which could be beneficial in the treatment of AML and LAM.

Materials and Methods

Cell Cultures

Human TSC-null cells, referred as 621-101, were isolated from an AML.^{27,28} In this cell line, both *tsc2* alleles are inactivated through a missense change from DNA sequence G to mutant A at position 1832 of the *tsc2* gene plus loss of heterozygosity at the chromosome 16p13 marker D16S291.^{27,28} The isolated 621-101 cells were immortalized by transfection of HPV E6/E7 (pLXSN 16E6E7-neo) and human telomerase (pLXSN hTERT-hyg). The immortalized cells were then transfected with empty vector (pcDNA3.1) or cDNA corresponding to the *tsc2* open reading frame (pcDNA3.1 TSC2-zeo).²⁹ The resulting cell lines were respectively referred to as 621-102 (TSC2[−] cells herein) and 621-103 (TSC2⁺ cells herein). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL of penicillin-streptomycin, and 2.5 g/mL of Fungizone.

Western Blot Analysis

Cell lysates from TSC2[−] and TSC2⁺ cells were resolved in SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) for immunoblot analysis with the following antibodies: anti-(x)-TSC2, x-CD31, x-zinc finger protein SNAI2 (SNAIL2), x-vimentin, x-N-cadherin, x-E-cadherin, x-zonula occludens protein 1, x-claudin, x- β -catenin, x-zinc finger E-box-binding homeobox 1 (TCF8/ZEB1), x-SNAIL1, x-P75R, x-COUP transcription factor 2 (COUP-TFII) (all from Cell Signaling Technology, Danvers, MA), x-podoplanin (antibody D2-40, from Dako, Carpinteria, CA), x-SRY-related HMG-box (SOX)-10, x-nestin, x-paired-like homeobox (PHOX)-2b, x-vascular endothelial growth factor receptor (VEGFR)-3, x-lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, x-prospero homeobox (PROX)-1 (all from Abcam, Cambridge, UK), x-CD133 (Millipore, Billerica, MA), x-forkhead box (FOX)-D3, x-SOX9, *Twist*-related protein (x-TWIST), x-CD34, x-VEGFR-2, x-SOX18, and x-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all from Santa Cruz Biotechnology, Santa Cruz, CA). An ECL plus Western blotting detection system (GE Healthcare, Little Chalfont, UK) was used for detecting the chemiluminescence signal.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4- μ m-thick, formalin-fixed, paraffin-embedded AML tissue sections using monoclonal mouse x-human podoplanin

antibody (Dako clone D2-40, code M3619) at 1:200 dilution and polyclonal rabbit x-human PROX1 antibody (Abcam, catalog no. 38692) at 1:200 dilution. The immunostaining was performed on a Bond 3 automated IHC/*in situ* hybridization system (Leica Microsystems, Wetzlar, Germany) using the Bond Polymer Refine detection system (Leica Microsystems), according to a modified manufacturer's protocol. After heat antigen retrieval in a high-pH buffer (ER2) for 20 minutes, sections were incubated with x-D2-40 antibody for 25 minutes and x-podoplanin for 50 minutes followed by incubation with postprimary reagent for 15 minutes, Bond polymer horseradish peroxidase for 25 minutes, and peroxidase block for 5 minutes. The peroxidase reaction was developed using diaminobenzidine provided in the kit.

RNA Isolation and Real-Time PCR

RNA was isolated from TSC2⁻ and TSC2⁺ cells using TRIzol (Life Technologies, Carlsbad, CA) and the iScript cDNA synthesis kit (Bio-Rad) was used for cDNA synthesis. Real-time PCR was conducted using iQ SYBR Green Supermix (Bio-Rad) and the following primers: *Prox1* sense, 5'-TCTCTAGTACAGGCTCCGAAG-3'; *Prox1* antisense, 5'-TTTGCTCTCAGGTGCTCATC-3'; *COUP-TFII* sense, 5'-GCCATAGTCCTGTTCACCTC-3'; *COUP-TFII* antisense, 5'-GGTACTGGCTCCTAACGTATTC-3'; *Sox18* sense, 5'-CGGTCTATTACAGCGGTG-3'; *Sox18* antisense, 5'-GACATGGAACCAAACATACACG-3'; *Lyve1* sense, 5'-TTAGCCCAAACCCCAAGTG-3'; *Lyve1* antisense, 5'-TCTGGAATGCACGAGTTAGTC-3'; *Vegfr-3* sense, 5'-CATCTACAAAGACCCCGACTAC-3'; *Vegfr-3* antisense, 5'-CCAGAGAAGCACCCCAAAG-3'; *Podoplanin* sense, 5'-TCAGAAAGCACAGTCCACG-3'; *Podoplanin* antisense, 5'-TATGATTCCAACCAGGGTCAC-3'; *CD34* sense, 5'-TCCCAAAGACCCTGATTGC-3'; *CD34* antisense, 5'-CTCCACCGTTTTCCGTGTAA-3'; *Phox2b* sense, 5'-ACTCACTACCCCGACATCTAC-3'; *Phox2b* antisense, 5'-CTCCTGCTTGCGAAACTTG-3'; *Nestin* sense, 5'-GGTCTCTTTTCTCTTCCGTCC-3'; *Nestin* antisense, 5'-CTCCCACATCTGAAACGACTC-3'; *Twist* sense, 5'-CTCAGCTACGCCTTCTCG-3'; *Twist* antisense, 5'-ACTGTCCATTTTCTCCTTCTCTG-3'; *P75R* sense, 5'-CCTGTCTATTGCTCCATCCTG-3'; *P75R* antisense, 5'-GGGCGTCTGGTTCACTG-3'; *Gapdh* sense, 5'-AATCCCATCACCATCTTCCA-3'; and *Gapdh* antisense, 5'-TGGACTCCACGACGTACTCA-3'.

Luciferase Reporter Assay

Promoters (2000 kb upstream of start codon) and 5'/3' untranslated regions (UTRs) of the *Lyve1* gene and *Vegfr-3* gene were cloned from TSC2⁻ cells and inserted into pGL3 firefly luciferase vector (Promega, Madison, WI) to generate reporter constructs. TSC2⁻ and TSC2⁺ cells were plated on 6-well plates and transfected with the reporter constructs and renilla luciferase internal control vector pRL-SV40.

Forty-eight hours after co-transfection, the Dual-Luciferase Reporter Assay System (Promega) was used to determine the ratio of firefly/renilla luciferase activity, as indicated by the manufacturer.

Transepithelial Electrical Resistance Assay

The transepithelial electrical resistance (TEER) assay was performed as previously described.^{31,32} Briefly, 1×10^5 TSC2⁻ and TSC2⁺ cells were seeded on 12-well Transwell plates (pore size, 0.4 μ m; Corning Inc., Corning, NY). Once the cell monolayers reached confluence, the TEER was measured every 24 hours using an Epithelial Volt/Ohm Meter (EVOM; World Precision Instruments, Sarasota, FL).

Size Differential Transport Assay

A size differential transport assay was performed as previously described.³³ Briefly, 1×10^5 TSC2⁻ and TSC2⁺ cells were seeded on 12-well Transwell plates (pore size, 0.4 μ m; Corning Inc.). Once the cell monolayers reached confluence, a final concentration of 100 ng/mL of fluorescein isothiocyanate (FITC) dextran of sizes 20, 70, and 150 kDa dissolved in dimethyl sulfoxide was added to the apical side of the wells. After 0, 5, 10, 15, 30, 45, 60, 90, and 120 minutes, samples were taken from the basolateral side of each well. The fluorescent signal of FITC-dextran of the samples was read at 520 nm with a Synergy H1 Multi-Mode Reader (BioTek, Winooski, VT).

In Vitro Tubulogenesis Assay

Fifty microliter of Matrigel (growth factor reduced and phenol red-free) (Corning Inc.) was spread on the bottom of each well in 12-well plates. The plates were incubated for 30 minutes at 37°C for Matrigel to solidify. TSC2⁻ and TSC2⁺ cells were then seeded onto the matrix at a concentration of 1×10^4 cells per well. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (charcoal stripped for growth factor reduction), 100 U/mL of penicillin-streptomycin, 2.5 g/mL of Fungizone, and 10 ng/mL of VEGF (from Life Technologies). After 48 hours, the cells were examined and photographed with a Leica DMIL microscope (Leica Microsystems).

Toll-Like Receptor Ligand Assay

Toll-like receptor (TLR) ligand assay was performed as previously described.³⁴ Briefly, TSC2⁻ and TSC2⁺ cells were exposed to the TLR ligands Pam3CSK4, heat-killed preparation of *Listeria monocytogenes*, Poly(I:C), Poly(I:C) low molecular weight, *Escherichia coli* K12 lipopolysaccharide, *Salmonella typhimurium* flagellin, FSL-1 (Pam2CGDPKHPKSF), Imiquimod, ssRNA40, and ODN2006 (all from InvivoGen, San Diego, CA). After 48 hours, the mRNA levels of proinflammatory cytokines

interferon (IFN)- α , IL-6, IL-10, Mx1, and tumor necrosis factor (TNF)- α , and chemokines CCL5, CCL20, and CXCL10 were detected using SYBR qPCR Supermix (Bio-Rad) and the following primers: *IFN α* sense, 5'-GAGTCACCCATCTCAGCAAG-3'; *IFN α* antisense, 5'-ACCAGGACCATCAGTAAAGC-3'; *IL6* sense, 5'-CCACTCACCTCTTCAGAACG-3'; *IL6* antisense, 5'-CATCTTTGGAAGGTTTCAGGTTG-3'; *IL10* sense, 5'-CATGTGAACCTCCCTGG-3'; *IL10* antisense, 5'-TAGATGCCTTTCTCTTGGAGC-3'; *Mx1* sense, 5'-GAAGATAAGTGGAGAGGCAAGG-3'; *Mx1* antisense, 5'-CTCCAGGGTGATTAGTCTCATG-3'; *TNF- α* sense, 5'-ACTTTGGAGTGATCGGCC-3'; *TNF- α* antisense, 5'-GCTTGAGGGTTTGCTACAAC-3'; *CCL5* sense, 5'-GATTTCCTGTATGACTCCCGG-3'; *CCL5* antisense, 5'-CCATCCTAGCTCATCTCCAAAG-3'; *CCL20* sense, 5'-AGCACTCCCAAAGAACTGG-3'; *CCL20* antisense, 5'-CTTGCTTGATTGCGCCG-3'; *CXCL10* sense, 5'-CC TTATCTTTCTGACTCTAAGTGGC-3'; and *CXCL10* antisense, 5'-ACGTGGACAAAATTGGCTTG-3'.

Transfection of Constitutively Active mTOR

To express constitutively active mTOR³⁵ in TSC2⁺ cells, mTOR mutant with four amino acids of mTOR changed (I2017T, V2198A, L2216H, L2260P) (kindly provided by Drs. Tatsuya Maeda and Goutam Ghosh Choudhury) was transfected into TSC2⁻ and TSC2⁺ cells using lipofectamine 2000 (Life Technologies). Empty vector was used as control.

Rapamycin and NCTD Treatments

1×10^3 TSC2⁻ or TSC2⁺ cells were seeded on 96-well plates. Twelve hours later, rapamycin (final concentration, 10 nmol/L), NCTD (an antilymphangiogenic drug that targets lymphatic endothelial cells through the suppression of VEGF-C and VEGF-D³⁶) (final concentration, 10 nmol/L), or a combination of both was directly added to the medium. Same volume of vehicle alone (dimethyl sulfoxide) was used as a negative control. Starting at time 0, 2 hours before treatment, and every 24 hours, cell proliferation assays were performed as described below.

Cell Proliferation Assay

Proliferation of TSC2⁻ and TSC2⁺ cells was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). Twenty microliters of CellTiter 96 AQueous One Solution Reagent was directly added into each well in 100 μ L of culture medium and incubated for 2 hours. The OD of samples was measured at 490 nm using an iMark Microplate Reader (Bio-Rad).

Statistical Analysis

All experiments were repeated at least three times, and data were analyzed using SPSS software version 23.0 (SPSS Inc.,

Chicago, IL) and R studio and are given as means $\pm$ SD. Differences between the two groups and treatments were determined by 2-tailed *t*-test. $P \leq 0.05$ was considered statistically significant.

Results

Western blot analysis confirmed the absence of TSC2 in the AML cells transfected with empty vector (TSC2⁻) and the presence of TSC2 in the AML cells transfected with the TSC2 expression plasmid (TSC2⁺) (Figure 1A). Consistent with their morphology *in vivo*, the TSC2⁻ cells had a spindle shape in culture, whereas the TSC2⁺ cells had a cuboidal or cobblestone phenotype, consistent with that produced by epithelial or endothelial cells (Figure 1B). Such change in cell morphology suggested the possibility of mesenchymal to epithelial transition. Therefore, we used immunoblot analysis to detect mesenchymal and epithelial markers. These studies found an increase in the epithelial marker E-cadherin and in the mesenchymal markers vimentin and TCF8/ZEB1 in TSC2⁺ cells (Figure 1C).

Western blot analysis demonstrated that the protein levels of the NCC markers SOX10, SNAIL2, FOXD3, and SOX9 were lower in TSC2⁺ cells than in TSC2⁻ cells, whereas the NCC precursor markers nestin and P75R and the NCC markers TWIST and PHOX2b were not detected (Figure 2), although real-time quantitative PCR revealed a decreased mRNA for PHOX2b and nestin and increased mRNA for P75R in TSC2⁺ cells (Supplemental Figure S1).

Western blot analysis revealed expression of LEC and LEC precursor markers PROX1, COUP-TFII, SOX18, LYVE1, VEGFR-3, and CD34 in both TSC2⁻ and TSC2⁺ cells. However, the protein levels of these markers were increased in TSC2⁺ cells with the exception of peripheral LEC/vascular endothelial precursor cell marker CD34, which was decreased. Peripheral LEC/vascular endothelial precursor cell marker CD133 was not detected in either cell line (Figure 3A). Traces of the LEC marker podoplanin were detected in TSC2⁻ cells, whereas TSC2⁺ cells had robust podoplanin expression (Figure 3A). The vascular endothelial cell (VEC) marker CD31 and the VEC precursor marker VEGFR-2 were not detected in either cell line (Figure 3B). PROX1 and podoplanin IHC performed on AML sections confirmed the Western results by revealing PROX1 weak nuclear and weaker cytoplasmic positivity in AML cells and negativity for podoplanin (Supplemental Figure S2). Real-time PCR revealed increased PROX1, LYVE1, podoplanin, and SOX18 mRNA levels in TSC2⁺ cells (all corresponding proteins increased in Western blots); however, the VEGFR-3 mRNA (corresponding protein level increased in Western blot) was decreased (Figure 3C), a finding inconsistent with TSC2 acting at the level of *Vefgr3*

Figure 1 TSC2 induces a change in the morphologic features of AML cells mimicking mesenchymal to epithelial transition. **A:** Western blot confirming the expression of TSC2 in AML cells transfected with a TSC2 expression plasmid (TSC2⁺) and the absence in expression of TSC2 in AML cells transfected with vector alone (TSC2⁻). **B:** Inverted microscope microphotographs showing spindle-shaped TSC2⁻, whereas TSC2⁺ cells are cuboidal. **C:** Western blots showing increased expression of both Mes (vimentin and TCF8/ZEB1) and Epi (E-cadherin) markers in TSC2⁺ cells. GAPDH detection indicates equal loading. Histograms show density of Western blot signals in TSC2⁻ cells relative to TSC2⁺ cells, as analyzed by ImageJ software version 1.47 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Scale bars = 20 μm. AML, angiomyolipoma; Epi, epithelial; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mes, mesenchymal; TSC2, tuberous sclerosis complex-2; ZO-1, zonula occludens protein 1.

transcription. Luciferase reporter assays designed to test the activity of the promoter and UTR of *Vegfr-3*, and *Lyve1* (control) confirmed that the differences in LYVE1 expression between TSC2⁻ and TSC2⁺ cells originated at the level of transcription, whereas the differences in VEGFR-3 expression occurred at the level of translation (Figure 3D).

LEC functional assays included tubulogenesis, TEER, and dextran differential transport assays. Tubulogenesis assays revealed that VEGF-C/VEGF-D induced tubule formation in TSC2⁺ cells plated in growth factor-depleted Matrigel, as opposed to the TSC2⁻ cells, which had spheroid growth (Figure 4A). TEER assays revealed higher *trans*-TSC2⁺ cell monolayer electrical resistance than TSC2⁻ cell monolayers (Figure 4B). Dextran differential

transport assays revealed that TSC2⁺ cells had higher differential transport of molecules than TSC2⁻ cells (Figure 4C). The expression of several cytokine mRNAs was up-regulated by exposure to TRL ligands in both TSC2⁻ and TSC2⁺ cells; however, the up-regulation was more pronounced in TSC2⁺ cells (Figure 4D). Proliferation assays revealed similarly decreased proliferation of TSC2⁻ and TSC2⁺ cells after treatment with NCTD (Figure 4E), whereas NCTD had no effect on epithelial and VEC controls (Supplemental Figure S3). Expression of hyperactive mTOR in TSC2⁺ cells down-regulated the expression of all LEC markers tested, namely, PROX1, SOX18, VEGFR-3, and LYVE1 (Figure 5). Finally, cell proliferation assays revealed that combined treatment with rapamycin and NCTD had an additive antiproliferative effect in TSC2⁻ and

Figure 2 Expression of NCC markers in AML cells with or without TSC2 correction does not support an NCC lineage derivation. Western blot showing absence of NCC precursor markers nestin and P75R, absence of NCC markers TWIST and PHOX2b in TSC2⁻ and TSC2⁺ cells, disappearance of NCC marker SOX10, and decreased expression of NCC markers SOX10, SNAIL2, FOXD3, and SOX9 in TSC2⁺ cells compared with TSC2⁻ cells. GAPDH detection indicates equal loading. Histograms show density of Western blot signals in TSC2⁺ cells relative to TSC2⁻ cells, as analyzed by ImageJ software version 1.47 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). AML, angiomyolipoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NCC, neural crest cell; TSC2, tuberous sclerosis complex-2.

TSC2⁺ cells that was of similar magnitude in the two cell lines (Figure 6, A and B).

Discussion

Characterization of AML

AML and LAM are considered different manifestations of the same disease. Although significant progress has been made in characterizing the underlying cause of these conditions and pharmacologically slowing their progression with rapamycin,^{23,37} our understanding of their pathogenesis remains incomplete because of the lack of an identified cell of origin. Because the only available bona fide cell lines to study these conditions are AML derived, we elucidated the source of these neoplastic cells by endowing them with TSC2 with the expectation that TSC2 reconstitution can revert the AML cell phenotype to that of its precursor cell type. To this end, we used a *tsc2* mutated immortalized AML cell line in which both *tsc2* alleles are inactivated,^{27,28} referred to as TSC2⁻ cells, and compared them with the same cell line stably transfected with TSC2 (TSC2⁺ cells) for multiple cell lineage markers. Cell type-specific functional studies were then conducted to validate our results.

The TSC2⁻ AML cells had the typical spindle shape characteristic of PEComa cells; however, on TSC2 correction, their morphologic features changed significantly as the cells became cuboidal and formed a cobblestone monolayer reminiscent of that produced by epithelial or endothelial

cells. Such a morphologic change suggested the possibility of TSC2-induced mesenchymal to epithelial transition. However, immunoblot analysis ruled out such a possibility by demonstrating an increase in the detection levels of several mesenchymal cell markers in TSC2⁺ cells, along with a concomitant increase in the epithelial marker E-cadherin.

An NCC origin for AML and LAM cells has been largely favored^{14,15,17} because of their coexpression of melanocytic and smooth cell markers, two cell lineages known to arise either fully, in the case of melanocytes,³⁸ or partially, in the case of smooth muscle cells,³⁹ from NCCs. However, because TSC2 down-regulated all the NCC markers expressed by TSC2⁻ cells without inducing the expression of NCC precursor markers, our findings were inconsistent with AML arising from NCCs. An alternative possibility to explain the observed melanocytic differentiation is through the occurrence of melanocytic metaplasia, a process known to take place in a variety of nonmelanocytic neoplasias, with the most common being breast,⁴⁰ thyroid,⁴¹ and skin.⁴² Similarly, smooth muscle metaplasia is known to occur in normal endometrium and female breast,⁴³ and it has been reported in extrauterine endometriosis⁴⁴ and renal carcinoma.⁴⁵ Interestingly, it has been reported that lymph node LECs express the melanocytic specific protein tyrosinase,⁴⁶ which catalyzes the production of melanin from tyrosine by oxidation. In addition, ultrastructural studies reveal that melanosomes transport by LECs to the lymph nodes.^{47,48} These mechanisms, rather than metaplasia, might explain

Figure 3 Expression of LEC markers in AML cells with or without TSC2 correction supports an LEC lineage derivation. **A:** Western blot showing expression of LEC and LEC precursor markers PROX1, COUP-TFII, SOX18, LYVE1, VEGFR-3, podoplanin, and CD34 in both TSC2⁻ and TSC2⁺ cells with increased expression in TSC2⁺ cells for all the markers, except peripheral LEC/vascular endothelial precursor cell marker CD34, which is decreased. Peripheral LEC/vascular endothelial precursor cell marker CD133 is negative in both cell lines. GAPDH detection indicates equal loading. Histograms show density of Western blot signals in TSC2⁻ cells relative to TSC2⁺ cells, as analyzed by ImageJ software version 1.47 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). **B:** Western blot showing absence of VEC marker CD31 and VEC precursor marker VEGFR-2. **C:** Real-time PCR revealing increased PROX1, SOX18, LYVE1, and podoplanin mRNA levels in TSC2⁺ cells along with decreased VEGFR-3 mRNA. Real-time PCR showing no difference in COUP-TFII and CD34 mRNA levels. **D:** Luciferase reporter assays consistent with TSC2 transcription regulation of *Lyve1* (control), additionally suggest that the increase in *Vegfr-3* expression in TSC2⁺ cells occurs posttranscriptionally. Real-time PCR data are expressed as means $\pm$ SD. * P < 0.05, ** P < 0.01, by t -test. AML, angiomyolipoma; COUP-TFII, COUP transcription factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LEC, lymphatic endothelial cell; LYVE1, lymphatic vessel endothelial hyaluronan receptor-1; PROX1, prospero homeobox-1; SOX18, SRY-related HMG-box-18; TSC2, tuberous sclerosis complex-2; VEC, vascular endothelial cell; VEGFR, vascular endothelial growth factor receptor.

the presence of melanocytic markers in AML and LAM cells and perhaps in all PEComas.

In a previous study, we found that, in addition to smooth muscle cell and melanocytic markers, lung LAM cells express LEC markers *in vivo*.¹² Therefore, we determined whether TSC2⁻ and TSC2⁺ also express markers indicative of a LEC lineage and whether these are affected by TSC2 reconstitution. Our studies indicate that TSC2⁻ and TSC2⁺ cells, as well as AMLs *in vivo*, express LEC markers, and we found that their expression is enhanced by TSC2.

Role of Lymphatic Epithelium in LEC Specification

Although until recently little was known about the lymphatic endothelium, our knowledge of this biological structure and its origins had increased exponentially in the last few years. It is now well established that LECs originate from the embryonic cardinal veins,^{49,50} from circulating VEC/LEC precursors, characterized by their expression of the LEC/VEC precursor markers CD34 and CD133,^{51–54}

and from other less defined sources.^{55,56} The best studied process of lymphangiogenesis is that undergone by LECs originated from the cardinal veins. In the latter, the first molecular indicator of LEC competence is the expression of the SOX family transcription factor SOX18 in the dorso-lateral wall.⁵⁷ SOX18 binds to *cis*-acting regulatory regions of the *Prox1* gene and activates its transcription.⁵⁷ PROX1 has long been considered the master regulator of LEC fate and serves as the most reliable marker of LEC identity.⁵⁸ This transcription factor is crucial for lymphatic development, and it is necessary for both LEC specification and ongoing maintenance of LEC identity in adults.^{49,50} The process of LEC fate specification also involves the activity of COUP-TFII, an orphan nuclear receptor transcription factor. COUP-TFII is a direct binding partner of PROX1 and works together with PROX1 to initiate the expression of known target genes. SOX18 is expressed during the initial stages of induction of PROX1 and probably plays no role in the maintenance of LEC identity.⁵⁷ The exit of LEC progenitor cells from the embryonic cardinal veins is dependent on the ligand for VEGF-C, VEGFR-3, and podoplanin

Figure 4 Functional LEC features in AML cells with or without TSC2 correction support an LEC lineage derivation. **A:** Tubulogenesis assay reveals tubule formation by TSC2⁺ cells (arrows) but not by TSC2⁻ cells when cultured in growth factor-depleted Matrigel in the presence of 10 ng/mL of vascular endothelial growth factor. **B:** TEER assays reveal higher transepithelial resistance in TSC2⁺ monolayers than in TSC2⁻ monolayers. **C:** Dextran differential transport assays reveal TSC2⁺ cells higher permeability compared with TSC2⁻ cells. **D:** The expression of several cytokine mRNAs is up-regulated by exposure to TLR ligands in both TSC2⁻ and TSC2⁺ cells. However, the increases in mRNA levels are higher in TSC2⁺ cells. **E:** Proliferation assay showing decreased proliferation of TSC2⁻ and TSC2⁺ cells after treatment with 10 nmol/L lymphangiogenesis inhibitor NCTD. Data are expressed as means ± SD. **P* < 0.05, ***P* < 0.01, by *t*-test for the mean of the end point. Scale bar = 100 μm. AML, angiomyolipoma; FITC, fluorescein isothiocyanate; IFN, interferon; LEC, lymphatic endothelial cell; NCTD, norcantharidin; TEER, transepithelial electrical resistance; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TSC2, tuberous sclerosis complex-2; V, vehicle alone.

expression and marks the stage in which the LECs are completely separated from the veins, allowing a molecular distinction between venous PROX1-expressing LEC progenitors and differentiating PROX1-expressing LECs.⁵⁰ LYVE1 is the LECs receptor for the mucopolysaccharide hyaluronan. LYVE1 is already present in LEC progenitors; its expression is maintained throughout lymphangiogenesis and persists in mature capillary LECs.⁴⁹ LYVE1 seems not to have a function in lymphangiogenesis,^{59,60} but it is implicated in the trafficking of cells within lymphatic vessels and lymph nodes.⁶¹ Regardless of their origin, mature capillary LECs express high levels of PROX1, COUP-TFII, VEGFR-3, LYVE1, and podoplanin.^{49,50}

LEC and LEC precursor markers PROX1, COUP-TFII, SOX18, LYVE1, and VEGFR-3 were expressed in both TSC2⁻ and TSC2⁺ cells, with significantly increased expression of all these markers in TSC2⁺ cells. Podoplanin was detected in trace amounts in TSC2⁻ cells and was induced by TSC2 transfection. IHC confirmed low levels of PROX1 and absence of podoplanin in AML cells *in vivo*, whereas both PROX-1 and podoplanin were strongly positive in LECs lining the lymphatic capillaries found in the tumor. The peripheral LEC/VEC precursor marker CD34 was also expressed in both TSC2⁻ and TSC2⁺ cells; however, unlike the other markers, it was decreased in the latter, whereas CD133 was absent in both cell types, as well as the VEC marker CD31⁶² and the VEC precursor marker VEGFR-2.⁶³ These findings suggested that TSC2⁻ cells are akin to LEC precursors, whereas TSC2⁺ cells have the immunophenotype of mature capillary LECs. In this regard,

AML cells differed from LAM cells in that the latter are positive for podoplanin—along with VEGFR-3, PROX1, and LYVE1¹²—and therefore LAM cells are similar to mature rather than immature LECs. Notably, the lymphatic markers LYVE-1, PROX-1, and VEGFR-3 have also been detected in mesothelial cells,⁶⁴ suggesting a common cellular origin for mesotheliomas, AMLs, and LAM from the mesoderm. However, a potential explanation to account for the differences in podoplanin expression between AML and LAM is that in the latter the PEComa cells are more migratory than in AML because podoplanin can be induced by cell migration.^{65,66} The multifocal nature of LAM is consistent with such a possibility.

We further observed that whereas the mRNA levels of PROX1, LYVE1, podoplanin, and SOX18 were increased in TSC2⁺ cells, VEGFR-3 mRNA level was decreased compared with that of TSC2⁻ cells. The last finding was inconsistent with TSC2 acting as a VEGFR-3 through transcription activator. We therefore performed luciferase reporter assays using the promoter and the 5'/3' UTR of *Vegfr-3* and of *Lyve1* for comparison purposes. These studies indeed suggested that whereas LYVE1 expression was regulated at the level of transcription—as expected from the protein and mRNA data—TSC2 up-regulates VEGFR-3 protein level by stimulating *Vegfr-3* gene translation. To the best of our knowledge, this represents the first reported instance of VEGFR-3 regulation at the translation level. This effect may be cell specific or may occur only in the experimental setting in which TSC2 is introduced into abnormal TSC2-deficient cells.

Figure 5 Transfection with hyperactive mTOR down-regulates LEC markers in TSC2^{+/+} cells, suggesting that TSC2 promotes LEC differentiation through mTOR inactivation. Western blot showing down-regulation of LEC markers PROX1, SOX18, VEGFR-3, and LYVE-1 in TSC2^{+/+} transfected with a hyperactive mTOR expression plasmid (AC-mTOR). GAPDH detection indicates equal loading. Histograms show density of Western blot signals in TSC2^{+/+} cells relative to TSC2^{-/-} cells, as analyzed by ImageJ software version 1.47 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LEC, lymphatic endothelial cell; LYVE, lymphatic vessel endothelial hyaluronan receptor; mTOR, mechanistic target of rapamycin; PROX1, x-prospero homeobox-1; SOX18, SRY-related HMGB-box-18; TSC2, tuberous sclerosis complex-2; VEGFR, vascular endothelial growth factor receptor.

Characterization of the Functional Properties of TSC2^{-/-} and TSC2^{+/+} Cells

We next conducted studies to determine whether TSC2^{-/-} and TSC2^{+/+} cells have functional properties characteristic of LECs. Consistent with their mature LEC immunophenotype, the TSC2^{+/+} cells exhibited robust tubulogenesis ability when cultured on Matrigel in the presence of VEGF,⁶⁷ a property lacked by TSC2^{-/-} cells. LECs are engaged in active molecular transport.⁵⁶ Therefore, we examined the transport properties of the TSC2^{+/+} and TSC2^{-/-} cell lines by measuring the TEER values and the permeability of the two cell lines to varying sizes of FITC-dextran molecules. The TEER essays indicate that there was an absence of tight junctions in TSC2^{-/-} and TSC2^{+/+} cells because both were far below the TEER values that characterize tight junction-bearing cells, such as epithelial cells and VECs.³³ Furthermore, the TEER values of TSC2^{-/-} cells were slightly below the reported 150 to 200 Ω/cm^2 values for LECs,³³ whereas the TEER values of TSC2^{+/+} cells were slightly above that range. These results further supported an LEC lineage. We next examined the paracellular transport of molecules within a wide variety of molecular weights, a feature of LECs⁵⁶ using FITC-labeled

dextran.³³ We observed that both cell types were capable of transporting dextran molecules, with a statistically significant increase in the level of transport for 20-kDa and 70-kDa molecules by TSC2^{+/+} cells. Such enhanced paracellular transport capability of TSC2^{+/+} cells directly correlates with their more LEC differentiated stage.

LECs play an important role in the initiation of host innate immune responses through sensing of pathogen-associated molecular patterns by pattern recognition receptors, including TLRs.³⁴ Therefore, as part of the LEC functional studies, we examined the expression of mRNA in proinflammatory cytokines (IFN- α , IL-6, IL-10, Mx1, and TNF- α) and chemokines (CCL5, CCL20, and CXCL10) in response to TLR ligands in TSC2^{-/-} and TSC2^{+/+} cells. We found that both cell lines responded to TLRs; however, the TLR ligands that triggered the response in each cell type were different, and the response was more pronounced in the more LEC-differentiated TSC2^{+/+} cells.

Finally, NCTD, a specific inhibitor of lymphangiogenesis,⁶⁸ halted the proliferation of both TSC2^{+/+} and TSC2^{-/-} cells, supporting their lymphatic endothelial nature. In this regard, TSC2^{-/-} cells were more responsive to NCTD than TSC2^{+/+} cells, suggesting that the effect of the drug is accentuated by LEC immaturity.

The constitutive activation of the mTOR pathway as a result of lack of active TSC is essential in the pathogenesis of AML. However, in addition to the canonical pathway, data from a variety of experimental systems have indicated the existence of noncanonical functions for TSC1,^{69,70} TSC2,^{11,71–78} and Rheb.^{79,80} Therefore, we assessed whether the LEC characteristics seen in TSC2^{+/+} cells were the result of TSC2-mediated inhibition of the mTOR pathway. We found that transfection of hyperactive mTOR down-regulated LEC markers in TSC2^{+/+} cells, suggesting that TSC2 correction promotes LEC differentiation through the inactivation of mTOR rather than through a noncanonical mechanism.

Clinical trials with the mTORC1 inhibitor rapamycin, the only US Food and Drug Administration–approved drug for the treatment of LAM and AML, have revealed only partial

Figure 6 Rapamycin and NCTD combined treatment has an additive antiproliferative effect of similar magnitude in TSC2^{-/-} and TSC2^{+/+} cells. Proliferation assays performed after treatment of TSC2^{-/-} (A) and TSC2^{+/+} (B) cells with 10 nmol/L NCTD, 10 nmol/L rapamycin, or a combination of both. Data are expressed as means $\pm$ SD. * P < 0.05, by t -test for the mean of the end point. NCTD, norcantharidin; TSC2, tuberous sclerosis complex-2; V, Vehicle alone.

efficacy in the treatment of both conditions^{22,37,81,82}; therefore, combined therapies with rapamycin followed by surgery,⁸³ in the case of AML, or including an adjuvant drug in addition to rapamycin, particularly in the case of LAM, are currently the focus of much attention.^{25,84,85} On the basis of the results obtained with NCTD in the treatment of TSC2⁻ cells, we treated these cells with a combination of rapamycin and NCTD and observed additive benefit. Interestingly, rapamycin has also been found to inhibit lymphangiogenesis.^{86,87} Therefore, our use of NCTD, another inhibitor of lymphangiogenesis, further supplemented the inhibition of lymphangiogenesis. Because our studies reveal that TSC2 correction induced a mature LEC phenotype, which may represent the AML cell of origin, we also treated TSC2⁺ with the same combinatory drug approach. These studies indicate an additive effect between rapamycin and NCTD in reducing the proliferative capabilities of TSC2⁻ and TSC2⁺ cells, revealing the preliminary effectiveness in repurposing an antilymphangiogenic drug as co-adjuvant therapy for rapamycin and presenting thereby as a potential new therapeutic approach for patients with AML and most likely for those patients with LAM.

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Supplemental Data

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