Modulation of Cell Migration and Invasiveness by Tumor Suppressor TSC2 in Lymphangioleiomyomatosis

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The loss of TSC2 function is associated with the pathobiology of lymphangioleiomyomatosis (LAM), which is characterized by the abnormal proliferation, migration, and differentiation of smooth muscle-like cells within the lungs. Although the etiology of LAM remains unknown, clinical and genetic evidence provides support for the neoplastic nature of LAM. The goal of this study was to determine the role of tumor suppressor TSC2 in the neoplastic potential of LAM cells. We show that primary cultures of human LAM cells exhibit increased migratory activity and invasiveness, which is abolished by TSC2 re-expression. We found that TSC2 also inhibits cell migration through its N-terminus, independent of its GTPase-activating protein activity. LAM cells show increased stress fiber and focal adhesion formation, which is attenuated by TSC2 re-expression. The small GTPase RhoA is activated in LAM cells compared with normal human mesenchymal cells. Pharmacologic inhibition of Rho activity abrogates LAM cell migration; RhoA activity was also abolished by TSC2 re-expression or TSC1 knockdown with specific siRNA. These data demonstrate that TSC2 controls cell migration through its N-terminus by associating with TSC1 and regulating RhoA activity, suggesting that TSC2 may play a critical role in modulating cell migration and invasiveness, which contributes to the pathobiology of LAM.

Keywords: lung; RhoA GTPase; smooth muscle cells; TSC1

Lymphangioleiomyomatosis (LAM) is a rare lung disease affecting primarily women of childbearing age; it is characterized by the abnormal and potentially neoplastic growth of smooth muscle-like cells within lungs, which leads to the cystic destruction of the lung interstitium and the loss of pulmonary function (1–3). LAM can be sporadic or manifested in association with tuberous sclerosis complex (TSC), an autosomal dominant inherited disorder affecting 1 in 6,000 individuals who develop hamartomas and benign tumors in the brain, heart, and kidneys; it is also manifested by cognitive defects, epilepsy, and autism (4, 5). Importantly, malignant tumors of the kidney, which develop either as malignant angiomyolipomas or renal cell carcinomas, occur in 50% of sporadic LAM (6) and in most patients with TSC (7). Circulating neoplastic smooth muscle-positive LAM cells show loss of heterozygosity (LOH) for TSC2 (8). To date, however, little information is available concerning whether the tumor suppressor protein TSC2 contributes to neoplastic LAM cell dissemination.

TSC2 forms a heterodimer with tumor suppressor TSC1 and functions as a TSC1/TSC2 protein complex (9, 10). TSC1 regu-

lates the activity of RhoA GTPase and associates with the cortical ezrin/radixin/moesin (ERM) proteins, which serve as molecular bridges between the plasma membrane and cortical actin filaments (11). Evidence that TSC2 binds TSC1 through the TSC1-binding domain located in the C-terminus of TSC2 (12–14), which overlaps with the Rho-activating domain of TSC1 (11), suggests that TSC1/TSC2 complex formation may be involved in the regulation of RhoA GTPase activity and, ultimately, cell migration. TSC2 functions as a modulator of the actin cytoskeleton and focal adhesion remodeling (15). TSC1 inhibits Rac1 and TSC2 blocks this activity of TSC1, which is a prerequisite for the activation of Rac1 and the subsequent inhibition of RhoA; this, in turn, promotes stress fiber disassembly and focal adhesion remodeling (15). Loss of function of either TSC1 or TSC2 due to inactivating mutations potentially promotes the deregulation of the TSC1/TSC2 complex formation followed by deregulation of Rac1 and RhoA activities, which, consequently, induces abnormal cell motility associated with the pathobiology of LAM.

Until recently LAM and TSC tumors were considered benign. Compelling evidence, however, supports a neoplastic model for LAM, suggesting a link between loss of TSC1/TSC2 function and cell invasion and metastasis (16). Identical TSC2 mutations and LOH patterns were detected in pulmonary LAM and in angiomyolipoma cells from the same patients with LAM with renal angiomyolipoma, suggesting that these cells have a common origin (17). Secondary tumor formations with the same TSC2 mutations were found in lymph nodes of patients with sporadic LAM (18). In addition, identical TSC2 mutations were found in native LAM cells and recurrent LAM cells after lung transplantation (19). The presence of the same TSC2 mutation in pulmonary LAM, LAM cells in the lymph nodes, and recurrent LAM after transplant strongly supports the notion that LAM cells migrate abnormally and metastasize *in vivo*. This was further supported by a recent report establishing the existence of disseminated, potentially metastatic LAM cells with TSC2 LOH in bodily fluids of patients with LAM (8). Thus, clinical and genetic evidence suggest that TSC2 loss of function is associated with neoplastic cell growth in LAM. However, the cellular and molecular mechanisms of these effects have not been established.

The goal of this study was to examine LAM cell migration and invasiveness, and to establish whether a link exists between TSC2 function and cell motility. We found that LAM cells from the lung tumors of patients with LAM show invasiveness, increased rate of migration, and RhoA activation; expression of TSC2 reverses these effects. The downregulation of TSC1 levels also inhibits RhoA activation; and the N-terminus of TSC2 is necessary and sufficient for inhibition of LAM cell migration, indicating that TSC1 and TSC2 are involved in the regulation of LAM cell migration.

MATERIALS AND METHODS

LAM Cell Culture

We used primary cultures of LAM-derived (LAM) smooth muscle-like cells dissociated from the LAM nodules of five patients who had

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undergone a lung transplant as we previously described (15, 20); all tissues were acquired in accordance with protocols approved by the University of Pennsylvania Institutional Review Board. Briefly, cells were dissociated by enzymatic digestion, plated on Vitrogen-coated plates (Cohesion Technologies Inc., Palo Alto, CA), and maintained in DF8 medium (equal amounts of Ham's F12 and Dulbecco's modified Eagle's medium with 1.6×10^{-6} M ferrous sulfate, 1.2×10^{-5} U/ml vasopressin, 1.0×10^{-9} M triiodothyronine, 0.025 mg/ml insulin, 1.0×10^{-8} M cholesterol, 2.0×10^{-7} M hydrocortisone, and 10 pg/ml transferrin) supplemented with 10% FBS. Before all experiments, cells were serum deprived for 24 h. In culture, LAM cells exhibited smooth muscle-like cell appearance: long spindle-shaped cells growing in parallel arrays that form a "hills-and-valleys" pattern. LAM cells from each patient with LAM were characterized on the basis of smooth muscle (SM) α -actin expression, S6K1 activity, ribosomal protein S6 phosphorylation, and DNA synthesis. All LAM cells used in this study had constitutively activated S6K1, hyperphosphorylated ribosomal protein S6, and a high degree of proliferative activity in the absence of any stimuli as well as a filamentous expression pattern of smooth muscle α -actin (20, 21). Mutational analysis of the *TSC2* gene was performed in LAM cells from two patients with LAM and was reported in our previously published study (20). As controls, we used primary cultures of normal human bronchus fibroblasts (HBFs) derived from the bronchus of the patients with LAM; two matched HBFs and LAM cells from the same patients were used in this study. In contrast to LAM cells, HBFs had low basal S6K1 activity and DNA synthesis (data not shown). As an additional control we used human airway smooth muscle (ASM) cells. ASM cells were dissociated from human trachea, which was obtained from human lung transplant donors and have been previously described (20, 22). We recognize that LAM cells have some limitations and show some degree of heterogeneity; for example, hyperphosphorylated ribosomal protein S6 is found in $\sim 80\%$ LAM cells. HBFs and ASM cells may also be not the best controls for LAM cells; however, HBFs and ASM cells may be reasonable controls for LAM cells, given that currently there is no perfect LAM cell model. All experiments were performed with a minimum of three different HBF and ASM cell lines.

Cell Migration Assay

Cell motility was examined using a Boyden chamber apparatus as we described previously (23–25). Serum-deprived LAM, HBF, and ASM cells were briefly trypsinized by 0.05% trypsin/0.53 mM EDTA, centrifuged at 900 rpm for 10 min, and resuspended in serum-free media supplemented with BSA. Cells (5×10^4) were then placed into the upper wells of the Boyden chamber fitted with an 8- μ m pore membrane coated with Vitrogen (100 μ g/ml). Agonists or vehicle in serum-free media supplemented with BSA were added to the lower chambers. Cells in the Boyden chamber were incubated for 4 h at 37°C in a 5% CO₂ incubator. Nonmigrated cells were scraped off; the membrane was fixed with methanol, stained with Hemacolor stain set (EM Industries, Inc., Gibbstown, NJ), and scanned. Cell migration was analyzed using the Gel-Pro Analyzer program (Media Cybernetics, Silver Spring, MD).

Collagen Invasion Assay

Confluent cells were trypsinized, resuspended in media with or without growth factors, and then 1×10^5 cells were placed in each well of a 6-well plate coated with collagen type I gel (Upstate Cell Signaling Solutions, Lake Placid, NY) as described previously (26). After 24 h the number of single individual cells that migrated into the collagen gel deeper than 10 μ m (deep cells), and the number of superficial cells, were counted using a Nikon Eclipse TE2000-E inverted microscope (Nikon, Melville, NY) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The invasion index was calculated according to the following formula: (deep cells) $\times$ 100/(deep + superficial cells), and is expressed as a percentage.

Transient Transfection and Immunostaining

Plasmids were prepared using the EndoFree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA). Transient transfection was performed using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Twenty-four hours after transfection, transfection efficiency was assessed and expressed as a percentage of transfected cells per total number of cells; then the cell migration assay was performed. The membrane with migrated cells was fixed with 3.7% paraformaldehyde (Polysciences, Inc., Warrington, PA) for 15 min, treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) for 20 min at room temperature, and then blocked with BSA in 20 mM TRIS (pH 7.5)–150 mM NaCl (TBS) for 1 h at 37°C. After incubation with primary anti-GFP rabbit serum (Molecular Probes, Eugene, OR), 1:200 dilution, and secondary Alexa Fluor 488 Goat anti-rabbit IgG conjugate (Molecular Probes), 1:400 dilution antibodies for 1 h at 37°C, respectively, the membrane was mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The cells were visualized on a Nikon Eclipse E400 microscope under $\times 200$ magnification. Cell migration was defined as the percentage of transfected cells migrated through the membrane compared with the total number of transfected cells plated on membrane.

Rho Activation Assay

Rho activity was measured using a Rho Activation Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's protocol. Cells were lysed in MLB buffer, then incubated with Rhotekin RBD-conjugated agarose for 1 h; beads were then collected, washed, resuspended in 2 $\times$ Laemmli reducing sample buffer, boiled, and SDS-polyacrylamide gel electrophoresis and immunoblotting analysis were performed. Rho was detected using anti-Rho (A, B, C) antibody.

Data Analysis

Statistical analysis of F-actin staining was performed by visual and quantitative analysis of digital images taken using a Nikon Eclipse E400 microscope at $\times 200$ magnification using Gel-Pro Analyzer software (Media Cybernetics) (15). Data points from individual assays represent the mean values $\pm$ SE. Statistically significant differences among groups were assessed with the ANOVA (Bonferroni-Dunn), with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All

Figure 1. LAM cell migration. Serum-deprived LAM, ASM cells, and HBFs were placed on collagen-saturated membranes in serum-free medium, and allowed to migrate in the Boyden chamber for 4 h in either the absence of any stimuli, in the presence of 10 ng/ml PDGF-BB, or 10% fetal bovine serum (FBS); membranes were then fixed, stained with Hemacolor stain set, and analyzed using Gel-Pro software (Media Cybernetics). Migration was assessed as the number of cells invaded into the membrane after 4 h of incubation. Migration of ASM cells without stimuli was taken as 1-fold, which was equal to 289.7 ± 16.4 cells per field. Data represent mean values $\pm$ SE from measurements performed in triplicate from three independent experiments using LAM cells, HBF, and ASM cells from different patients in each experiment by ANOVA (Bonferroni-Dunn).

experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

RESULTS

Increased Migratory Activity and Invasiveness of LAM Cells

Abnormal cell migration and invasiveness are among the characteristic features of tumors, which may occur, in part, due to the deregulation of TSC2 signaling, and could be augmented by promigratory growth factors, such as PDGF and serum containing the critical promigratory factor lysophosphatidic acid (LPA) (11, 23, 25, 27). Since the difference between normal and tumor cells lies in their ability to migrate continuously and invade extracellular matrixes (28–31), we examined the migration and invasiveness of LAM cells. The LAM cells used in this study had constitutively activated S6K1 and hyperphosphorylated ribosomal protein S6, which are molecular signatures of TSC2 dysfunction, and increased DNA synthesis, compared with nor-

mal HBF and ASM cells used as control cells (data not shown). Migration of LAM cells was examined using the Boyden chamber migration assay. The serum-deprived LAM, ASM cells, and HBFs were plated on the collagen-saturated membrane of the Boyden chamber in the absence of any stimuli, and were allowed to migrate for 4 h. Unstimulated LAM cells had significantly higher migratory activity compared with the chemokinesis of HBFs and ASM cells (Figure 1). Importantly, when LAM cells were stimulated with either PDGF or FBS, their migratory activity was further increased compared with both unstimulated LAM cells, ASM cells, and HBFs (Figure 1), indicating that promigratory growth factors enhance LAM cell migration.

Next we examined the ability of LAM cells to invade a collagen matrix. As seen Figure 2A, LAM cells invaded the collagen and after 24 h were found at the 20- μ m depth in the collagen. In contrast, human ASM cells were found only in the top collagen layer, which remained unchanged for 48 h and 72 h (data not shown). Similar experiments were performed using HBFs, which are summarized in Figure 2B: $40.5 \pm 7.3\%$ LAM cells invaded collagen matrix; in contrast, only $3.9 \pm 2.3\%$ HBF and $3.6 \pm 3.6\%$ ASM cells were found in the collagen layer. Collectively, our data show that primary human LAM cells, which carry TSC2 gene mutations but retain TSC1 expression (15, 20), have increased migratory activity and invade the collagen matrix, which may be critical in the neoplastic cell dissemination in LAM disease.

Expression of TSC2 Inhibits the Increased Migratory Activity and Invasiveness of LAM Cells

To determine whether LAM cell invasiveness is associated with TSC2 dysfunction, we examined whether TSC2 expression is sufficient for the inhibition of LAM cell invasiveness. As shown in Figure 3, transfection of LAM cells with TSC2 significantly inhibited invasiveness, indicating that TSC2 may be involved in regulating LAM cell invasiveness.

To define whether TSC2 is required for the regulation of cell migration, we examined whether re-expression of TSC2 will affect increased LAM cell migration. Expression of TSC2 significantly inhibited LAM cell migration compared with cells transfected with control GFP plasmid. As seen in Figure 4, most of the LAM cells transfected with GFP ($99.7 \pm 9.2\%$) maintained invasive motility in the absence of any stimuli. In contrast, only $37.1 \pm 5.1\%$ of the total number of cells expressing GFP-tagged TSC2 migrated into the collagen-saturated matrix in serum-free medium. These data demonstrate that TSC2 may regulate cell migration, and supports our hypothesis that loss or mutation of TSC2 may promote abnormal cell motility in LAM.

Figure 2. LAM cell invasiveness. (A) LAM or ASM cells were plated on 6-well plates coated with collagen type I. After 24 h, cell invasion was analyzed using a Nikon Eclipse TE2000-E inverted microscope and Image-Pro Plus software. The collagen gel was traversed from top to bottom, and images were taken in 5- μ m steps. Images are representative of three independent experiments. White arrows indicate cells located at the top collagen layer; black arrows indicate cells located at the 20- μ m depth of the collagen layer. The quantity of invaded ASM cells did not increase after 48 h and 72 h of incubation (data not shown). (B) Statistical analysis of the invasion assay of LAM, ASM cells, or HBFs. Single individual cells found deeper than 10 μ m were interpreted as cells migrated into the gel, or “deep” cells. The invasion index was calculated as the percentage of “deep” cells per total number of cells taken as 100%. Eight to ten fields containing 80–120 cells per field were analyzed for each cell type. Data represent mean values $\pm$ SE from three independent experiments by ANOVA (Bonferroni-Dunn).

Figure 3. TSC2 inhibits LAM cell invasiveness. Cells were transfected with GFP-TSC2 or GFP as a control followed by the cell invasion assay. The invasion index was calculated as the percentage of GFP-positive “deep” cells, found deeper than 10 μ m, per total number of GFP-positive cells taken as 100%. Data represent mean values $\pm$ SE from two independent experiments by ANOVA (Bonferroni-Dunn).

Figure 4. TSC2 inhibits LAM cell migration, and the N-terminus (but not the C-terminus) of TSC2 is sufficient for this inhibition. Quantitative analysis of the migration assays of LAM cells transfected with GFP, GFP-TSC2, GFP-C-terminus TSC2 (TSC2-C), or GFP-N-terminus TSC2 (TSC2-N). Cells were transiently transfected with plasmids; cell transfection efficiency was then assessed, and transfected cells were allowed to migrate in a Boyden chamber for 4 h; membranes were fixed, then immunostained with anti-GFP antibody to detect GFP-tagged TSC2 constructs and GFP. Data represent the percentage of migrated transfected cells per total number of transfected cells plated on the membrane. Data represent mean values $\pm$ SE from two experiments. * $P < 0.001$ for TSC2-N or TSC2 versus GFP by ANOVA (Bonferroni-Dunn).

ber for 4 h; membranes were fixed, then immunostained with anti-GFP antibody to detect GFP-tagged TSC2 constructs and GFP. Data represent the percentage of migrated transfected cells per total number of transfected cells plated on the membrane. Data represent mean values $\pm$ SE from two experiments. * $P < 0.001$ for TSC2-N or TSC2 versus GFP by ANOVA (Bonferroni-Dunn).

To further address the mechanism of invasive LAM cell motility, we next examined which structure-functional domain of TSC2 is important for inhibiting LAM cell migration. We found that the N-terminus of TSC2 was sufficient, and as effective, in inhibiting cell migration as full-length TSC2 (Figure 4). Importantly, the C-terminus, which contains the GAP domain that is involved in regulating TSC2-dependent protein translational regulation and cell proliferation (32), has little effect on cell migration. Our data demonstrate the functional role of the N-terminal of TSC2 in regulating cell migration.

RhoA GTPase Modulates LAM Cell Migration

Small GTPase RhoA has a well-established role in regulating cell migration; the constitutive activation of RhoA results in cell

transformation and metastasis (33). Importantly, RhoA activity is regulated by TSC1, which forms a cytosolic complex with TSC2 (11). Because stress fiber formation correlates with RhoA activation and its downstream effector RhoA kinase (ROCK), we examined whether inhibition of RhoA or ROCK will modulate the actin cytoskeleton and LAM cell migration. Tat-C3 toxin (a cell-penetrating form of *Clostridium botulinum* exoenzyme, which specifically catalyzes the inactivation of RhoA [34]) and Y27632 (a specific inhibitor of ROCK [35]) were used to inhibit RhoA and ROCK activity. Both Tat-C3 and Y27632 abolished RhoA-dependent stress fiber formation (Figure 5A), which correlates with the inhibition of LAM cell migration (Figures 5B and 5C). In contrast, rapamycin (200 nM), a specific inhibitor of the mTOR/S6K1 signaling pathway, had little effect on LAM cell migration (Figure 5B). Importantly, this concentration of rapamycin is sufficient to abrogate LAM cell proliferation (20). To further examine the involvement of RhoA in LAM cell migration, we treated LAM cells with ROCK inhibitors Y27632 and HA-1077 at the range of concentrations that inhibit ROCK activity in other cell types (36). As seen in Figure 5C, Y27632 and HA-1077 significantly inhibited LAM cell migration in a concentration-dependent manner. In contrast, the same concentrations of Y27632 and HA-1077 had little effect on the migration of HBFs (data not shown). These data demonstrate that RhoA and ROCK activation is critical for LAM cell migration.

TSC2 and siRNA TSC1 Inhibit the Constitutive Activation of RhoA in LAM Cells

Because Tat-C3 inhibited LAM cell migration, we examined RhoA activity in LAM cells. As seen in Figure 6A, RhoA is constitutively activated in the absence of any stimuli in LAM cells; in contrast, HBFs and ASM cells showed a significantly lower level of basal RhoA activity.

Next, we examined whether TSC2 expression would affect RhoA activation in LAM cells. As seen in Figure 6B, expression of TSC2 significantly inhibited RhoA activity compared with cells transfected with control plasmid. To investigate whether

Figure 5. RhoA modulates LAM cell invasiveness. (A) Tat-C3 and Y27632 promote stress fiber disassembly. Serum-deprived cells were treated with either 0.5 μ M Tat-C3, 20 μ M Y27632, or DMSO as a control for 12 h. Stress fiber formation was detected by rhodamine phalloidin staining of F-actin. Images are representative of three separate experiments. Scale bar is 20 μ m. (B) Tat-C3, but not rapamycin, inhibits LAM cell migration. Serum-deprived cells were treated with either 0.5 μ M Tat-C3 or 200 nM rapamycin; the migration assay was then performed using a Boyden chamber. (C) Y27632 and HA-1077 inhibit LAM cell migration. Serum-deprived cells were treated with either Y27632 or HA-1077, followed by the migration assay. Data are representative of six replicates for each condition in two independent experiments. * $P < 0.001$ for Y27632- or HA-1077-treated cells versus control by ANOVA (Bonferroni-Dunn).

TSC1 is also required for TSC2-dependent RhoA inhibition, we used siRNA directed against TSC1 and downregulated TSC1 levels as we described previously (15). As seen in Figure 6B, downregulation of TSC1 levels resulted in significant inhibition of the constitutive RhoA activation in LAM cells. Because TSC2 inhibits RhoA activity and siRNA TSC1 is sufficient for this inhibition, we conclude that TSC1 is required for the TSC2-dependent regulation of RhoA activity. These data and our published study (15) show that TSC2 acts upstream of RhoA, and suggest that TSC2-dependent inhibition of LAM cell migration occurs due to the TSC2-dependent inhibition of RhoA activity.

TSC2 Modulates Stress Fiber Formation in LAM Cells

Because RhoA activation correlates with stress fiber formation, we next performed F-actin staining on LAM cells to detect stress fibers in LAM cells. We found that LAM cells had significantly increased stress fiber formation; the quantity of F-actin per cell in LAM cells was $44.3 \pm 2.3\%$ and $58.7 \pm 1.9\%$ higher compared with that in ASM cells and HBFs, respectively (Figure 7A). We next examined whether expression of TSC2 affects stress fiber formation in LAM cells. As shown in Figure 7B, expression of TSC2 leads to stress fiber disassembly compared with control GFP-expressed cells. Quantitative analysis of F-actin staining revealed that expression of TSC2 markedly reduced the quantity of stress fibers per cell by $27.9 \pm 15.4\%$ compared with control GFP (Figure 7D). Statistical analysis demonstrated that $98.2 \pm 5.1\%$ of GFP-transfected cells had stress fibers; in contrast, only $45.3 \pm 4.2\%$ cells transfected with TSC2 showed stress fibers (Figure 7C). These data demonstrate that TSC2 promotes stress fiber disassembly, and correlates with the inhibitory effect of TSC2 expression on RhoA activity.

DISCUSSION

The tumor suppressor proteins TSC1 and TSC2 have well-established roles in regulating cell growth, and loss of their functions is associated with LAM disease. Little, however, is known about the role of TSC1 and TSC2 in neoplastic LAM cell dissemination. This study shows that TSC2 plays a critical role in regulating LAM cell migration and invasiveness. Primary cultures of LAM cells show increased migratory activity and invasiveness. These effects are abolished by TSC2 re-expression. Importantly, TSC2-dependent regulation of LAM cell migration involves the modulation of RhoA activity, inhibition of which results in the abrogation of LAM cell migration. We also found that TSC1 is required for the regulation of RhoA activity in LAM cells. Collectively, these data suggest that loss of TSC2 may promote cell migration and invasiveness by specifically upregulating RhoA activity, which, in turn, requires TSC1.

Although the pathobiology of LAM is generally linked to abnormal cell growth, the neoplastic nature of LAM has been suggested by recent clinical and genetic evidence (16). Until recently LAM and TSC tumors were considered benign. Compelling evidence, however, supports a neoplastic model for LAM (17–19), suggesting a link between loss of TSC2 function and cell invasion and metastasis. Thus, the naturally occurring *TSC2* mutations and loss of heterozygosity (LOH) for *TSC2* in the Eker rat leads to renal adenoma and carcinoma, some of which become malignant and metastasize to the lung, pancreas, and liver (37). The hematogenous lung metastases of malignant uterine leiomyosarcoma were also identified in the Eker rat (38). Established *TSC2*-deficient ELT3 smooth muscle cells derived from Eker rat uterine leiomyomas develop tumors in nude mice (39). *TSC1*^{-/-} and *TSC2*^{-/-} embryonic fibroblasts showed anchorage-independent growth and colony formation in soft agar, indicating that loss of TSC2 induces growth independent

Figure 6. (A) Rho activity is increased in LAM cells. Serum-deprived LAM cells, ASM cells, and HBFs were subjected to the Rho activation assay. *Top panel* represents Rho-GTP pulled down with Rhotekin-RBD agarose and total Rho; images are representative from three independent experiments. *Bottom panel* shows the statistical analysis of basal Rho activity in LAM cells, ASM cells, and HBFs. Data represent mean values $\pm$ SE from three independent experiments by ANOVA (Bonferroni-Dunn). (B) TSC2 and siRNA TSC1 inhibit Rho activity in LAM cells. Cells were transfected with either GFP-TSC2 and control GFP, or siRNA TSC1 and siGLO RISC-Free siRNA control, followed by the Rho activity assay. *Top panel* represents Rho-GTP pulled down with Rhotekin-RBD agarose and total Rho detected with anti-Rho antibodies. *Bottom panel* shows quantitative analysis of three independent experiments performed using the Gel-Pro Analyzer Software. * $P < 0.001$ for GFP-TSC2 versus control GFP; siRNA TSC1 versus control siRNA by ANOVA (Bonferroni-Dunn).

Figure 7. TSC2 modulates stress fiber formation in LAM cells. (A) LAM cells have increased stress fiber formation. Serum-deprived LAM cells, HBFs, and ASM cells were subjected to staining with rhodamine-phalloidin to detect stress fibers. Quantitative analysis of F-actin staining was performed using a Nikon Eclipse TE2000-E Microscope and the Gel-Pro Analyzer Software. F-actin optical density of LAM cells was taken as 100%. Data represent mean values $\pm$ SE from three separate experiments by ANOVA (Bonferroni-Dunn). (B) TSC2 expression promotes stress fiber disassembly. Cells were transiently transfected with control GFP or GFP-TSC2 plasmids; cells were then serum deprived and were stained with rhodamine-phalloidin to detect F-actin (red) and immunostained with anti-GFP antibody to detect transfected cells (green). Images are representative of three separate experiments. (C) Statistical analysis of stress fiber formation in LAM cells transfected with TSC2. F-actin staining was quantified by visual analysis of digital images taken using a Nikon Eclipse TE2000-E microscope. Data represent the percentage of cells with stress fibers per total number of cells expressing GFP or GFP-TSC2 taken as 100%. Data represent mean values $\pm$ SE from three separate experiments by ANOVA (Bonferroni-Dunn). (D) Quantitative analysis of stress fiber disassembly induced by TSC2 expression. The stress fiber formation in cells expressing GFP or GFP-TSC2 was assessed by analyses of F-actin optical density using the Gel-Pro Analyzer Software. F-actin optical density of cells transfected with GFP was taken as 100%. Data represent mean values $\pm$ SE from three separate experiments by ANOVA (Bonferroni-Dunn).

of adhesion, which is one of the characteristics of the invasive cell phenotype (40, 41). Metastatic tumors in lung were found in TSC1 $^{+/-}$ mice with renal carcinoma (42). We demonstrate that primary cultures of human LAM cells have increased migratory activity and invasiveness that is abrogated by TSC2 re-expression, which indicates that loss of TSC2 function promotes invasive, neoplastic cell migration associated with LAM.

Little is known about the involvement of TSC2 in the regulation of cell motility. TSC2 forms a cytosolic heterodimer with TSC1 encoded by the tumor suppressor gene *TSC1*. Erk-dependent phosphorylation of TSC2 leads to dissociation of the TSC1/TSC2 complex, and promotes oncogenic transformation (43). It is shown that TSC2 binds TSC1 through its TSC1-binding domain, which overlaps with the Rho-activating domain of TSC1 (11), suggesting that TSC1/TSC2 complex formation may be involved in the regulation of RhoA GTPase activity and, ultimately, cell migration. Dysregulation of TSC2 function due to TSC2 loss or mutation may result in dysregulation of TSC1/TSC2 complex formation, upregulation of RhoA GTPase, and, finally, enhanced cell motility. Our study shows that unstimulated LAM cells show increased RhoA activity, and inhibition of either RhoA GTPase or its downstream effector ROCK abrogates the increased migratory activity of LAM cells. These data correlate with studies

demonstrating that RhoA activity is necessary for increased migration of human tumor cells (33). Further, upregulation of RhoA GTPase as well as RhoA effector protein ROCK are commonly observed in human cancers and are often associated with more invasive and metastatic phenotypes (33). Importantly, our data demonstrate that both TSC2 re-expression and down-regulation of TSC1 levels with specific siRNA abrogate abnormal RhoA activity in LAM cells. Collectively, our data suggest that loss of TSC2 function leads to TSC1-dependent RhoA activation, potentially through the dysregulation of the TSC1/TSC2 complex, which, in turn, promotes LAM cell migration and invasiveness. Our finding that expression of TSC2 inhibits RhoA activity in LAM cells contradicts a previous paper by Astrinidis and coworkers which demonstrated that re-expression of TSC2 in TSC2-deficient ELT3 cells results in RhoA activation and inhibition of cell migration (44). These differences in experimental data may occur due to differences in experimental approaches. We used *transient* TSC2 expression, which promotes the "classical" lamellopodial or mesenchymal motility driven by Rac1 activation and inhibition of RhoA. Astrinidis and colleagues used ELT3 and MDCK clones with *stable* overexpression of TSC2. Because RhoA activation is required to promote a rounded bleb-associated mode of motility in some tumor cell

lines, it is possible that stable overexpression of TSC2 had changed the cell phenotype, which requires activation of RhoA (33).

TSC1/TSC2 regulates cell growth and proliferation through the mTOR/S6K1 signaling pathway, which is abrogated by rapamycin, thus inhibiting mTOR. In TSC2-deficient cells, actin remodeling is insensitive to rapamycin, suggesting that the rapamycin-sensitive mTOR pathway does not contribute to biochemical events that occur as a part of the regulatory mechanisms of cell dynamics (15). The role of TOR in regulating the actin cytoskeleton has been well known in yeast (45); in mammals, however, this function of mTOR has remained an enigma. Recently, it was shown that mTOR, as a part of the rapamycin-insensitive mTOR complex 2 (mTORC2), regulates the actin cytoskeleton through Rac1 and PKC α (46, 47). Our current study also shows that LAM cell migration is rapamycin-independent. Further studies are needed to establish the relationship between the TSC1/TSC2-dependent regulation of the actin cytoskeleton, cell adhesion and motility, and mTORC2, and their relative contribution to LAM pathobiology.

Until recently TSC1 and TSC2 were generally considered to be regulators of protein translation and cell growth, and loss of function mutations of *TSC1* and *TSC2* promote abnormal cell growth associated with LAM pathology. However, loss of only TSC2 is associated with disease severity (5). Our study identifies that TSC2 functions not only as a negative regulator of cell growth, but also as critical regulator of actin remodeling, cell migration, and invasiveness. Thus, loss of TSC2 function may not only results in abnormal cell growth, but also in increased cell migration and invasiveness, which may lead to LAM disease severity. Our data further suggest that tumors characterized by TSC2 dysfunction may be amenable to therapies that abrogate activated RhoA. Further studies are necessary to determine the mechanism(s) by which TSC2 suppresses RhoA activity, cell migration, and invasiveness. Although LAM is a rare interstitial lung disease, in terms of research, its importance cannot be overstated because these studies specifically improve our understanding of the molecular signaling pathways regulating mesenchymal cell migration, such as vascular or airway smooth muscle cell or interstitial lung fibroblast migration (48); this is critically important in diseases such as pulmonary arterial hypertension, asthma, idiopathic pulmonary fibrosis, and interstitial lung disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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