Journal of Cellular Biochemistry 103:369–382 (2008)

PROSPECTS

# Pulmonary Lymphangioleiomyomatosis (LAM): Progress and Current Challenges

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**Abstract** Lymphangioleiomyomatosis (LAM), a rare lung disease, is characterized by the progressive proliferation, migration, and differentiation of smooth muscle (SM)-like LAM cells, which lead to the cystic destruction of the lung parenchyma, obstruction of airways and lymphatics, and loss of pulmonary function. LAM is a disease predominantly affecting women and is exacerbated by pregnancy; only a lung transplant can save the life of a patient. It has been discovered that in LAM, somatic or genetic mutations of tumor suppressor genes *tuberous sclerosis complex 1* (*TSC1*) or *TSC2* occur and the TSC1/TSC2 protein complex functions as a negative regulator of the mTOR/S6K1 signaling pathway. These two pivotal observations paved the way for the first rapamycin clinical trial for LAM. The recent discoveries that TSC1/TSC2 complex functions as an integrator of signaling networks regulated by growth factors, insulin, nutrients, and energy heightened the interest regarding this rare disease because the elucidation of disease-relevant mechanisms of LAM will promote a better understanding of other metabolic diseases such as diabetes, cancer, and cardiovascular diseases. In this review, we will summarize the progress made in our understanding of TSC1/TSC2 cellular signaling and the molecular mechanisms of LAM; we will also highlight some of the lesser explored directions and challenges in LAM research.
J. Cell. Biochem. 103: 369–382, 2008. 

**Key words:** TSC1; TSC2; smooth muscle; lung; tumor; signal transduction

The short history of lymphangioleiomyomatosis (LAM) for the last 10 years includes raising disease awareness by The LAM Foundation, linking somatic TSC2 gene mutations to LAM pathology [Carsillo et al., 2000], understanding that TSC2 loss of function mutations lead to the constitutive activation of p70 S6 kinase (S6K1) and abnormal LAM cell proliferation [Goncharova et al., 2002, 2006c], which provided the foundation for the development of new therapeutic strategies for LAM [Juvet et al., 2006; Taveira-DaSilva and Moss, 2006]. During the last 7 years, the TSC1/TSC2 signal transduction pathway has been the focus of extensive genetic and biochemical investigations, leading to a growing appreciation of the intricate mechanisms of its regulation and information transmission into biological processes. However, the inherent complexity of the TSC1/TSC2 signaling pathways and the pleiotropy of their regulation are such that despite intensive efforts, we still do not fully understand how dysregulation of TSC1/TSC2 leads to LAM disease. The complexity of TSC1/TSC2 signaling is rooted in the fact that it is not regulated in linear fashion, but instead functions as part of an integrated network interacting at multiple levels both through internal feedback loops and with other signaling pathways. The goal of our review is not to attempt a comprehensive coverage of the substantial body of work that has been done in TSC1/TSC2 signaling and LAM. Rather, while reviewing the most important observations, we have attempted to highlight the peculiarities in the pathology and genetics of LAM disease and in the TSC1/TSC2 signaling in LAM, that may be indicative of fundamental underlying cellular and molecular mechanisms, pursuit of which may lead to finding novel therapies for LAM.

Grant sponsor: NIH/NHLBI; Grant number: 2RO1HL071106; Grant sponsor: American Lung Association; Grant number: CI-9813; Grant sponsor: The LAM Foundation; Grant number: LAM048F01-05 and LAM059P07-06.

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Received 19 April 2007; Accepted 20 April 2007

DOI 10.1002/jcb.21419

## WHAT IS LAM DISEASE?

LAM is a rare progressive cystic lung disease affecting primarily women of childbearing age; it is characterized by the abnormal and potentially metastatic growth of atypical smooth muscle (SM)-like LAM cells within lungs and axial lymphatics [Johnson, 2006; Juvet et al., 2006; Taveira-DaSilva and Moss, 2006]. Growth of LAM cells leads to the cystic destruction of the lung interstitium, obstruction of airways and pulmonary lymphatics, and formation of fluid-filled cystic structures (lymphangioleiomyomas); these destructive changes of the lungs ultimately lead to the loss of pulmonary function and only a lung transplant can save the patient's life. Importantly, other rare diseases such as Birt-Hogg-Dube syndrome, Largerhans cell histiocytosis, and Sjogren's syndrome are presented with lung cysts [Taveira-DaSilva and Moss, 2006]. However, LAM disease, in addition to the lung cysts, is also characterized by lymphatic abnormalities and predominantly renal or abdominal angiomyolipomas (AML), which are vascular tumors consisting of LAM cells and adipocytes [Johnson, 2006; Taveira-DaSilva and Moss, 2006]. LAM is predominantly sporadic; however, it can be manifested in association with tuberous sclerosis complex (TSC), an autosomal dominant inherited disorder affecting 1 in 5,800 individuals. TSC patients develop hamartomas and benign tumors in the brain, heart, and kidneys; it is also manifested by cognitive defects, epilepsy, and autism; the prevalence of LAM among women with TSC is 26–39%. Importantly, malignant tumors of the kidney, which develop either as malignant AML or renal cell carcinomas, occur in about 63% of sporadic LAM [Astrinidis et al., 2000] and in most TSC patients [Kwiatkowski, 2003].

LAM is predominantly a disease of women and can be exacerbated by pregnancy; importantly, steroid receptors for estrogens were detected in LAM lungs while they were absent in normal lungs [Juvet et al., 2006], which is indicative that steroid hormones play a role in LAM pathology. Growing LAM cells, which infiltrate lungs, consist of two cell subpopulations: myofibroblast-like spindle-shaped cells and epithelioid-like polygonal cells; LAM cells predominantly form nodules, but also small clusters of cells can be found dispersed within lung parenchyma [Taveira-DaSilva and Moss, 2006]. Spindle-shaped cells expressing SM-specific proteins SM α-actin, desmin, and vimentin, form the core of the nodule surrounded by epithelioid-like cells, which have immunoreactivity for human melanoma black 45 (HMB45) antibody which binds glycoprotein gp100, a marker of melanoma cells and immature melanocytes [Juvet et al., 2006; Taveira-DaSilva and Moss, 2006]. Within the nodule, the spindle-shaped SM-like cells are haphazardly distributed [Krymskaya, 2007], in contrast to SM cells in airways and vasculature, where SM cells form well-organized SM layers [Taveira-DaSilva and Moss, 2006]. Interestingly, the dispersed LAM cells, which do not form larger nodules, also express HMB45, suggesting that some of the SM-positive LAM cells have melanocytic differentiation [Zhe and Schuger, 2004]. LAM cells also express another two melanocyte-specific proteins, CD63, a melanoma-associated protein, and PNL2, an uncharacterized melanocytic protein. Interestingly, the PNL2-positive LAM cells had decreased DNA synthesis detected by Ki67 immunostaining compared to PNL2-negative LAM cells [Zhe and Schuger, 2004]. Similarly, SM-like LAM cells show high immunoreactivity for proliferating cell nuclear antigen (PCNA), a marker of DNA synthesis and cell proliferation, compared to the epithelioid-like HMB45-positive cells [Taveira-DaSilva and Moss, 2006], suggesting the existence of two cell subpopulations with two different proliferative potentials, and that SM-positive LAM cells represent the proliferating component of the LAM nodules.

The issue of the origin of LAM cells is still debated: the first assumption might be that LAM cells are either airway or vascular in origin. However, this is a misnomer because LAM cells are found throughout the lungs without any predominant localization in close proximity to upper airway, bronchus, or vasculature. LAM cells also have the appearance of "immature" SM-like cells: they are irregularly distributed within the nodule without forming well-organized layers of SM cells, whereas SM cells in airways and vasculature form organized layers. One of the recent hypotheses, which is based on some genetic and clinical evidence [Henske, 2003; Crooks et al., 2004], is that LAM cells can originate from AML and can be brought into the lungs. The metastatic or neoplastic dissemination of LAM cells from AML is one of the possibilities.

Until recently, LAM and TSC tumors were considered benign. However, clinical and genetic data suggest a link between the loss of *TSC2* function and cell invasion and metastasis. Thus, mutational analysis of pulmonary LAM cells and AML cells from renal tumors of TSC patients with LAM showed identical TSC2 mutations [Yu et al., 2001]. Formation of secondary tumors with identical TSC2 mutations in the lymph nodes of LAM patients have also been reported [Sato et al., 2002; Karbowniczek et al., 2003]. Furthermore, LAM cells were found in blood, urine, and chylous fluids of LAM patients with AML [Crooks et al., 2004]. If the metastatic hypothesis for LAM is correct, then AML or renal tumors are the focal points of primary tumors, and pulmonary LAM cells represent metastasized secondary tumors. The metastatic origin of pulmonary LAM is an attractive hypothesis, which awaits experimental confirmation before it could become a valid model for LAM. Conspicuously, about a third of sporadic LAM cases are without AML, and in such cases LAM cell origin cannot be explained by metastatic or neoplastic cell dissemination. There is a single case reported about LAM nodule recurrence after single-lung transplantation in patient without renal AML [Karbowniczek et al., 2003]. This indicates that LAM cells may have an inherent potential to disseminate; however, more in vivo evidence are needed to uncover the origin of LAM cells.

## GENETICS OF LAM

Until recently, LAM was considered an "orphan" life-threatening disease of unknown etiology, with uncertain clinical prognosis, and no effective treatment. The key advance came with the discovery that the somatic or germline mutations of the tumor suppressor gene *TSC2* were associated with the progressive growth of SM-like LAM cells in the lungs of LAM patients [Smolarek et al., 1998; Carsillo et al., 2000]. The mutations in another tumor suppressor gene *TSC1* were also linked to the LAM disease [Sato et al., 2002]; however, mutations in the *TSC2* gene arise more frequently than *TSC1* mutations (about 60% of TSC cases, and the majority of LAM) [Kwiatkowski, 2003; Crooks et al., 2004; Johnson, 2006; Taveira-DaSilva and Moss, 2006]. The prevailing model for LAM is that the disease develops through a two hit mechanism: a mutation in either *TSC1* or *TSC2* is followed by a second hit referred to as loss of heterozygosity (LOH) leading to the loss of function of either TSC1 or TSC2 proteins. Thus, somatic LAM develops due to two acquired mutations (predominantly in *TSC2*), and LAM patients with TSC have one germline mutation (again predominantly in *TSC2*) and one acquired mutation [Juvet et al., 2006]. Until recently, the prevailing opinion was that mutational inactivation of either *TSC1* or *TSC2* genes results in a similar phenotype of the disease. However, genetic analysis [Dabora et al., 2001; Sancak et al., 2005] and some animal studies [Wilson et al., 2006] challenge this paradigm. Thus, TSC patients with *TSC1* mutations exhibited a milder disease phenotype, on average, compared to TSC patients with *TSC2* mutations in similar age groups [Dabora et al., 2001; Sancak et al., 2005]. Mutational analysis of familial and sporadic cases of TSC found a similar predominance of *TSC2* mutations and TSC2-associated disease severity compared to *TSC1* mutations and a milder disease phenotype [Sancak et al., 2005]. Another challenge in LAM relates to linking the distribution and type of *TSC1* or *TSC2* mutations to a specific region of the gene [Cheadle et al., 2000; Kwiatkowski, 2003], and linking these specific mutations to the disease phenotype and severity. Prognostic significance of mutations in different structural and functional regions of *TSC1* or *TSC2* are critically important for predicting disease outcome and, if identified, may be decisive for the selection of therapeutic approaches to treat the disease.

## LINKING TSC2 MUTATIONS TO ABNORMAL GROWTH OF SM-LIKE LAM CELLS

The next breakthrough in LAM came with the discovery of the function of TSC2 as a negative regulator of the mammalian target of rapamycin (mTOR)/p70 S6 kinase (S6K1) signaling. In vivo SM-positive cells in the LAM nodule showed high immunoreactivity for phosphorylated ribosomal protein S6 which correlates with its activation [Goncharova et al., 2002]. Ribosomal protein S6 is phosphorylated by an activated S6K1, an effector of mTOR and a component of the phosphatidylinositol 3-kinase (PI3K) signaling cascade [Krymskaya, 2003; Um et al., 2006]. In contrast, in vivo normal SM cells, for example, vascular SM cells in the blood vessels, show no immunoreactivity for

phospho-S6; this demonstrates that ribosomal protein S6 is hyperphosphorylated only in LAM cells and indicates that mTOR/S6K1 signaling is constitutively activated in SM-positive LAM cells in vivo [Goncharova et al., 2002]. These immunohistochemical data were confirmed by immunoblot analysis of LAM tissue lysates. However, to establish that the constitutive activation of mTOR/S6K1 signaling in LAM cells in vivo occurs as a result of the deregulation of TSC2 function, cell, and molecular biological studies were needed. Consequently, we have established primary cultures of LAM-derived cells from different sporadic LAM patients without TSC and identified the mutations in the TSC2 gene in LAM-derived cell cultures [Goncharova et al., 2002, 2006c]. In LAM cells, ribosomal protein S6 was hyperphosphorylated and S6K1 was also constitutively activated; TSC2 re-expression inhibited these both effects, thus, establishing that TSC2 is an upstream regulator of mTOR/S6K1 signaling. LAM cell cultures also showed increased proliferation under serum-free conditions, which was inhibited by TSC2 re-expression or treatment with rapamycin. Rapamycin (i.e., sirolimus, an antifungal macrolide antibiotic approved by the US Food and Drug Administration drug for immunosupression) is a specific inhibitor of mTOR, a serine-threonine kinase, an obligatory upstream regulator of S6K1. Thus, the linkage between the mutational inactivation of TSC2 and the constitutive activation of S6K1 in LAM cells [Goncharova et al., 2002, 2006c] identified a potential molecular target to treat LAM, which led to the first rapamycin clinical trial. While the results of the first pilot studies using rapamycin in clinic at the University of Cincinnati are eagerly awaited, the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) randomized controlled Trial has already begun enrollment.

## TSC1/TSC2 TUMOR SUPPRESSOR COMPLEX

In the last few years, dramatic advances were made in defining the roles of tumor suppressors TSC1 and TSC2 in cellular signaling networks and in understanding how loss of their functions results in dysregulating multiple critical cellular processes including cell cycle, cell size, cell proliferation, actin dynamics, cell motility, microtubule organization, and adhesion. Here, we will highlight major signaling networks modulated by TSC1/TSC2 signaling and summarize the currently established regulators and effectors of TSC1/TSC2 tumor suppressor activity. Because of space constrains, we will cite only the most recent original studies; references to previously published original studies can be found in the following excellent comprehensive reviews [Cheadle et al., 2000; Findlay et al., 2005; Nobukini and Thomas, 2005; Inoki et al., 2005a, 2005b; Avruch et al., 2006; Johnson, 2006; Juvet et al., 2006; Shaw and Cantley, 2006; Taveira-DaSilva and Moss, 2006; Um et al., 2006; Vivekanand and Rebay, 2006; Lee et al., 2007].

Tumor suppressor genes *TSC1* and *TSC2* encode TSC1 and TSC2 proteins, also known as hamartin and tuberin, respectively [Krymskaya, 2003]. TSC1, a 130 kDa protein, and TSC2, an approximetely 200 kDa protein, form a physical and functional complex, in which TSC1 apparently functions as the regulatory component stabilizing TSC2, and facilitating TSC2 catalytic function as a GTPase activating protein (GAP) for the small GTPase Rheb (Fig. 1). A recent study suggests that in the absence of growth factor or insulin stimulation,

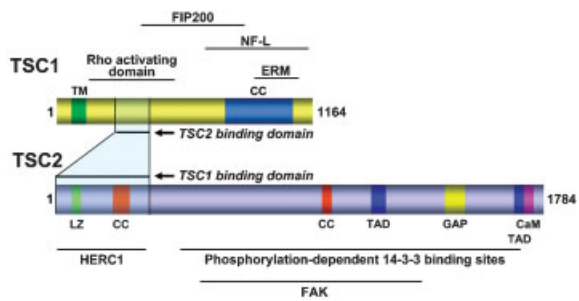


**Fig. 1.** Structural and functional domains of TSC1 and TSC2. TSC1 has putative structural *trans*membrane [TM, amino acids (aa) 127–144] and *c*oiled-*c*oil [CC, aa 730–996] domains; functional domains of TSC1 include overlapping Rho activating domain [aa 145–510], TSC2-binding domain [aa 302–430], and *f*ocal adhesion kinase family *i*nteracting *p*rotein of *200* kD binding domain [FIP200, aa 403–787], *n*euro*f*ilament *l*ight chain [NF-L, aa 674–1164], and *e*zrin-*r*adixin-*m*oesin [ERM, aa 881–1084] binding domains. TSC2 includes putative *l*eucine *z*ipper [LZ, aa 81–98], two CC domains [aa 346–371 and 1008–1021], two *t*ranscription-*a*ctivated *d*omains [TAD, aa 1163–1259 and 1740–1755], GAP homology [GAP, aa 1517–1674] and *cal*modulin-binding [CaM, aa 1740–1755] domains. Functional domains of TSC2 include TSC1-binding domain [aa 1–418], *h*omologous to *E6*-AP carboxyl-terminus and *RCC1*-like binding domain [HERC1] domain, *f*ocal *a*dhesion *k*inase binding domain [FAK, aa 609–1080] and multiple phosphorylation-dependent 14-3-3 binding sites.

TSC1 localizes TSC2 at the intracellular membrane, which is required for the stabilization of TSC2 and the efficiency of TSC2 function as a RhebGAP [Cai et al., 2006] (Fig. 2). In response to growth factor stimulation, TSC2 is inactivated by Akt-dependent phosphorylation, which disrupts its interaction with TSC1. The dissociation of the TSC1/TSC2 complex promotes re-localization of the phosphorylated TSC2 into the cytosol, where it binds different isoforms of 14-3-3 proteins and, apparently, becomes highly ubiquitinated [Cai et al., 2006; Chong-Kopera et al., 2006]. Dissociation of TSC2 from TSC1 makes TSC2 also available for binding with HERC1, a protein with an E3 ubiquitin ligase *h*omology to the *E*6AP *c*arboxyl *t*erminal (HECT) domain [Chong-Kopera et al., 2006]. Importantly, both HERC1 and TSC1 bind to the *N*-terminal region of TSC2 [Krymskaya, 2003; Goncharova et al., 2004, 2006b; Chong-Kopera et al., 2006] (Fig. 2), and TSC1 prevents the interaction of TSC2 and HERC1. Thus, a possible mechanism by which TSC1 binding stabilizes TSC2 is that TSC1 inhibits the interaction between TSC2 and HERC1, thereby inhibiting TSC2 ubiquitination and degradation [Chong-Kopera et al., 2006]. Indeed, in TSC1−/− mouse embryonic fibroblasts (MEFs) TSC2 levels are markedly decreased [Kwiatkowski et al., 2002]; in contrast, when TSC2 is knocked-down in TSC2−/− MEFs, TSC1 levels are unchanged [Zhang et al., 2003]. Another ring-finger containing E3 ligase *p*rotein *a*ssociated with *m*yc (PAM) also binds TSC2 and may potentially also target TSC2 for ubiquitination and proteasomal degradation [Murthy et al., 2004]. Ubiquitination and proteasomal degradation of TSC2 is potentially a critical factor in the regulation of TSC2 cell functions. It is a possibility that TSC2 mutations that weaken its interaction with TSC1, destabilize TSC2 and target it for degradation, thus, promoting activation of small GTPase Rheb and mTOR/S6K1 signaling leading to increased cell growth and potentially to the development of disease-relevant pathologies.

## TSC1/TSC2 INTEGRATES GROWTH FACTOR, INSULIN, AND ENERGY SIGNALING NETWORKS IN COORDINATED REGULATION OF TORC1 AND CELL GROWTH

Compelling genetic and biochemical studies in *Drosophila* and mammals demonstrate that the PI3K-Akt and the TSC1/TSC2-Rheb-mTOR-S6K1 signaling cascades constitute branches of a highly connected and conserved signal transduction network regulating cell growth and proliferation [Avruch et al., 2006; Shaw and Cantley, 2006; Lee et al., 2007]. Consequently, the majority of inhibitory TSC2 phosphorylation sites are regulated by Akt activation (Table I). While the multiple Akt-dependent sites that inactivate TSC2 RhebGAP activity leading to mTOR/S6K1 activation have been identified, the physiological significance of Akt-dependent TSC2 phosphorylation had not been questioned. Conflicting evidence comes from studies in *Drosophila* showing that mutation of Akt phosphorylation sites on TSC2 neither prevented S6 activation by insulin nor affected *Drosophila* development [Dong and Pan, 2004]. A very recent publication provides convincing data that novel protein *p*roline-*r*ich *A*kt/PKB *s*ubstrate *40* kDa (PRAS40) mediates Akt-dependent insulin-induced activation of

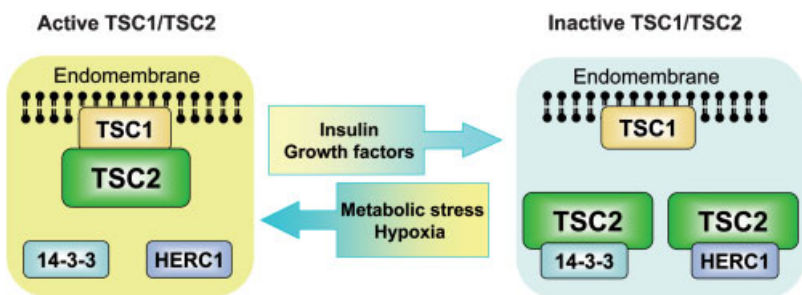


**Fig. 2.** Schematic representation of the potential regulatory mechanism of TSC1/TSC2 by growth factors, metabolic stress and energy. Growth factor input induces phosphorylation of TSC2 by various protein kinases followed by dissociation of TSC2 from TSC1 and its translocation into the cytosol, where TSC2 binds to 14-3-3 or HERC1 proteins, which leads to TSC2 inactivation and/or degradation. Upon metabolic stress or under hypoxic conditions, TSC1 and TSC2 in the form of an active complex localize at the endomembrane.

**TABLE I. The Ins and Outs for TSC2 Regulation by Phosphorylation**

| Upstream effectors/ Protein kinases | Phosphorylation sites | Effect of phosphorylation |
|---|---|---|
| I. ENHANCED TSC2 TUMOR SUPPRESSOR ACTIVITY | | |
| AMPK | S1345 | Stabilization of TSC1/TSC2 complex under energy stress conditions |
| GSK3 | S1337 S1341 | |
| RTP801/REDD1 | Not known | Stabilization of TSC1/TSC2 complex under hypoxic conditions |
| RTP801L/REDD2 | | |
| II. INACTIVATED TSC2 TUMOR SUPPRESSOR ACTIVITY | | |
| PKB/Akt | S939 S981 S1086 S1088 S1130 S1132 T1422 T1462 T1798 | TSC1/TSC2 complex dissociation; TSC2/14-3-3 complex formation; TSC2 degradation |
| RSK1 | S1798 | TSC1/TSC2 complex dissociation |
| ERK1/2 | S540 S664 S939 T1462 | |
| p38 MAPK/MK2 | S1210 | TSC2/14-3-3 complex formation |
| FAK | Not known | Promotes S6K1 activation |

mTORC1 in the absence of TSC2 [Haar et al., 2007; Sancak et al., 2007]. However, further studies are needed to determine whether Akt phosphorylates TSC2 in parallel to PRAS40 phosphorylation; also whether there is a differential effect of Akt phosphorylation on TSC2 RhebGAP activity depending on the species, that is, *Drosophila* versus mammals; or the specific agonists, for example, PDGF, EGF, IGF, insulin, or the time of stimulation; or Akt-TSC1/TSC2 cellular localization.

In addition to PI3K-Akt-dependent inactivating phosphorylation, the activity of the TSC1/TSC2 tumor suppressor complex is also regulated by other upstream kinases which relay the signals from growth factors, nutrients, and energy levels (Table I and Fig. 3). *E*xtracellular signal-*r*egulated *k*inase (Erk1/2) [Ma et al., 2005] and Erk-activated p90 ribosomal S6 kinase 1 (RSK1) phosphorylate TSC2 on amino acid residues, which overlap with ones phosphorylated by Akt; others are Erk-specific [Shaw and Cantley, 2006]. Importantly, phosphorylation by Erk suppresses TSC2 tumor suppressor function not only in vitro but also in vivo as demonstrated using a xenographic animal model [Ma et al., 2005]. Furthermore, immunohistochemical analysis of *sub*ependymal *g*iant cell *a*strocytomas (SEGA), a common TSC-associated brain lesion, shows activation of Erk and Akt in parallel to inactivating phosphorylation of TSC2 on Ser1462 and activation of S6K1 [Han et al., 2004]. Thus, in addition to classical LOH of *TSC2* and consequent loss of tumor suppressor function of TSC2, inhibitory phosphorylation of TSC2 may serve as another critical mechanism for TSC2 inactivation in vivo, which may contribute to the development of pathological conditions and cancer.

The p38-activated kinase MK2 (also known as MAPKAPK2) directly phosphorylates TSC2 on Ser-1210, which creates a binding site for 14-3-3 [Li et al., 2003]. The p38 MAPK-dependent phosphorylation of TSC2 is required for anisomysin-induced activation of S6K1, demonstrating additional mechanisms of TSC2 regulation, which may act independently and/or in parallel to TSC2 phosphorylation by PI3K/Akt and Erk; although, the functional significance of TSC2 phosphorylation by MK2 remains to be determined.

Another pathway of TSC2-depedent regulation of S6K1 activity involves focal adhesion kinase (FAK) and cell adhesion [Gan et al., 2006b]. FAK directly associates with TSC2 without apparent interaction with TSC1 and promotes tyrosine phosphorylation of TSC2, which is required for FAK-dependent S6K1 activation. It is important to note, that FAK-dependent activation of S6K1 is critical for cell adhesion-induced S6K1 phosphorylation; in contrast, in cell suspension, S6K1 activity is inhibited by TSC2. This study indicates that TSC2 tumor suppressor activity can be negatively regulated not only by growth factors, but also by cell adhesion, which is known to play a critical role in regulating cell growth, cell cycle progression, and proliferation.

A recent study demonstrates that TSC1 binds to a FAK family interacting protein of 200 kD (FIP200), a newly identified protein that binds to the kinase domain of FAK and inhibits its kinase activity [Gan et al., 2005]. FIP200 binds TSC1 at its 403–787 region [Gan et al., 2005], which partially overlaps with the TSC1 domain binding for TSC2 (amino acids 302–430) [Goncharova et al., 2004, 2006b] (Fig. 1). The interaction of TSC1 with FIP200 prevents

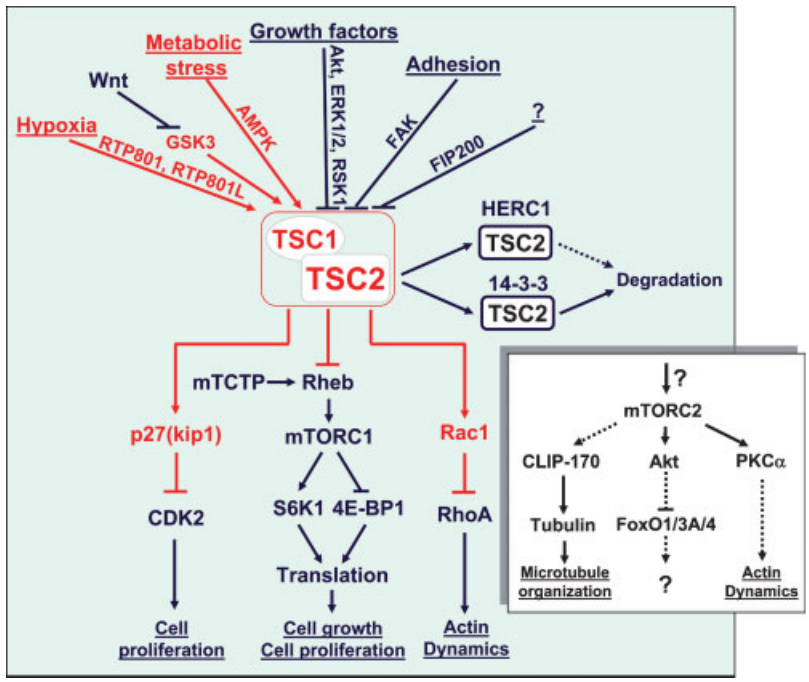


**Fig. 3.** Schematic representation of TSC1/TSC2 signaling. The TSC1/TSC2 complex controls cell growth depending on inputs from growth factors, nutrients, cellular energy levels, oxygen levels, and cell adhesion. Under metabolic stress or hypoxic conditions TSC1/TSC2 is positively regulated by the phosphorylation of RTP801 (REDD1) and RTP801L (REDD2) or AMPK, respectively, which augment TSC1/TSC2 complex stability. This leads to inhibition of mTOR-dependent phosphorylation of S6K1 and 4EBP1 and blockade of protein translation, cell growth and proliferation. TSC1/TSC2 regulates the activities of Rac1 and RhoA GTPases, actin cytoskeleton, and cell adhesion. Under growth factor stimulation, TSC2 is subjected to inhibitory phosphorylation by Akt, ERK1/2, and ERK1/2-dependent RSK1, which results in the potential dissociation of the TSC1/TSC2 complex and the binding of TSC2 with 14-3-3 protein or a HERC1 ubiquitin ligase. This, in turn, promotes activation of Rheb GTPase, which activates mTORC1 by directly binding to the Raptor. mTORC1 positively regulates protein translation by phosphorylation translation control proteins S6K1 and 4E-BP1, which promote cell growth and proliferation. TSC1/TSC2 down-regulation also leads to reduction of p27(kip1) levels, increasing CDK2 activity and cell proliferation. FAK and FIP200 phosphorylate TSC2, which leads to inhibition of S6K1 activity. Wnt-dependent GSK3 inhibition results in suppression of TSC1/TSC2 activity and stimulation of protein translation and cell growth in a mTORC1-dependent manner. In addition to mTORC1, mTOR is a member of the functionally distinct complex mTORC2 (mTOR/rictor/mLST8/Sin1); upstream regulators of mTORC2 are currently unknown. mTORC2 apparently controls microtubule organization and actin dynamics through CLIP-170 and PKCα, respectively. Additionally, mTORC2 phosphorylates Akt at Ser473, which promotes Akt-dependent phosphorylation of FoxO1, FoxO3A and FoxO4 transcription factor. Red or blue coloring indicates regulatory events which are either positively or negatively, respectively, involved in TSC1/TSC2 signaling. Arrows indicate functional enhancement; flat bars indicate functional suppression.

TSC1/TSC2 complex formation leading to increased S6K1 activity and cell growth [Gan et al., 2005], which further supports the notion that TSC2 activity as a tumor suppressor is regulated by TSC1 binding. This study identifies FIP200 as a putative inhibitor of TSC1/TSC2 tumor suppressor activity [Gan et al., 2006a].

Growth factor- or insulin-induced inactivating phosphorylation of TSC2 inhibits its Rheb-GAP activity and leads to activation of small GTPase Rheb, which directly binds to and activates mTOR [Nobukini and Thomas, 2005; Avruch et al., 2006]. Rheb activity is also positively regulated by the recently discovered guanine nucleotide exchange factor activity of translationally controlled tumor protein (TCTP) [Hsu et al., 2007]; whether TCTP acts downstream or parallel to TSC1/TSC2 remains to be elucidated. The role of TSC2 in regulating the mTOR/S6K1 signaling pathway has been intensively studied, and it is clearly demonstrated that the TSC1/TSC2 complex is a key regulator of mTOR/S6K1 signaling. Thus, phosphorylation-induced inhibition of TSC2 Rheb-GAP activity releases Rheb GTPase activity, which directly activates mTOR in the mTOR/Raptor complex (TORC1). Serine-threonine kinase mTOR, an upstream activator of S6K1, is part of both complexes: the rapamycin-sensitive

mTOR/Raptor (mTORC1) phosphorylating S6K1, and the rapamycin-insensitive mTOR/Rictor (mTORC2). Comprehensive reviews of the manner in which dysregulation of TSC1/TSC2-mTORC1 modulates intracellular signaling, for example, negative feedback inhibition of insulin signaling and Akt activation, are published in [Harrington et al., 2005; Inoki et al., 2005a].

The recent discovery that mTORC2 phosphorylates Akt at Ser473 suggested the possibility that mTORC2 may regulate the activation of TSC2 and mTORC1 [Sabatini, 2006]. While Akt phosphorylation on Ser473 has been widely used as a key indicator of its activation, the physiological function of this phosphorylation remains illusive. The kinases that phosphorylate Akt on Ser473 were generally named phosphoinositide-dependent kinase 2 (PDK2) in contrast to PDK1, which phosphorylates Akt-Thr403, another site that is critical for Akt activation. In addition to mTOR/Rictor, there are a group of kinases with PDK2 activity including ILK, DNA-PK, and PKC [Woodgett, 2005]. Another member of the mTORC2 complex has been identified, Sin, which also regulates Akt-Ser473 phosphorylation and maintains mTOR/Rictor complex integrity [Frias et al., 2006; Shiota et al., 2006; Yang et al., 2006; Guertin et al., 2006b]. Knockout of Rictor and Sin in mice solved a puzzle about the role of mTORC2 in the regulation of Akt [Polak and Hall, 2006; Bhaskar and Hay, 2007]. Rictor and Sin are essential for normal growth and development of the mouse embryo and are critical for Akt phosphorylation at Ser473 during embryogenesis [Shiota et al., 2006; Yang et al., 2006; Guertin et al., 2006b]. Importantly, knockdown of mTORC2 proteins had little effect on TSC2, mTOR, S6K1, and GSK1, which are known effectors of Akt [Jacinto et al., 2006]. In contrast, Rictor and Sin knockdown affected phosphorylation levels of the Forkhead transcription factors FoxO1, FoxO3A and FoxO4, which are negatively regulated by Akt and required for cell survival [Sarbassov et al., 2005; Jacinto et al., 2006; Yang et al., 2006; Guertin et al., 2006a,b]. Collectively, these studies demonstrate that Akt-Ser473 is not essential for Akt-dependent regulation of TSC1/TSC2 and that mTORC2 does not modulate the activity of TSC1/TSC2-mTORC1.

In addition to growth factors and insulin, TSC1/TSC2 activity is regulated by cellular energy, that is, the availability of glucose and ATP. Energy starvation results in decreased ATP levels and increased AMP levels leading to activation of AMP-activated protein kinase (AMPK) [Hardie, 2007]. In mammalian cells, AMPK is activated by glucose depravation and other stresses, which leads to ATP depletion such as attenuation of ATP synthesis during hypoxia or increased ATP consumption during physical exertion. Activated AMPK initiates two major signaling cascades, one is to switch on catabolic pathways to generate ATP, and another is to switch off ATP-consuming processes, which are not essential for short-term survival, that is, protects against apoptosis during glucose starvation. Thus, decreased cellular ATP levels activate AMPK, which directly phosphorylates TSC2; this is followed by inhibition of mTOR/S6K1 leading to inhibition of translation and attenuation of protein synthesis [Um et al., 2006]. AMPK phosphorylation primes TSC2 for its subsequent phosphorylation by GSK3 [Inoki et al., 2006]. Activity of GSK3 is negatively regulated by the Wnt signaling pathway, which plays a critical role in cellular proliferation, especially during development [Clevers, 2006]. Wnt-dependent activation of S6K1 requires inhibition of GSK3 and TSC1/TSC2 activity [Inoki et al., 2006]. Inhibition of GSK3 activity by Wnt is specific and distinct from its inhibition by Akt and RSK1 [Choo et al., 2006]. Thus, TSC2 integrates both energy and Wnt signaling in coordinated inhibition of mTOR/S6K1 signaling, protein translation, and cell growth.

Prolonged stressors, for example, hypoxia, also regulate TSC1/TSC2 by upregulating TSC1/TSC2 complex activity through two stress-induced proteins, RTP801/REDD1 and RTP801L/REDD2 [Corradetti et al., 2005; Ellisen, 2005]. These two proteins have approximately 50% homology to each other but have little homology to other known proteins. RTP801/REDD1 and RTP801L/REDD2 are mammalian orthologs of *Scylla* and *Charybdis*, which were identified as negative regulators of the TOR pathway in *Drosophila* [Reiling and Hafen, 2004]. Activated *Scylla* and *Charybdis* inhibited S6K with concomitant inhibition of cell size and growth, and acted upstream of TSC1/TSC2. The mechanism of RTP801-dependent regulation of TSC1/TSC2 complex activity remains unknown.

Thus, the role of TSC1/TSC2 tumor suppressor complex in integrating signaling networks

from growth factors, nutrients, and energy has been clearly demonstrated. However, most of the findings were based on data obtained using biochemical and molecular in vitro models; the relative contribution of potentially differential regulation of TSC1/TSC2 by growth factors, nutrients, and energy in LAM remains to be determined. The one of the major challenges in this direction of LAM research is a lack of cell model for LAM [Krymskaya, 2007]. LAM cell model is urgently needed not only to validate TSC1/TSC2 signaling in physiologically relevant cells, but also to serve as preclinical LAM cell model for validation of therapeutic approaches to treat LAM disease.

## TSC1/TSC2 AND RHO GTPASES

While the link between the loss of TSC2 function and abnormal cell growth is well established, little information is available about the exact role of TSC1 and TSC2 in regulating cell migration and metastatic LAM cell dissemination. Thus, primary LAM cell cultures show increased migration and invasiveness that are abolished by the re-expression of TSC2 [Goncharova et al., 2006a,b]. Increased RhoA activity was also found in serum-deprived primary LAM cell cultures; and re-expression of TSC2 inhibited RhoA activity [Goncharova et al., 2006b]. It is well established that the Rho family of small GTPases plays a critical role in regulating cell motility, transformation, invasion, and metastasis. Pharmacological inhibition of RhoA GTPase or its downstream effector ROCK abolished the increased migratory activity of LAM cells, indicating that RhoA activation is critical for the upregulation of LAM cell migration [Goncharova et al., 2006b]. These data suggest that LAM cells have the potential to migrate abnormally and metastasize in vitro, which may support the metastatic model for LAM [Henske, 2003]. Furthermore, RhoA GTPase may serve as a novel target for a therapeutic approach in LAM.

Findings that RhoA is activated in primary cultures of LAM cells [Goncharova et al., 2006b] suggest a potential link between TSC1/TSC2 signaling and Rho GTPases. Indeed, the role of TSC1 in regulating RhoA activity and cell adhesion was demonstrated [Lamb et al., 2000] prior to the discovery that the interaction between TSC1 and TSC2 stabilizes both proteins in a functional complex, in which TSC2 has the catalytic GAP activity toward Rheb GTPase. Thus, overexpression of TSC1 in NIH 3T3 fibroblasts activated RhoA and promoted stress fiber formation [Lamb et al., 2000]. Membrane binding of TSC1 through its *C*-terminus with the ezrin-radixin-moesin (ERM) family of actin-binding proteins is a prerequisite for TSC1-dependent RhoA activation (Fig. 1). However, the mechanism of RhoA activation by TSC1 is not known.

Importantly, the Rho-activating domain of TSC1 (amino acids 145–510) overlaps with the domain that binds to TSC2: the amino acids 302–430 of TSC1 associate with amino acids 1–418 of TSC2 and are required for TSC1/TSC2 complex formation. These data suggested that the interaction of TSC1 with TSC2 is critical for TSC1-dependent RhoA activation and focal adhesion formation; and this has already been demonstrated experimentally [Goncharova et al., 2004]. Thus, re-expression of TSC2 in TSC2-null cells inhibited RhoA activation, stress fiber, and focal adhesion formation. Importantly, only the TSC1-binding domain of TSC2 or siRNA-induced TSC1 depletion produced similar effects, indicating that increased RhoA activity, stress fiber, and focal adhesion formation requires TSC1 and is regulated by the formation of the TSC1/TSC2 complex. Our studies also showed that TSC1-induced RhoA activation is regulated by the TSC1-dependent reciprocal inhibition of Rac1 by an as yet unidentified mechanism. These studies suggest that TSC1 and TSC2 modulate the activities of small Rho GTPases and may also regulate cell adhesion and motility.

## TUMORS IN HETEROZYGOUS *TSC2* MUTANT MICE AND EKER RAT RESEMBLE TSC BUT DO NOT REPRODUCE LAM

One of the major challenges in LAM research is a lack of an animal model that phenotypically mimics the disease. While *TSC1* and *TSC2* heterozygous mice develop tumors, which resemble the TSC phenotype, this animal model does not develop a phenotype that mimics LAM disease. Thus, knock-out of either *TSC1* or *TSC2* in the mice produces a lethal phenotype, while heterozygous animals develop bilateral renal cystadenomas, which progress at low frequency to renal carcinoma and liver hemangiomas due to LOH of these genes [Kobayashi et al., 1999; Onda et al., 1999]. Interestingly,

*TSC1* mutations in mice caused the development of similar, but not identical tumors compared to tumors developed in *TSC2* heterozygote mice, for example, the development of renal tumors in *TSC1*+/− mice were slower compared to *TSC2*+/− mice [Kobayashi et al., 2001; Wilson et al., 2006]. While, these data might be indicative of the differential effects of *TSC1* or *TSC2* loss on tumor pathology, further studies are needed to confirm these notions.

Before discovery of the *TSC2* gene, Eker rat model of familial renal tumors was one of the earliest examples of rodent models of hereditary tumors, and served to validate Knudson's two-hit hypothesis [Yeung, 2004]. Eker rats carry a spontaneous germline mutation in the *TSC2* gene caused by the insertion of an approximately five kilobase DNA fragment in the codon corresponding to codon 1272 in human *TSC2* [Kobayashi et al., 1995]. In addition to renal tumors, the Eker rat also develops tumors in spleen, uterus, pituitary gland and brain due to LOH [Yeung, 2004].

*TSC1* and *TSC2* heterozygous mice and Eker rat resemble the disease phenotype for TSC; however, these animals do not develop lung tumors. Compelling epidemiological evidence support the link between TSC and LAM disease, thus, about 40% of adult females with TSC develop cystic lung disease consistent with symptoms of LAM [Johnson, 2006]. Genetic evidence also unequivocally shows that *TSC2* and, in rare cases *TSC1*, gene mutations are susceptibility factors for LAM. This raises the question why TSC1 or TSC2 animal models do not translate into the LAM disease phenotype. The LAM animal model is urgently needed to serve as a preclinical model for the study of disease pathophysiology and for the development of potential therapies.

## TOWARD COMBINATIONAL THERAPY IN LAM

While the critical role the mTOR-S6K1 signaling pathway plays in regulating cell growth makes mTOR-S6K1 a logical therapeutic target in LAM, recent studies suggest caution should be taken in the use of mTOR inhibitors to treat cancers that result from the activation of this signaling pathway. The continuous activation of mTOR-S6K1 in cells in culture can lead to a negative feedback loop of the upstream activators of PI3K [Findlay et al., 2005]. Similarly, in vivo, tumors that arise in mice due to loss of TSC2 exhibit constitutive activation of the mTOR-S6K1 pathway but not activation of the PI3K pathway [Manning et al., 2005]. This feedback appears to explain why tumors that lose TSC2 are predominantly benign [Cheadle et al., 2000; Kwiatkowski, 2003; Yeung, 2003]. The constitutive activation of the mTOR-S6K1 signaling pathway due to TSC2 loss in the context of PI3K activation induces much more aggressive tumors leading to the death of the mice [Manning et al., 2005]. Additionally, prolonged treatment with rapamycin may lead to the development of drug-resistance [Sabatini, 2006].

A central therapeutic question for treating LAM is whether targeting other signaling cascades can be more effective than a single agent alone. Rapamycin targets only the mTOR-S6K1 signaling pathway, which predominantly controls protein synthesis and cell growth. Recent compelling evidence in cancer research demonstrates that statins inhibit the activity of RhoA, cancer cell invasion, and have synergistic anticancer activity with EGF receptor inhibitors [Demierre et al., 2005]. Statins were developed to treat cardiovascular disease, and, surprisingly, randomized controls trials for preventing cardiovascular disease indicated that statins had provocative and unexpected benefits for reducing colorectal cancer and melanoma. Statins are small molecule inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, which plays a critical role in the cholesterol metabolic pathway called mevalonate cascade. As well as reducing cholesterol levels, statins can inhibit other products and reactions in the mevalonate pathway, including the generation of mevalonate and downstream isoprenoids. Isoprenoids are long, hydrophobic molecules — for example, farnesyl and geranylgeranyl groups — that attach to various proteins such as members of the Rho family of small GTPases. This attachment allows them to anchor to cell membranes and perform their normal functions. Inhibition is this hydrophobic modification of Rho GTPases by statins has significant effects on cancer growth. Preclinical models of colorectal and breast cancer, and melanoma show that statin anticancer effects involve the inhibition of geranylgeranylation of Rho GTPases [Demierre et al., 2005]. Thus, statins demonstrate the beneficial effects on inhibiting carcinogenesis; however, little is known about the effects of

statins on LAM. There is evidence that simvastatin inhibits Rho activity and migration of primary human LAM cell cultures [Goncharova et al., 2007]. Importantly, simvastatin also inhibits LAM cell proliferation; furthermore, combined treatment of LAM cells with simvastatin and rapamycin abrogated cell proliferation to a greater degree compared to inhibitory effects of each agent alone [Goncharova et al., 2007]. Thus, simultaneous inhibition of mTOR/S6K1 and RhoA GTPases may have potential as a therapeutic strategy for combinational treatment in LAM.

## CONCLUDING REMARKS

As we stated before, LAM disease is characterized by abnormal growth, proliferation, and migration of atypical SM-like cells in the lungs leading to cystic destruction of the lungs. Compelling evidence shows that the dysfunction of TSC1/TSC2 signaling contributes to abnormal growth, proliferation, and, potentially, to metastatic dissemination of LAM cells. However, it is not known whether TSC1/TSC2 dysfunction leads to cystic destruction of the lungs or whether other yet unidentified signaling molecules contribute to this aspect of LAM pathology. For example, high levels of serum-response factor (SRF) were detected in LAM lungs [Zhe et al., 2005], which may produce a pro-proteolitic imbalance in several matrix metalloproteinases (MMPs) and their natural inhibitor, a tissue inhibitor of metalloproteinase (TIMP)-3, and this imbalance may contribute to the progressive cystic destruction of the lungs [Krymskaya and Shipley, 2003; Zhe et al., 2003]. Further investigations will be required to determine whether there is a relationship between TSC1/TSC2 dysfunction and SFR-MMP imbalance in LAM. Evidence also points to the putative link between *TSC2* gene dysfunction and polycystic kidney disease (PKD), caused, in part, by mutations in the *PKD1* gene [Cai and Walker, 2006]. Human *TSC2* and *PKD1* genes are adjacent to each other in tail-to-tail orientation on chromosome 16, and large DNA deletion involving both *TSC2* and *PKD1* occurs in TSC patients with severe renal cystic disease. TSC2 defines cellular localization of PDK1 [Kleymenova et al., 2001]; however, whether TSC2 and PKD1 contribute to LAM pathology remains to be established.

The one of the most challenging and lesser explored directions in LAM research is the role of estrogen in the etiology and pathology of LAM. LAM predominantly develops in premenopausal women and can worsen during pregnancy; exogenous estrogen also exacerbates LAM [Johnson, 2006; Juvet et al., 2006; Taveira-DaSilva and Moss, 2006]. Various anti-estrogen strategies have been used in the treatment of LAM; however, their effectiveness has not been demonstrated. The importance of further investigations into the role of estrogen in etiology, pathology, and cell signaling of LAM cannot be overestimated, not only because it may uncover mechanisms of LAM pathology, but also because this is the potential to develop novel therapeutic strategies for LAM treatment.

In summary, it is apparent that TSC1/TSC2 plays multiple roles in controlling LAM cell behavior, including growth, proliferation, adhesion, and motility. However, it remains to be established whether only TSC1 or TSC2 dysfunction accounts for multisystem pathological changes seen in pulmonary LAM. LAM pathology provides clues to the enigmatic complexity of LAM disease, solving of which may lead not only to elucidating cellular and molecular mechanisms, but also may pave the way to finding the cure for LAM.

## ACKNOWLEDGMENTS

We apologize for not being able to cite many important studies due to restrictions on the number of references. We thank Dr. Reynold A. Panettieri, Jr. and Mr. Andrew Eszterhas for helpful discussions and critical reading of the manuscript.

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