Molecular Pathogenesis of Lymphangiomyomatosis Lessons Learned from Orphans

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Lymphangiomyomatosis (LAM) is a rare progressive cystic lung disease affecting young women. The pivotal observation that LAM occurs both spontaneously and as part of the tuberous sclerosis complex (TSC) led to the hypothesis that these disorders share common genetic and pathogenetic mechanisms. In this review we describe the evolution of our understanding of the molecular and cellular basis of LAM and TSC, beginning with the discovery of the TSC1 and TSC2 genes and the demonstration of their involvement in sporadic (non-TSC) LAM. This was followed by rapid delineation of the signaling pathways in *Drosophila melanogaster* with confirmation in mice and humans. This knowledge served as the foundation for novel therapeutic approaches that are currently being used in human clinical trials.

Keywords: tuberous sclerosis; TSC1; TSC2; mTOR; signal transduction; estrogen

Lymphangiomyomatosis (LAM) is a rare progressive cystic lung disease of uncertain etiology that affects young women. LAM cysts are formed as a result of the proliferation of an abnormal smooth muscle–like cell, the LAM cell. The mechanism by which LAM cells cause this architectural distortion of the lung is unknown. The clinical course of LAM is frequently inexorable, leading to death or lung transplantation in 10 to 15 years, although recent studies indicate that there is much variability in the natural history of the disorder (1, 2). The most common clinical manifestation is the insidious onset of exertional dyspnea; patients may also experience a nonproductive cough. Other common features include spontaneous pneumothorax, which results from cyst rupture, and chylothorax, which results from obstruction of pulmonary lymphatics and hilar lymph nodes by the slowly proliferating LAM cells. Less frequently, hemoptysis or chyloptysis may occur (1, 2).

Most of the current therapies for LAM are supportive in nature. Bronchodilators are offered because many patients have obstructive physiology, often with some degree of reversibility, on pulmonary function testing. Oxygen is provided to patients with significant hypoxia. Current recommendations stipulate

CLINICAL RELEVANCE

This review will help clinicians understand the current concepts of the pathogenesis of LAM and apply this knowledge to currently available and experimental treatments for this devastating condition.

pleurodesis for the first pneumothorax, as recurrent pneumothoraces are likely to occur (3). The occurrence of LAM in premenopausal women, and observations that LAM may worsen in pregnancy and with exogenous estrogen therapy, has prompted many clinicians to consider anti-estrogen therapy for this condition. However, whether this approach is effective remains uncertain, as the published studies are retrospective and therefore have the potential for selection bias. Moreover, anti-estrogen therapy may be associated with significant untoward effects. Finally, lung transplantation is a consideration when the forced expiratory volume in one second (FEV₁) approaches 30% of predicted, with rapidly declining lung function associated with a poor quality of life, or when pneumothoraces are recurrent and refractory to pleurodesis.

While LAM occurs spontaneously in otherwise healthy women, it is also observed in as many as 34% of patients (including males) with tuberous sclerosis complex (TSC) (4–6), a congenital disorder associated with multifocal hamartomas, including tumors of the central nervous system, and renal angiomyolipomas (7). The finding that LAM in patients with TSC (TSC-LAM) and sporadic LAM (S-LAM) are histologically indistinguishable has significantly aided research into the cellular and molecular underpinnings of LAM.

In this article, we will describe the current state of knowledge regarding the biology of the abnormal smooth muscle cells observed in LAM lesions, and explore how our evolving understanding of this rare disease has led to novel therapeutic strategies.

CHARACTERIZATION OF THE LAM CELL

The first description of the LAM cell was published in 1966 by Cornog and Enterline (8), who concluded that the abnormal cells observed in lung tissue specimens from their patients with LAM had a smooth muscle phenotype. They also noted that these cells were indistinguishable from the pulmonary lesions found in patients with TSC. Furthermore, they hypothesized that the cells represented a clonal population despite lacking other features of malignancy. These early insightful and prophetic

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observations awaited confirmation through the use of modern immunohistochemical, biochemical, and molecular genetic methods. Indeed, many years elapsed before further progress was made in the characterization of the LAM cell.

Ultrastructural studies in the late 1970s and early 1980s further demonstrated the similarity between the pulmonary lesions of TSC and those of sporadic LAM (9, 10). The exclusive occurrence of S-LAM in females in association with the observations that S-LAM may be exacerbated by pregnancy (11) and by exogenous estrogens led to a variety of hormone-based therapies (12–15). These developments were paralleled by the finding that LAM cells express sex steroid receptors (16–18). Subsequent immunohistochemical studies of lung tissue specimens from patients with LAM demonstrated that the cells express α -smooth muscle actin (19), confirming Cornog and Enterline's (8) original description of the LAM cell as a smooth muscle cell.

In 1991, histologic heterogeneity was described in LAM cells (20). Specifically, two subsets of LAM cells were defined: one with myofibroblast-like, spindle-shaped features, and another with larger, polygonal, epithelioid characteristics. Subsequent studies demonstrated that LAM cells stained positively with human melanoma black (HMB45) antibodies, which bind to a glycoprotein, gp100, expressed by melanoma cells and immature melanocytes (21, 22). The significance of gp100 expression by LAM cells is unclear. Moreover, its expression is variable (22, 23) and appears to correlate inversely with the expression of proliferating cell nuclear antigen (PCNA), a marker of active cell proliferation (23). Interestingly, gp100 and PCNA expression delineate the two subtypes of LAM cell: the spindle-shaped cells exhibit low gp100 expression and high PCNA expression, while the large, epithelioid cells exhibit the reverse pattern. Although the functional differences of these two subpopulations remain uncertain, the spindle-shaped cells may represent the proliferative component of LAM lesions (24).

What do these observations tell us about the nature of LAM? The answer to this question remains elusive. The two subpopulations of LAM cells may represent sequential stages of differentiation downstream of an LAM stem cell. An alternate possibility is that the two cell types represent alternative phenotypes, and that differentiation into one or the other phenotype is under the control of hitherto unknown stimuli.

GENETICS OF TSC AND LAM

Early investigators recognized a striking similarity between the pulmonary lesions seen in otherwise healthy women with LAM, and those seen in patients with TSC and lung involvement (8, 10). This led to the hypothesis that TSC-LAM and S-LAM might share common pathogenetic mechanisms.

TSC is an autosomal dominant disorder characterized by hamartoma formation in multiple organs and tissues, including the brain, skin, heart, kidneys, and gastrointestinal tract. Brain involvement by cortical tubers accounts for the major neurological abnormalities in TSC, including seizures, mental retardation, and developmental disorders. TSC occurs in 1 in 6,000 to 1 in 10,000 births (25). Interestingly, about two-thirds of cases of TSC are *de novo*; that is, they originate as a germline mutation that affects the offspring but is not present in the somatic cells of either parent (26). Linkage analysis followed by positional cloning efforts led to the cloning of the TSC2 gene in 1993 (27) and the TSC1 gene in 1997 (28). The names of the gene products of TSC1 and TSC2 are derived from characteristic phenotypic features of patients with TSC: hamartin is the protein product of the TSC1 gene, and tuberlin is the protein product of the TSC2 gene.

The focal and variable nature of the hamartomas seen in TSC have long suggested that these tumors develop following the now classic two-hit model originally proposed for retinoblastoma by Knudson (29). Formal evidence for this hypothesis was provided by genetic analyses of tissue obtained from patients with TSC, which demonstrated loss of heterozygosity (LOH) for each of TSC1 (30, 31) and TSC2 (32). These findings also provided support for the hypothesis that TSC1 and TSC2 have tumor suppressor-like effects, which are lost in the brain lesions, renal angiomyolipomas (AMLs), and other hamartomas observed in these patients. Thus, an inherited or germline mutation in TSC1 or TSC2 constitutes the first hit, while second hits that occur in the various tissues affected by TSC give rise to hamartoma formation. Although this model is well proven for renal AMLs, it remains possible that other mechanisms are at play in TSC. Haploinsufficiency, effects due to the presence of a single functioning TSC1 or TSC2 allele, may be important in some of the brain and other lesions observed in TSC (25). Evidence for two-hit inactivation of TSC1 or TSC2 in cortical tubers is very limited, but may be due to the cellular heterogeneity of those lesions. LOH has been shown convincingly for TSC subependymal giant cell astrocytomas (33).

To date, over 300 mutations in TSC1 and TSC2 have been reported (25, 34–38). TSC2 mutations are about four times more common than TSC1 mutations, likely reflecting a higher intrinsic rate of mutation in the former gene (35). There are no mutational hotspots identified in either gene, with no specific mutation accounting for more than 2% of all observed mutations (25).

The observations that two-hit inactivation of TSC2, or less commonly TSC1, occurred in TSC lesions suggested that involvement of these same genes might occur in S-LAM. Through diligent sample collection from both pulmonary and AML lesions, Smolarek and coworkers showed that 8 of 14 (57%) of AML/lymph node samples from patients with S-LAM had LOH of TSC2 (39). None of these patients had evidence of TSC on the basis of a careful clinical examination, neuroimaging (brain CT or MRI), and retinal examination. In contrast, no abnormalities in the TSC1 gene were identified in these patients. Moreover, no evidence of *germline* mutations in TSC2 could be found in patients with S-LAM, with or without AMLs (34). Using single-strand conformation polymorphism (SSCP) analysis of all 41 exons of TSC2, the same investigators then demonstrated TSC2 mutations in five of seven AMLs from patients with S-LAM. Pulmonary LAM tissue was available in four of these five patients, and in all four cases, the same mutation was detected. TSC2 mutations were not detectable in normal lung, kidney, or blood from these patients (40). A Japanese study of 6 patients with TSC-LAM and 22 patients with S-LAM confirmed these findings (41). No germline mutations of either TSC1 or TSC2 were detectable in 21 of the 22 patients with S-LAM. They also demonstrated the same mutation in more than one anatomical site, supporting the notion that LAM cells may spread via a metastatic mechanism.

In summary, this series of meticulous experiments demonstrated that germline mutations in TSC1 and TSC2 are not present in patients with S-LAM; in contrast, TSC-LAM is characterized by germline mutations in TSC2. However, LAM cells in both TSC-LAM and S-LAM carry mutations. Patients with S-LAM have two acquired mutations (typically in TSC2) whereas patients with TSC-LAM have one germline and one acquired mutation (again, typically in TSC2). These findings explain why LAM occurs frequently in patients with TSC (a prevalence of approximately 34% [4–6]), while S-LAM is extremely rare. Together, these reports support a Knudson-type model (29) of LAM pathogenesis.

HAMARTIN AND TUBERIN PHYSICALLY INTERACT

Genetic studies of TSC laid the groundwork for functional studies of the proteins hamartin and tuberin. These proteins are highly conserved and ubiquitously expressed. Hamartin and tuberin have molecular weights of 130 kD and 198 kD, respectively. Hamartin has a potential transmembrane domain near its N-terminus (28), but is mostly located in the cytosol rather than in association with the cell membrane (42–44). Toward its C-terminus, there is a coiled-coil domain (28). In addition, hamartin contains Rho GTPase-activating (GAP), tuberin binding, and ezrin-radixin-moesin (ERM) family binding domains (45). Finally, hamartin has a C-terminal domain that binds to neurofilament L (NF-L) in cortical neurons (46).

Tuberin, a larger protein, binds to hamartin via its N-terminus (47, 48). Tuberin has a region of homology with the Rap 1 GTPase-activating protein (Rap1GAP) (27, 49). Tuberin also has multiple potential phosphorylation sites for the serine-threonine kinase, protein kinase B (Akt/PKB) (50–54). Protein kinase C (PKC), cyclic nucleotide-dependent kinases, casein kinase 2, and tyrosine kinases also have potential phosphorylation sites on tuberin (27, 50–52, 54–57). The many and varied domains in the hamartin and tuberin proteins strongly suggested an important role for the two proteins in the transduction of signals from cell membrane-associated receptors. However, confirmation of this assertion had to await further functional studies.

Hamartin and tuberin are closely associated *in vivo*. This association has been demonstrated by immunohistochemical colocalization studies (58–63) as well as by coimmunoprecipitation from subcellular fractions (64). In their elegant series of experiments, Nellist and colleagues (44) showed that hamartin and tuberin are binding partners that coelute from subcellular fractions in a complex with a molecular weight of 450 kD, larger than the combined molecular weights of hamartin and tuberin. Furthermore, they demonstrated that the coiled-coil domain of hamartin mediates its homodimerization *in vitro*, forming large insoluble complexes. Finally, they showed that tuberin binds hamartin shortly after the latter's synthesis, suggesting that it acts as a molecular chaperone that can prevent the formation of hamartin aggregates. However, TSC2^{-/-} cells, which lack tuberin, did not exhibit hamartin self-aggregation in another study by Yamamoto and colleagues (43). Although some controversy remains in this area, the data clearly reflect a close association between the two proteins.

FUNCTIONS OF HAMARTIN AND TUBERIN IN CELLULAR SIGNALLING

Characterization of the TSC1 and TSC2 genes and their products permitted the functional studies that have led to our current understanding of the signaling pathway in which hamartin and tuberin participate. The major role of the hamartin–tuberin complex is to inhibit the mammalian target of rapamycin (mTOR). The net result is inhibition of protein synthesis and cell growth when compelling extracellular growth signals are absent. The major components of this pathway are illustrated in Figure 1.

Regulation of Protein Synthesis and Cell Growth

An important series of observations resulted from studies conducted in *Drosophila melanogaster* that documented increased cell size, without an increase in DNA content, in the eyes and wings of TSC1^{-/-} and TSC2^{-/-} mutants (65–67). Furthermore, in both mammalian and *Drosophila* cells, Gao and Pan observed that TSC1^{-/-} and TSC2^{-/-} mutants were resistant to amino acid starvation and exhibited increased activity of ribosomal S6 kinase (S6K), an enzyme that stimulates protein synthesis at the

ribosome (67). In the study by Tapon and associates (65), cells with loss-of-function mutations in TSC1 or TSC2 inappropriately entered S phase, indicating continued cell growth. As a result, the cells were larger and progressed through the cell cycle more quickly than wild-type cells. Furthermore, the investigators were able to demonstrate that overexpression of TSC1 and TSC2 in *Drosophila* resulted in a reduction in both cell size and number, and that this was not related to an increase in cell death. In contrast, the cells cycled more slowly and took longer to proliferate than wild-type cells. Importantly, overexpression of both genes simultaneously (but not one or the other alone) was required to produce this effect. Overexpression of S6K abrogated this phenomenon, but had no effect on eye size in the absence of TSC1 and TSC2 overexpression. Finally, increased expression of the PI3 kinase antagonist PTEN resulted in a dramatic decrease in cell size, enhancing the effect of combined TSC1 and TSC2 overexpression (65).

These observations provided the basis for subsequent experiments in which hamartin was shown to inhibit S6K and its target, ribosomal protein S6. Phosphorylation of S6 is required for ribosome assembly, and therefore plays a central role in the regulation of cell growth (reviewed in Ref. 68). Kwiatkowski and colleagues demonstrated enhanced S6K phosphorylation on threonine residue 389, and S6 phosphorylation on serines 240 and 244, in TSC1^{-/-} mouse embryo fibroblasts (69). Further, data from tumor tissue of patients with TSC (70) and from LAM cells (71) revealed that S6 was activated via hyperphosphorylation in these cells. Inhibition of S6K concomitant with decreased S6 phosphorylation is observed when tuberin or hamartin are overexpressed in TSC2^{-/-} or LAM cells (71, 72).

It is now recognized that S6K phosphorylation is influenced by multiple upstream signals, such as insulin, amino acids, and mitogens (73–75). The evidence described above reveals that hamartin and tuberin can inhibit S6K, via a mechanism involving inhibition of phosphorylation of threonine 389 (69), and thereby act as negative regulators of ribosome assembly and cell growth. S6K is also phosphorylated, and thereby activated, by other kinases (76), but a full discussion of these pathways is beyond the scope of this review.

Regulation of mTOR by the Hamartin–Tuberin Complex

Administration of the immunosuppressive agent rapamycin (sirolimus) is able to prevent phosphorylation of S6K by all known stimuli. This drug is currently in use for the prevention of allograft rejection because of its profound inhibitory effect on lymphocyte activation. Its mechanism of action is the inhibition of a kinase known as the mammalian target of rapamycin (mTOR) (77). Rapamycin produces this effect by binding to the cyclophilin protein known as FKBP12; the rapamycin–FKBP12 complex directly inhibits mTOR (77).

mTOR is a central regulator of cell growth, chiefly through its effects on protein synthesis (78). One mechanism by which mTOR accomplishes its effects is through phosphorylation and thus activation of S6K, thereby initiating ribosomal assembly. However, mTOR also phosphorylates 4E-BP1, a protein that binds the eukaryotic translation initiation factor eIF4E, thereby releasing the latter from a state of inhibition, permitting protein synthesis at the ribosome to begin (79).

How do mTOR and hamartin and tuberin interact to modulate protein synthesis? This question has been addressed via genetic epistasis methods in *Drosophila* (reviewed by Kwiatkowski [25]), and in a number of molecular studies. For example, cultured mammalian cells lacking hamartin or tuberin have constitutively high levels of S6K and 4E-BP1 phosphorylation; subsequent treatment of these cells with rapamycin reverses this phosphorylation (80). Despite supplementation with amino acids, no

Figure 1. Schematic overview of the signal transduction pathways involving the TSC1 and TSC2 gene products, hamartin and tuberin. Arrowheads indicate activating or facilitating influences; flat-headed lines indicate inhibitory influences. (See text for details.) The hamartin–tuberin dimer maintains Rheb in a GDP-loaded state, thereby preventing activation of mTOR, which requires activated Rheb-GTP. Growth and energy signals tend to inhibit this function of the hamartin–tuberin complex, permitting mTOR activation. In addition, dissociated hamartin and tuberin have several effects on cytoskeletal remodeling and estrogen signaling, respectively. The sites of action of several drugs with therapeutic potential in LAM are indicated in red. AA, amino acids; FT, farnesyltransferase. The question mark downstream of the tuberin–CaM complex indicates the possibility for additional interactions that have not yet been identified. Other potentially important pathways, such as the IFN- γ –JAK–STAT and PDGFR signaling cascades, are not shown.

further S6K or 4E-BP1 phosphorylation occurs in these cells in the presence of rapamycin (80). Another key observation was the finding by Inoki and colleagues (52) that the ability of mTOR to phosphorylate both itself and S6K is diminished in the presence of overexpressed hamartin and tuberin. These findings indicate that the hamartin–tuberin complex acts upstream of mTOR, maintaining it in a deactivated state.

This model of mTOR suppression by the hamartin–tuberin complex was confirmed in 2003 when several groups identified tuberin's function as a GTPase-activating protein (GAP) for Rheb (ras homologue expressed in brain), a member of the Ras family of small GTPases (81–87). By stimulation of GTP hydrolysis by Rheb, hamartin–tuberin functions as a brake to reduce the level of mTOR activity. In the absence of a functional hamartin–tuberin complex, Rheb remains in a GTP-loaded state in which it activates mTOR in an uncontrolled fashion (82). Mutations in the GAP domain of tuberin have been identified in patients with TSC (37), resulting functionally in an inability to inactivate Rheb (88). The presence of hamartin is also essential for maximal tuberin GAP activity, as shown by the observation that mutations disrupting the hamartin–tuberin complex lead to enhanced Rheb activation (89). These observations indicate that the mechanism of inhibition of mTOR by the hamartin–

tuberin complex is through tuberin's ability to maintain Rheb in an inactivated, GDP-loaded state.

Regulation of Tuberin by Akt/PKB and Other Kinases Activated by Growth Factors

Akt/PKB is a cytosolic kinase recruited to the membrane upon ligation of membrane receptor tyrosine kinases such as the insulin receptor. The cytoplasmic catalytic domains of these receptors activate PI3-kinase, which in turn phosphorylates membrane lipids resulting in the formation of phosphatidyl inositol trisphosphate (PIP₃) and related compounds. Akt/PKB is recruited to the membrane via its pleckstrin homology (PH) domain, which binds to modified phospholipids, where it is phosphorylated by PDK-1, another PH domain containing protein kinase. Consequently, multiple downstream pathways relating to translation, transcription, and cell cycling, as well as a variety of anti-apoptotic mechanisms, are activated (90).

A pivotal discovery was the observation that Akt/PKB phosphorylates tuberin, promoting dissociation of the hamartin–tuberin complex (50–52). Furthermore, expression of a mutant form of tuberin resistant to Akt/PKB-mediated phosphorylation prevents S6K phosphorylation. In contrast, expression of a mutant tuberin that mimics the Akt-phosphorylated wild-type

protein enhances S6K phosphorylation (52). Overexpression of Akt/PKB leads to accelerated degradation of hamartin and tuberlin via a ubiquitination-mediated pathway (51). These data demonstrate that extracellular growth-promoting signals, such as insulin, act to remove the inhibitory influence of the hamartin-tuberlin complex on the mTOR pathway, creating permissive conditions for cell growth.

Interestingly, an intact and functional hamartin-tuberlin complex also appears to be required for activity of Akt/PKB. Cultured murine embryo fibroblasts lacking either hamartin or tuberlin exhibit a marked reduction in PI3 kinase and Akt/PKB activity (91). This phenomenon has recently been shown to be the result of a negative feedback loop in which S6K phosphorylates and thereby inhibits insulin receptor substrate (IRS) function (92, 93), and a separate process downstream of mTOR in which expression of platelet-derived growth factor receptors (PDGFRs) is reduced (91). Both series of events lead to reduced Akt/PKB activation in response to insulin, IGFs, and PDGF. This observation may explain the benign nature of tumors observed in TSC, and of LAM cells; loss of hamartin and tuberlin causes S6K activation, but the effect of this activation on cell growth would subsequently be mitigated by down-regulation of Akt/PKB.

Several other kinases are known to phosphorylate tuberlin and hence regulate the activity of the hamartin-tuberlin complex. Most of these interactions inactivate the complex, leading to increased mTOR activity and cell growth. When tuberlin is phosphorylated by mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2), the result is the binding of tuberlin by 14-3-3 proteins (56). This effect results in sequestration of tuberlin, thus permitting mTOR to become activated. Moreover, tuberlin is also a target for phosphorylation by the larger p90 ribosomal S6K (57), which must be distinguished from the smaller p70 S6K, discussed above. Furthermore, phosphorylation of tuberlin by extracellular signal-regulated kinase 2 (ERK2) disrupts the hamartin-tuberlin complex, and a tuberlin mutant resistant to ERK2 phosphorylation blocked tumorigenesis in a TSC2^{+/-} cell line in which ERK2 was constitutively activated (55). Finally, under conditions of energy deprivation, tuberlin is phosphorylated on a site that results in increased activity of the hamartin-tuberlin complex and hence inactivation of mTOR. The kinase that mediates this phosphorylation event is AMP-activated protein kinase (AMP kinase) (94).

These observations place the hamartin-tuberlin complex in a key regulatory position downstream of multiple growth signaling pathways, including those linked to the insulin receptor-Akt/PKB pathway, as well as the Ras-MAPK pathway. Moreover, activation of the hamartin-tuberlin complex by AMP kinase supports its role as an inhibitor of cell growth and protein synthesis under conditions of energy starvation.

IFN- γ -JAK-STAT Pathway

The signaling pathway downstream of the interferon γ (IFN- γ) receptor has recently been implicated in the molecular pathogenesis of TSC and LAM. Janus kinases (JAK) are activated upon ligation of the IFN- γ receptor, which in turn phosphorylates members of the signal transducers and activators of transcription (STAT) family of transcriptional activators. El-Hashemite and coworkers (95) demonstrated that TSC1^{-/-} and TSC2^{-/-} mouse embryo fibroblasts and TSC1^{+/-} and TSC2^{+/-} renal and liver tumor cells display decreased levels of IFN- γ expression and increased STAT phosphorylation. Treatment of the cells with IFN- γ resulted in increased apoptosis, and this effect was synergistic with rapamycin (95). A subsequent immunohistochemical and immunoblotting study of S-LAM cells and TSC-LAM cells from pulmonary lesions and AMLs revealed decreased IFN- γ

and increased STAT phosphorylation (96). IFN- γ has been shown to have activity both alone and in combination with rapamycin in mouse models of TSC (97, 98). These studies indicate that IFN- γ and rapamycin should be considered as potential therapies for TSC and LAM.

The Role of Estrogen in LAM Pathogenesis

As S-LAM occurs exclusively in women of childbearing age, it has been hypothesized that the pathogenesis of the disorder may be linked to estrogen-mediated signaling events (1). Although there is no animal model of LAM, TSC1^{+/-} and TSC2^{+/-} mice frequently develop liver hemangiomas (99). Similar to the abdominal AMLs observed in LAM and TSC, these tumors are comprised of cells of varying types with a predominant endothelial component and a smaller number of smooth muscle cells. The latter have a very similar phenotype to LAM cells (99) in that they stain positively for HMB45 antigen, estrogen, and progesterone receptors. Estrogen treatment of TSC1^{+/-} and TSC2^{+/-} mice with liver hemangiomas resulted in more rapid tumor growth than observed controls. In contrast, treatment of the mice with the anti-estrogen tamoxifen retarded tumor growth (99). Hence, tamoxifen therapy may ultimately prove beneficial in the treatment of LAM. However, it must be recognized that this mouse liver hemangioma model does not necessarily reflect events occurring in the lungs of patients with LAM, and therefore caution is warranted in generalizing the findings to LAM.

How might estrogen interact with signaling events in LAM cells? In this regard, tuberlin interacts directly with the intracellular receptor for estrogen, estrogen receptor alpha (ER α) through a domain localized at the carboxy terminus of tuberlin. Direct interaction between these two proteins was first identified *in vivo* in HEK 293 and ELT-3 smooth muscle cell lines (100). Tuberlin's interaction with ER α resulted in growth inhibition in these cells; this phenomenon was attributable to a reduction in estrogen-induced activation of a signaling pathway including PDGFR β and extracellular signal-regulated kinase 1/2 (ERK 1/2) (100). Overexpression of tuberlin led to a reduction in the ability of estrogen to activate this PDGFR β -ERK 1/2 pathway. It has been shown subsequently that in TSC2^{-/-} cells, mitogen activated protein/extracellular signal regulated kinase kinase (MEK1)-independent signaling from PDGFR β can occur, resulting in phosphorylation of MAPK. This process is dependent on the presence of superoxide anion (O $_2^-$) (101). These findings indicate that there are growth-promoting pathways aside from the mTOR pathway that may play a role in the pathogenesis of TSC and LAM.

A number of studies have suggested that binding between calmodulin (CaM) and tuberlin may influence tuberlin's effects on estrogen-mediated signaling (102-104). Together, these studies suggest that CaM may act to sequester tuberlin from ER α , thereby preventing inhibition of estrogen-mediated signaling by free tuberlin. Further investigation will be required to determine the significance of these findings in LAM and TSC.

In addition to the PDGFR β pathway mentioned above, there are a number of additional nongenomic estrogen-activated signaling pathways that depend on ER α . Another such pathway of potential significance to the pathogenesis of LAM is the PI3K-Akt signaling cascade. An estrogen-dependent direct interaction of ER α with the p85 regulatory subunit of PI3K was reported several years ago (105). Moreover, estrogen-induced activation of endothelial nitric oxide synthase (eNOS) was abolished when PI3K was inhibited, indicating that this signaling pathway is entirely nongenomic and non-nuclear in nature (105). Of particular interest to the pathogenesis of LAM is that estrogen, acting via a nongenomic pathway, can promote protein tyrosine

phosphatase activity that dephosphorylates tuberlin, resulting in its degradation (106).

The role of estrogen in the pathogenesis of LAM remains one of the least understood aspects of this disorder. Nevertheless, anti-estrogen therapies are commonly used in the treatment of LAM. The importance of further investigation in this area cannot be overemphasized because of the potential therapeutic benefits and adverse effects of this readily available therapeutic modality.

THE LAM CELL AND ITS MICROENVIRONMENT

The Actin Cytoskeleton and LAM Cell Migration

An additional role of the hamartin–tuberlin complex appears to be regulation of the actin cytoskeleton and cell migration. This function is mediated through the interaction of hamartin with members of the Rho GTPase family, which includes Rho, Rac, and Cdc42. These small GTPases play a crucial role in the regulation and remodeling of the actin cytoskeleton.

Lamb and colleagues demonstrated increased focal adhesion formation when hamartin is overexpressed and conversely, disruption of focal adhesion formation when hamartin is absent (45). The activation of Rho in these studies was dependent on an interaction of hamartin with ERM proteins. The ability of tuberlin to activate Rho and focal adhesion kinase (FAK), and to promote cellular migration, was demonstrated by Astrinidis and colleagues (107). This is in contrast to a subsequent report by Goncharova and colleagues (108), who demonstrated that hamartin inhibits Rac1, but that tuberlin, whose binding site on hamartin overlaps the Rho-activating domain of Rac1 (44), prevents this inhibition. In their model, hamartin on its own can inhibit Rac1, permitting activation of Rho and the formation of stress fibers and focal adhesions leading to cell stability. In contrast, hamartin–tuberlin interaction tends to promote cellular migration through the inhibition of Rho via increased Rac1 activity. The authors hypothesized that loss of hamartin or tuberlin could lead to a dysregulation of Rac1–Rho signaling, cytoskeletal remodeling, and abnormal cell motility. Differences in their findings from those of Astrinidis and associates (107) may be attributable to differences in experimental methods.

Further experiments have implicated mTOR in actin cytoskeleton and focal adhesion remodeling. Formation of stress fibers and focal adhesions is promoted by siRNA inhibition of mTOR, or its novel binding partner, rictor (109). The mTOR–rictor complex is rapamycin-insensitive (110), whereas the formation of a complex between mTOR and an alternative binding partner, raptor (111), makes it susceptible to rapamycin. However, it appears that the mTOR–rictor complex is uniquely involved in cytoskeletal rearrangement (110). The role of raptor and rictor in hamartin–tuberlin signaling via mTOR is still incompletely understood (68) and much remains to be learned with respect to altered cytoskeletal remodeling in the pathogenesis of LAM.

A Possible Role for Matrix Metalloproteinases in LAM Pathogenesis

In malignant tumours, altered metabolism of the extracellular matrix contributes to invasion and metastasis (112). The up-regulation of a variety of matrix metalloproteinases (MMPs) in LAM in concert with the genetic data described above, supports the view that AMLs and pulmonary LAM cells have a common origin, and that one lesion is a “benign metastasis” from the other (40, 41, 113).

Spindle phenotype LAM cells have been shown to express membrane type 1 matrix metalloproteinase (MT1 MMP) (114). The latter is a membrane-associated enzyme that activates MMP-2, which is also secreted in excess by LAM cells in comparison

with normal bronchial and vascular smooth muscle cells (115, 116). Interestingly, MMPs, which degrade extracellular matrix proteins thereby facilitating cell migration, may also enhance LAM cell growth via inactivation of insulin-like growth factor (IGF) binding proteins (24). The latter are an inhibitory influence on cell growth because they are capable of binding IGF, thereby inhibiting IGF-mediated stimulation of cellular proliferation. Indeed, cleavage of IGF-binding proteins by MMP-1 has been shown to promote human airway smooth muscle growth (117).

Doxycycline, an inhibitor of MMPs (118), may be efficacious in the treatment of pulmonary capillary hemangiomatosis (119), a disorder in which angiogenesis is dependent upon MMP activity. Recently, Moses and colleagues (120) reported a case of a patient with advanced pulmonary LAM in whom treatment with doxycycline resulted in a reduction in urinary MMPs that was associated with improvement in FEV₁ and levels of oxygen saturation with exercise over 6 months. However, as this is a report of a single case, further studies are required to confirm this intriguing observation.

THE BIGGER PICTURE: PATHOGENESIS OF CYSTIC LUNG DISEASE IN LAM

LAM is characterized by the replacement of normal pulmonary parenchyma by thin-walled cysts, which result in the respiratory manifestations of the disease, including progressive dyspnea, recurrent pneumothorax, and chylous effusions. An important but unanswered question is why cystic destruction of the lung, as opposed to another pattern, is observed in LAM. Figure 2 illustrates how the unique biology of LAM cells might contribute to the development of pulmonary cysts.

LAM cells proliferate along lymphatic channels in the lung and in extrapulmonary sites, including the mediastinal, retroperitoneal, and pelvic lymphatics. In these locations, LAM cells are divided into fascicles or bundles by channels lined by lymphatic endothelial cells. Kumasaka and colleagues have reported that LAM cells produce vascular endothelial growth factor (VEGF)-C, and that the degree to which it is produced by LAM cells correlated with the degree of lymphangiogenesis observed in six autopsy cases (121). Subsequent work by the same group revealed that the lymphatic channels recruited by LAM cells tend to divide the cells into clusters that are then shed from the lesion, a phenomenon they were able to observe *in vitro* (122). The authors postulated that this mechanism may account for the ability of LAM cells to metastasize to distant sites. In addition, it seems plausible that the recruitment of new lymphatic channels may similarly facilitate the progressive invasion of the lung parenchyma by LAM cells.

The proliferation of LAM cells along lymphatic channels puts them in proximity to both airways and blood vessels. Obstruction of blood vessels results in focal areas of hemorrhage and hemoptysis, while obstruction of lymphatics leads to the development of chylothorax. In addition, it has been speculated that a constrictive effect of bundles of LAM cells on airways results in airflow obstruction, leading to air trapping and ultimately cystic changes in the pulmonary parenchyma (123). An alternative or coexisting mechanism for cyst development may be the elaboration of MMPs by LAM cells as described above, leading to the degradation of the extracellular matrix of the pulmonary parenchyma (115, 116) in a manner similar to the protease-mediated development of emphysema. Intriguingly, the TSC2 gene is adjacent to the PKD1 gene on chromosome 16p 13.3 and mutations in PKD1 are responsible for autosomal dominant polycystic kidney disease (ADPKD), in which large renal cysts displace normal renal tissue and lead to progressive renal failure (68, 124, 125). The

Figure 2. Possible mechanisms of lung cyst formation in LAM. Spindle phenotype LAM cells express matrix metalloproteinases (MMPs), which may degrade the supporting architecture of the pulmonary interstitium. In addition, LAM cells secrete VEGF-C, which specifically directs the growth of new lymphatic channels that may promote further invasion of lung tissue by LAM cells. LAM cells proliferating in lymphatics may cause airway obstruction by local proliferation and perhaps, through cytoskeletal reorganization, contraction. This could lead to air trapping in distal airspaces resulting in their dilatation, contributing to cyst formation. Replacement of the pulmonary parenchyma by cysts may then lead to hypoxemia and pneumothorax. Obstruction of lymphatics and blood vessels by a similar process could conceivably lead to hemoptysis, chyloptysis, and chylothorax. Possible therapeutic agents are indicated in highlighted text. FT, farnesyltransferase.

occurrence of renal cysts associated with mutations in TSC2 and PKD1 suggests common pathogenic mechanisms that may also be at play in the formation of lung cysts. Further study in this area is warranted, but the lack of an animal model of LAM makes this line of research difficult to pursue.

POTENTIAL THERAPEUTIC STRATEGIES DIRECTED AT SIGNALING PATHWAYS

Rapid progress in our understanding of the molecular basis of LAM has led to rational hypotheses regarding viable treatment options. Some potential treatments are extant drugs, such as sirolimus, that are already in use for other indications. In fact, an NIH-sponsored randomized controlled trial of sirolimus in LAM has begun enrollment at the time of this writing.

In other cases, drugs have yet to be developed. One potential target for future consideration in the treatment of LAM is angiogenesis. In the study of TSC1^{+/-} and TSC2^{+/-} mice by El-Hashemite and colleagues (99), VEGF levels were elevated in the mice receiving estrogen therapy but were reduced in the mice receiving tamoxifen, suggesting that this crucial angiogenic factor may be important in the progression of LAM lesions. In support of this assertion is the recent finding in a series of surgical and autopsy cases of LAM, that VEGF-C is overexpressed in LAM lesions and is associated with the excessive growth of lymphatics in the lesions (121). A variety of small molecule and antibody inhibitors of VEGF pathways have been developed and some are in clinical trials (126–128).

Other possible targets in LAM include growth factor receptor tyrosine kinases located upstream of the deregulated hamartin-tuberin-mTOR pathway. Although it is unclear that this strategy will be beneficial, the advent of effective tyrosine kinase inhibitors such as imatinib in the therapy of malignant diseases makes this approach an intriguing possibility worth further investigation. Moreover, targeting Rheb, a farnesylated protein, through inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase with the use of HMG-CoA inhibitors (“statins”) or with other inhibitors of the mevalonic acid pathway, or with farnesyltransferase inhibitors, may prove useful (88, 129, 130). The sites of action of these therapeutic strategies are illustrated in Figures 1 and 2.

CONCLUSIONS AND FUTURE DIRECTIONS

Remarkable advances in the genetics of TSC and knowledge of the cellular signaling pathways modulated by hamartin and tuberlin have enabled unprecedented advancements in the understanding of the biology of LAM and provided several potential therapeutic strategies currently being studied. Hamartin and tuberlin play central roles in cell growth and proliferation via their influence on mTOR and the actin cytoskeleton. The powerful effects of mTOR on protein synthesis and cell growth necessitate the presence of a strict negative regulator, namely the hamartin-tuberlin dimer, in order to prevent the dysregulated cell growth observed in LAM, AMLs, and TSC tubers. The recognition that multiple protein kinases activated by growth factor receptor

ligation are inhibitors of the hamartin–tuberin complex serves to underscore the vital role that these two proteins play in the regulation of cell proliferation. Research on the biology of LAM and TSC has greatly advanced our understanding of other hamartomatous syndromes such as Cowden disease, Peutz-Jeghers syndrome, and Proteus syndrome (131).

Despite these advancements, much remains to be learned regarding the biology and treatment of LAM. There are two major impediments to further basic research progress. First is the lack of an established tissue culture model system that permits cloning and analysis of purified LAM cell populations. The second is the lack of an animal model of LAM.

Clinically, there are also major issues. One is the slow progression of this disease, measured in decades in many patients, which makes assessment of any clinical intervention very difficult. The recognition of the potential for rapamycin (sirolimus) to directly block the growth of LAM cells is extremely exciting, enhanced by its major therapeutic activity in a variety of TSC animal models. However, the potential necessity for long-term treatment, and side effects, clearly mandate a carefully done randomized clinical trial, as is currently underway. In addition, it is possible that mTOR inhibition by rapamycin will abrogate the negative feedback loop, hence counteracting potential benefits, although this has not been seen in animal models (132).

Nonetheless, rapid progress in our understanding of the fundamental biology of LAM has given hope to patients with LAM and to clinicians caring for them. We anticipate continued progress in this area that will lead to novel therapeutic insights for this devastating illness in the near future.

Conflict of Interest Statement: D.J.K. is receiving \$461,000 in 2006–2008 from Novartis as a research grant for preclinical studies of RAD001 in treatment of TSC mouse models. F.X.M. received Rapamune and \$200,000 in trial funding for an upcoming RCT for LAM from Wyeth Pharmaceuticals. G.P.D. and S.C.J. do not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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