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The Loss of Tuberin Promotes Cell Detachment and Invasion Through the Beta-Catenin Pathway

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Mutations in the tumor suppressor tuberin are a common factor in the development of Tuberous Sclerosis Complex (TSC) and Lymphangiomyomatosis (LAM). TSC is an autosomal dominant syndrome characterized by hamartomas in multiple organs. LAM is a cystic lung disease that is characterized by the infiltration of smooth muscle-like cells into the pulmonary parenchyma. The mechanism by which the loss of tuberin promotes the development of LAM has yet to be elucidated, though several lines of evidence suggest it is due to the metastasis of smooth muscle-like cells from tuberin-deficient hamartomas. Here we show that tuberin-deficient cells lose E-cadherin and gain smooth muscle actin (SMA) expression, promoting a migratory phenotype. Migratory tuberin-deficient cells accumulate caspase-cleaved forms of beta-catenin that are transcriptionally active and unable to associate with GSK3-beta. In reporter assays, cleaved forms of beta-catenin promote MMP7 expression, a component of cell invasion. In cell invasion assays, non-adherent tuberin-deficient cells are invasive and express MMP7. Altogether, these results suggest that tuberin-deficient cells undergo a type of epithelial-mesenchymal transition (EMT), which may underlie the development of LAM.

Prof. Judy Black

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The Anti-Angiogenic Factor Tumstatin Is Absent In Lung Tissue From Patients With Lymphangiomyomatosis (LAM).

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Remodeling of the airways of which angiogenesis is a prominent feature, occurs in patients with LAM. The regulation of angiogenesis is controlled by both pro-angiogenic factors (eg vascular endothelial growth factor, VEGF) and anti-angiogenic factors (eg the non-collagenous 1 (NC1) domains of the collagen IV molecule). The collagen IV molecule consists of 6 alpha chains. The cleaved non-collagenous domain of both the alpha 3 chain (tumstatin) and the alpha 5 chain have anti-angiogenic properties. In order to gain a greater understanding of the homeostatic control of angiogenesis we investigated whether the NC-1 domain of the 6 alpha chains of the collagen IV were present in the LAM airway. We also studied the effect of transforming growth factor-beta (TGF-beta), which is increased in the airways of LAM, on the release of VEGF from LAM cells.

We investigated the presence of the NC1 domain of the six collagen IV alpha chains, and the 7S domain of the alpha 3 chain in LAM and non-LAM airways using immunohistochemistry and TGF-beta-induced VEGF release was measured by ELISA.

The NC1 domains of collagen IV alpha1-6 chains were all present in non-LAM airway tissue (n=4), however both alpha 3 and alpha 5 were absent in LAM airways (n=8). In contrast, the amino terminal 7S domain of the alpha 3 chain was present in both LAM and non-LAM sections. TGF-beta induced greater release of VEGF from airway smooth muscle cells from LAM when compared to non-LAM (345 (5.7(SEM)) versus 230.3(18.1) pg/ml p<0.003, n=4 for both).

The results of this study show an imbalance in the distribution of tumstatin and VEGF in the LAM airway. The absence of tumstatin and over expression of VEGF may be directly contributing to the aberrant angiogenesis seen in LAM.

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Doxycycline inhibits proliferation and MMPs in LAM-derived cells

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Lymphangiomyomatosis (LAM) is associated with abnormal airway smooth muscle pathology and destruction of lung tissue. Levels of matrix metalloproteinases (MMPs) have been reported to be altered in LAM, and altered MMP expression is thought to play a prominent role in the tissue degradation. Drugs which reduce cell proliferation and MMP expression could have therapeutic potential in preventing the progressive lung damage associated with LAM. The current study was undertaken to investigate whether doxycycline can inhibit LAM-derived smooth muscle cell proliferation and reduce the level of MMPs.

Lung tissue was obtained from subjects undergoing resection for lung lesions or transplantation. Smooth muscle cells were isolated from the airways of female subjects with LAM (n=4) or from age, sex-matched non-LAM (control, n=4) subjects. LAM-derived smooth muscle cells stained positive for HMB-45. FBS-induced proliferation of LAM and control smooth muscle cells was assessed in the presence or absence of doxycycline (0.1-100µg/ml) by MTT assay. Zymography was used to examine pro-and active MMP-2 expression in cell supernatants (days 3, 5 and 7).

Proliferation of LAM-derived smooth muscle cells was attenuated by doxycycline (100µg/ml) following stimulation with 5% FBS for 9 days (n=4, p<0.05; 43.4 ± 12.3% reduction). Doxycycline had no effect on proliferation of control cells (n=4, p>0.05). Levels of pro-MMP-2 were unaltered by doxycycline treatment (n=3, p>0.05) in both LAM and control cell supernatants. Expression of active MMP-2 in LAM cell supernatants (day 5) appeared reduced, though this did not reach statistical significance (p>0.05, n=3). Whether doxycycline can modulate the expression of other MMPs, including MMP-9 is currently being investigated.

The results of this study suggest that doxycycline may have a role in regulating LAM cell proliferation and MMP expression which contribute to the tissue destruction associated with LAM.

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Lymphangioliomyomatosis Avoiding an Unnecessary Biopsy A Case Report

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It is rare for extra-pulmonary symptoms to lead to diagnosis of Lymphangioliomyomatosis (LAM), with only 4 case reports reporting such diagnosis.^{1,2,3,4} We describe how diagnosis of LAM avoided unnecessary abdominal lymph node biopsy.

Case Report:

A 30-year-old Caucasian female was admitted with complaints of abdominal pain and dysuria. Her review of systems was significant for dyspnea on exertion. She suffered from menorrhagia secondary to von Willebrand's disease (VWD); after failing medical therapy she underwent total hysterectomy and RSO one month ago. The patient was admitted to GYN service with suspicion of post-operative pelvic abscess. A CT scan of the abdomen was done, which showed bulky pelvic lymphadenopathy as well as cystic lung lesions raising the suspicion of LAM, Histiocytosis X, or other interstitial lung disease. She underwent VATS biopsy, which came back positive for LAM. There was suspicion of lymphoma secondary to bulky pelvic lymphadenopathy, and the plan was made to biopsy the lymph nodes.

Discussion:

The diagnosis of LAM is nearly always delayed, as the most common symptoms are vague.⁵ Often a CT that shows the lungs leads to a radiological diagnosis. Typically, the cyst walls range from 2 mm to a nearly imperceptible thickness.⁶

Enlarged abdominal lymph nodes are a documented part of the multisystem nature of LAM. In one series, 39% of LAM patients had abnormal lymphadenopathy.⁷

The biopsy in this case was avoided after a careful review of the literature described lymphadenopathy as one of the constellation of signs of LAM.⁸ This was particularly important in our patient's case, as her VWD placed her at increased risk of bleeding from an invasive procedure.

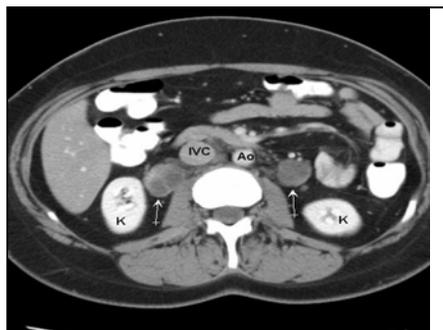


Figure 1. CT scan of abdomen showing para-aortic lymphadenopathy.



Figure 2. CT scan of lungs showing cystic lung disease.

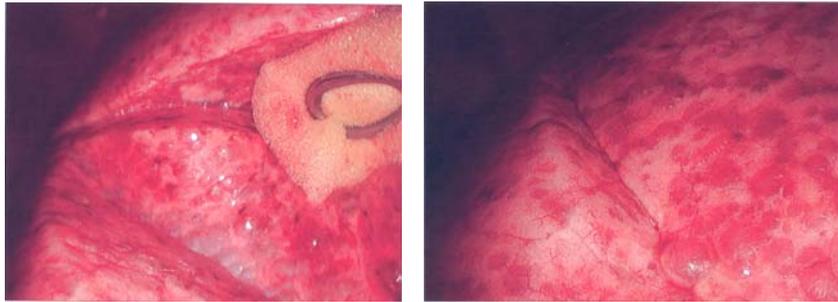


Figure 3. Intraoperative picture during VATS biopsy. Pictures show cystic lesions covering the lung surface.

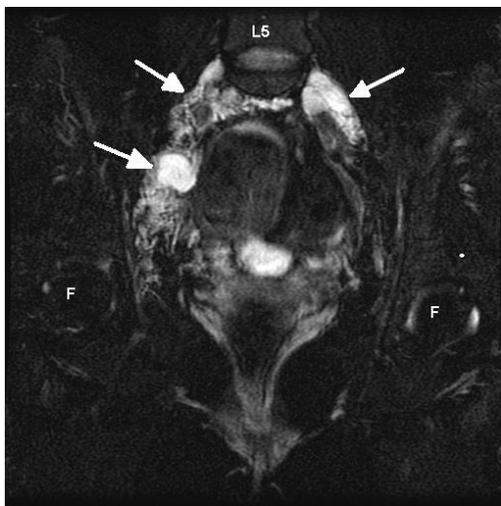


Figure 4. MRI of the abdomen illustrating lymphangiomyomas.

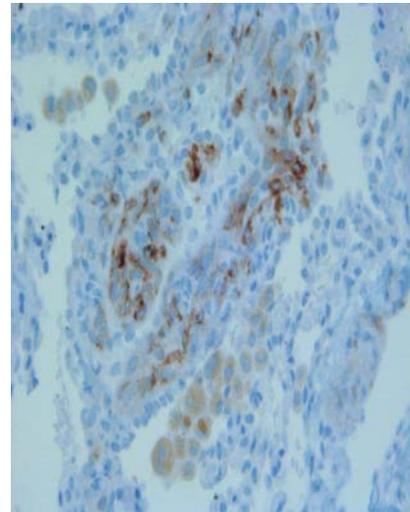


Figure 5. Tissue sample stained for HMB45.

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⁸ Ryu JH, et al. The NHLBI lymphangiomyomatosis registry: characteristics of 230 patients at enrollment. *Am J Respir Crit Care Med* 2006; 173:105-111.

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Phenotype Reversion Of Human *Tsc2*^{-/-} Smooth Muscle Cells

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Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome resulting from the loss of function of the hamartin/tuberin complex. Two genes are implicated in TSC, *TSC1* and *TSC2*, respectively encoding hamartin and tuberin. The complex inhibits target of rapamycin (TOR)-mediated signaling to S6 kinase and participates in the control of cellular growth. From a surgically removed angiomyolipoma of a *TSC2* patient we have isolated alpha-actin-positive smooth muscle cells, named A⁺, bearing mutation on exon 18, with loss of heterozygosity for *TSC2* gene. S6K1 is constitutively activated, while IGF-1 is abundantly released and involved in cell survival rather than proliferation. EGF is necessary to promote proliferation of *TSC2*^{-/-} A⁺ cells. Following retroviral transduction of *TSC2* gene in A⁺ cells, tuberin was successfully expressed and EGF-growth dependency was lost, thus it appears that EGF requirement for A⁺ cell growth is due to lack of tuberin. *TSC2*^{-/-} A⁺ cells were positively labeled by HMB45 antibody but, following *TSC2* transduction, A⁺ cells were negative. In *TSC2*-transfected cells phosphorylation of Akt and S6 was reduced, with the expression of Akt and S6 unaltered. Also PTEN phosphorylation decreased drastically. Exposure for 2 hours with IGF-1 (50ng/ml) slightly activated PI3K in *TSC2*^{-/-} A⁺ cells; this was not affected by inhibitors such as LY294002 (LY) and wortmannin. After reintroduction of *TSC2* gene the sensitivity to these inhibitors was restored and LY (20μM) addition reduced basal and IGF-1-induced Akt phosphorylation, and inhibited activation of S6K1 and PTEN. The phenotype of *TSC2*^{-/-} A⁺ cells was also assessed after antiEGFR and rapamycin incubation. When A⁺ cells were exposed at plating time to antiEGFR (5μg/ml) labelling with HMB45 was down-regulated within 5 days. A smaller effect was observed with rapamycin (5ng/ml). The proliferation rate of *TSC2*^{-/-} A⁺ cell was reduced by rapamycin when added at plating time, but a delay of 3 hours caused the loss of rapamycin effect. AntiEGFR was effective when added in both conditions. In conclusion our study shows that lack of tuberin affects greatly the EGF dependency for growth and IGF-1 related metabolism. The insensitivity to IGF-1 pathway inhibitors is reversed by the reintroduction of *TSC2* gene. The superior effects of antiEGFR versus rapamycin suggest a novel therapeutic strategy for TSC and LAM.

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Rapamycin-resistant production of matrix metalloproteinases by TSC-null MEF lines and a human AML line.

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Introduction: Matrix metalloproteinases (MMPs) are a family of enzymes that degrade components of the extracellular matrix (ECM), and have been implicated in the pathogenesis of many chronic lung diseases and tumors. MMPs, especially MMP-2, have been identified at increased levels in the pulmonary lesions of lymphangiomyomatosis (LAM) patients. In addition to pulmonary LAM lesions, renal angiomyolipoma (AML) lesions may also express high levels of MMP-2. We hypothesized that loss of TSC1/TSC2 would lead to increased MMP production.

Methods: Serum-free conditioned media from *Tsc1*^{-/-} and *Tsc2*^{-/-} murine fibroblast (MEF) cells and their corresponding wildtype controls were collected after 24 hours of serum starvation. Similar conditioned media from human AML *Tsc2*^{-/-} cells and the corresponding *Tsc2*-reconstituted cells (provided by Rachel Squillace) were also collected. Gelatinase activity reflecting MMP-2 and MMP-9 was assayed by zymography using gelatin-containing gels. Cell proliferation was measured by mitochondrial activity using MTT assay. pS6 expression was measured by immunoblotting.

Results: *Tsc1*^{-/-} and *Tsc2*^{-/-} MEFs produced higher levels of MMP-2 than their wildtype counterparts. Similarly, conditioned media from human AML (*Tsc2*^{-/-}) cells had higher levels of MMP-2 activity than did media from *Tsc2*-reconstituted AML cells. Rapamycin treatment at 20nM for 24 hours effectively suppressed cell proliferation assessed by MTT assay and dramatically decreased pS6 levels by immunoblotting; however, rapamycin treatment did not significantly impact MMP-2 production from TSC-deficient cells.

Conclusion: These preliminary findings indicate that TSC1/TSC2 loss leads to increased MMP-2 production. This increase appears to be mTORC1 independent. Additional studies are planned to evaluate MMP expression differences in greater detail in these cells. However, these data suggest a potential limitation to rapamycin therapy for LAM.

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Role of Myosin Light Chain Kinase in LAM Cell Physiology

Olga Chenaya and Primal de Lanerolle

The cytoskeleton plays a key role in cell proliferation and migration, signature features of LAM cells. The organization of the cytoskeleton is largely determined by forces generated by actin and myosin II. Actin-myosin II interactions are regulated by phosphorylation of the 20 kDa myosin light chains (MLC) by myosin light chain kinase (MLCK). Previous studies showed increased levels of MLC phosphorylation and MLCK expression in proliferative cells and that destabilizing the cytoskeleton, by inhibiting MLCK or disrupting actin filaments, resulted in apoptosis of cancer cells *in vitro* and *in vivo*. Because targeting cytoskeletal stability could be beneficial in treating proliferative diseases like LAM, we have begun characterizing the mechanisms that regulate the organization of the cytoskeleton in LAM cells by focusing on MLCK and MLC phosphorylation.

Using Western blot analysis on LAM cells from 5 different patients, we confirmed the smooth muscle origin of LAM cells by demonstrating the expression of smooth muscle II heavy chain, calponin and smooth muscle alpha actin. Surprisingly, we found that LAM cells express almost exclusively the long, non-muscle form MLCK. This is in contrast to other smooth muscle cells that express primarily the shorter, smooth muscle form of MLCK. To investigate the molecular basis for this decrease in expression, we isolated the smooth muscle MLCK promoter from LAM cells. Reporter gene assays demonstrated a decrease in activity of the smMLCK promoter isolated from LAM cells. We are currently characterizing mutations that we have discovered in the promoter to determine if they affect the expression of MLCK. Other experiments have shown that LAM cells are more resistant to apoptosis induced by MLCK and other cytoskeletal disruptors.

Current studies are investigating the mechanisms that down regulate the expression of smooth muscle MLCK, how the absence of smooth muscle MLCK affects cytoskeletal dynamics and why LAM cells are more resistant to pro-apoptotic agents. These experiments may reveal unique features of the cytoskeleton in LAM cells and suggest novel ways to treat LAM disease.

Treatment of refractory lymphangiomyomatosis (LAM)-associated Chylous Effusion with a pleuroperitoneal window and omental flap: a case report. David J. Kwiatkowski¹ MD, PhD, Subroto Paul, MD, Stacey Su, MD, Heather Edenfield, and Raphael Bueno, MD. Division of Thoracic Surgery, Department of Surgery, and ¹Division of Translational Medicine, Department of Medicine, Brigham & Women's Hospital, Boston, Massachusetts

Chylous pleural effusions are seen in 3 – 10% of LAM patients, and are often refractory to medical and surgical treatment. Our patient was a 74-year-old woman who presented with recurrent chylopericardium and chylothorax. Thirty years earlier, she reported sustaining 3 pneumothoraces, which led to a left pleurodesis procedure, with findings of 'blisters' on her lung. She was then relatively well until age 71 when she developed right-sided lower anterior chest discomfort, and abdominal CT scan showed an abdominal mass, reported on biopsy as plexiform neurofibroma. The discomfort improved, and she did well for a further three years.

Sixteen months ago, she noted the onset of progressive dyspnea. Echocardiogram revealed tamponade physiology and pericardiocentesis yielded 600 ml of chylous fluid. She was treated with the creation of a subxiphoid pericardial window and laparoscopic ligation of the thoracic duct tributaries. Persistent bilateral chylous effusions developed post-operatively. Abdominal magnetic resonance imaging (MRI) showed a complex 14 cm x 5 cm retroperitoneal mass, encasing the aorta, IVC, bilateral renal arteries, and left renal vein. Chest CT scan demonstrated the presence of numerous diffusely-distributed bilateral thin-walled lung cysts and bilateral pleural effusions. Drainage of 2-3 liters of chyle per day (90% from the R pleural space) persisted despite treatment with total parental nutrition (TPN) and bowel rest. Chest tube dysfunction and/or removal led to prompt re-accumulation of fluid with dyspnea at rest. Right thoracotomy with pleurectomy and talc poudrage, and mass ligation of the thoracic duct provided little benefit.

Review of her previous abdominal biopsy material showed lymphangiomyoma, leading to a diagnosis of abdominal lymphangiomyoma and pulmonary LAM. Doxycycline 100 mg PO BID; rapamycin, with dosage adjusted to achieve a trough level of 7 – 15 ng/ml; and provera 400 mg IM every month were all given without major improvement.

A transdiaphragmatic window was created, by laparoscopic resection of a portion of diaphragm to the right of the crus and posterior to the liver. An omental flap was mobilized and placed into the right pleural space with the hope that the omentum might both absorb chyle and keep the pleuroperitoneal channel patent. Chest tube output promptly ceased. Abdominal drains were removed 4 weeks later. She was discharged five weeks after surgery without drains. The patient continues to do well five months after creation of the pleuroperitoneal window, is eating a 20 gram fat/day diet with slow regain of weight, and is on an active exercise program including treadmill walk, with SaO₂ of 92% on room air after exercise. Follow-up chest films have shown tiny right and small left pleural effusions, with continuing cystic disease. She continues on doxycycline, rapamycin (trough levels 3-5 ng/ml), and progesterone therapies.

In summary, we report a case of advanced LAM in a 74 year old woman in whom refractory chylothorax was leading to a seemingly inexorable downhill course. Treatment with a pleuroperitoneal window and omental flap, combined with triple drug therapy, led to dramatic and sustained clinical improvement.

Masatoshi Kurihara, MD

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Thoracoscopic surgery for preventing recurrent pneumothorax in LAM patients requiring lung transplantation in the future (Total pleural covering method) Clinical and experimental research

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Introduction:

LAM is a disorder characterized by recurrent pneumothorax.

Talc, which is used in chemical pleurodesis is effective in stopping recurrent pneumothorax, but the severe and broad adhesion of the lung to the intrathoracic wall is serious problems when undergoing lung transplantation. Use of anti-coagulants in particular leads to more serious bleeding when separating the lung from the intrathoracic wall.

This ground breaking technique for thoracoscopic surgery, can both prevent adhesion and recurrence of pneumothorax.

Patients and Methods:

For 20 LAM patients (20-51 years old), thoracoscopic surgery called total pleural covering technique (TPC) was performed. After the entire visceral pleura was covered with a regenerated oxidized cellulose mesh (Surgicel Ethicon), it was then coated with fibrin glue.

Selection of covering material to be used is a key factor to success in TPC.

An experimental study using dogs was performed to determine which of the following three materials are best suited for covering lung surfaces; Regenerated Oxidized Cellulose mesh(ROC), Vicril mesh(VIC) or Polyglycolic Acid mesh(PGA). Suitability was considered for prevention of adhesion as well as thickening of pleura.

Results:

There was no recurrence of pneumothorax in 18 of 20 cases (90%) over a period of 56 months after TPC. There was a recurrence of limited pneumothorax in two cases (10%). The thoracoscopic and microscopic findings show that the pleural surface had been thickened and covered with collagen fibers and that it had no adhesion to the thoracic wall postoperatively.

According to experimental data, ROC and PGA coated with fibrin glue are not adhesive to the thoracic wall whereas uncoated VIC and uncoated PGA are both severely adhesive.

Discussion:

The total pleural covering technique causes thickening of visceral pleura comprised of collagen fibers and does not raise adhesion, resulting in a 90% effective prevention of recurring pneumothorax. Therefore lung transplantation can be more easily performed after TPC, should it be necessary in the future. Regarding non-adhesive properties, ROC is the most effective material. The mechanism that prevents adhesion is that ROC coated with fibrin glue appears to not invade fibroblasts in the thoracic wall. Therefore TPC is the better choice to prevent recurrent

pneumothorax in LAM patients when compared to chemical pleurodesis.

Conclusions:

Based on the observed performance, the TPC technique is the superior method in preventing recurrent pneumothorax for LAM patients that may require lung transplantation in the future. Additionally, regenerative oxidized cellulose mesh is the best TPC covering material.

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Cytological, Immunocytochemical, and Ultrastructural Characterization of LAM Cell Clusters in Chylous Effusion in Patients with Lymphangiomyomatosis.

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[Introduction] Lymphangiomyomatosis (LAM) affects exclusively women of reproductive age, involves the lungs and axial lymphatic system, and is frequently complicated with chylous effusion. In the previous study, we reported that LAM cell cluster (LCC), a cluster of LAM cells enveloped by a monolayer of lymphatic endothelial cells, played the central role in the dissemination of LAM cells. This study examined cytological, immunocytochemical, and ultrastructural features of LCC in order to clarify its diagnostic significance for LAM.

[Materials and Methods] We evaluated LAM-associated chylous effusion including nine pleural effusions, five ascites, and one pericardial effusion obtained from 12 patients with LAM. We performed Papanicolaou staining and immunocytochemistry for muscular antigens (alpha-smooth muscle actin, desmin), melanoma-related antigens (HMB45, PNL-2, CD63), female hormone receptors (estrogen and progesterone receptors), and markers for lymphatic endothelial cells (VEGFR-3, Podoplanin, Prox-1, D2-40) if amounts of specimen are enough for the examination. Furthermore, electron microscopic analysis was performed in one specimen.

[Results] We detected LCC in all of chylous effusion we tested. LCC seemed to be more abundant in ascites than pleural effusion. Cytological features of LCC were a globular cluster consisting of cells in the center enveloped by a monolayer of flattened cells. LAM cells form a tightly cohesive core and have a moderate nuclear/cytoplasmic ratio, oval or elliptic nuclei with finely granular chromatin pattern. Immunocytochemical examination revealed that LAM cells forms a core of cluster and are positive for muscular antigens, melanoma-related antigens, and progesterone receptor, but only two of 7 specimens were positive for estrogen receptor. Surface monolayer cells were

lymphatic endothelial cells. Electron microscopy revealed abundant cytoplasmic filaments in inner core cells, many pinocytotic vesicles in an outer monolayer cells and a poorly formed basement membrane between inner and outer cells.

[Conclusions] LCC shows a uniquely organized structure consisting of LAM cells and lymphatic endothelial cells that is totally different from cancer cell clusters. Therefore, cytological examination of chylous effusion has a diagnostic significance for LAM if demonstrates LCC and is likely to be able to avoid invasive tests such as lung biopsy.

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TSC deficiency may cause misregulation of cellular asymmetry through adenomatous polyposis coli tumor suppressor protein and microtubule

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Cellular asymmetry is crucial for directional cell migration, immune response, and neuronal development. The establishment and maintenance of this asymmetry is thought to be mediated by local regulation of cytoskeletal dynamics and signaling including Rho family GTPase activities. This is often misregulated in tumor cells including LAM disease; therefore, it is important to understand how the cells properly coordinate cellular asymmetry. Microtubules (MTs) has been implicated in the polarization of migrating cells through local regulation of Rho family GTPases including Rac1. Recent studies including our studies have shown that MT plus end binding proteins (+TIPs) seem to be important to regulate plus end dynamics of small subsets of MTs. Among several +TIPs, adenomatous polyposis coli (APC) is a good candidate for MT-mediated Rac1 activation due to its unique distribution in cellular protrusions and ability to bind to the Rac1-specific guanine nucleotide exchange factor, Asef. Therefore, we have begun to explore the role of APC in local regulation of MT dynamic and MT-mediated Rac1 activation and membrane protrusion. We used a nocodazole-washout assay to induce MT growth-mediated activation of Rac1 and found that the kinetics of APC localization on MT plus ends was correlated with the onset of membrane protrusion during MT regrowth. Next, we tested if APC deficiency alters MT regrowth-mediated membrane protrusions. Wild type mouse primary fibroblast showed robust membrane protrusions after nocodazole washout, whereas APC-deficient cells showed significantly less membrane protrusion. APC-deficient cells also showed impaired cell migration. Immunostaining of MTs showed comparable MT arrays in both APC-deficient and wild-type cells. This result suggests that impaired membrane protrusion and cell migration in APC-deficient cells may be due to some problem of Rac regulation.

Because some phenotypes of APC-deficient cells and tuberous sclerosis complex (TSC)-null cells seem to be strikingly similar and Yeung's group showed that TSC could interact with β -catenin, we attempted to explore the possible link between TSC and APC. To test if TSC is important regulator of APC and MTs, we compared APC localization and MT array in wild type and TSC-null mouse primary fibroblast by wound healing assay and immunostaining. Very interestingly, TSC-null cells showed less APC clusters at the leading edge of cells. In addition, TSC-null cells seem to have a problem to orient MT arrays toward a wound. These results imply that TSC could participate in cell polarity through the regulation of APC and MTs.

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The Birt-Hogg-Dube and Tuberous Sclerosis Complex homologs have opposing roles in amino acid homeostasis in *Schizosaccharomyces Pombe*

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Birt-Hogg-Dube (BHD) syndrome is a tumor suppressor gene disorder characterized by benign tumors (hamartomas) of the skin, cystic lung disease, spontaneous pneumothorax, and renal cell carcinoma. Germline BHD mutations have also been identified in families with hereditary spontaneous pneumothorax, without dermatologic or renal disease. BHD encodes folliculin, a protein with no significant homology to other human proteins. Very little is known about the molecular pathogenesis of BHD or the function of folliculin.

The clinical hallmarks of BHD resemble certain aspects of tuberous sclerosis complex (TSC), a tumor suppressor gene disorder caused by mutations in the TSC1 or TSC2 gene, suggesting that the BHD and TSC proteins may function in similar cellular pathways. If this is correct, then investigation of the BHD signaling pathway may elucidate the pathogenesis of cystic lung disease in LAM.

To evaluate the relationship between BHD and TSC, we identified and deleted the BHD homolog in *Schizosaccharomyces pombe* (*S. pombe*), and compared the phenotypes with those of *S. pombe* lacking *Tsc1* or *Tsc2*, which we have previously reported (van Slegtenhorst et al., *J. Biol. Chem.* 2004). Expression profiling revealed that six permease and transporter genes, known to be downregulated in *delta tsc1* and *delta tsc2*, were upregulated in *delta bhd*. This “opposite” expression profile was unexpected, given the partially overlapping phenotypes in human BHD and TSC. The intracellular levels of specific amino acids known to be low in *delta tsc1* and *delta tsc2*, including ornithine and citrulline, were elevated in *delta bhd*.

The TSC1/2 proteins inhibit the small GTPase Rheb in mammals and *S. pombe*. Expression of a hypomorphic allele of *rhb1+*, the *S. pombe* Rheb homolog, dramatically increased permease expression levels in *delta bhd* but not in wild-type yeast. Loss of Bhd sensitized yeast to Rapamycin-induced increases in permease expression levels, and Rapamycin induced lethality in *delta bhd* yeast expressing the hypomorphic *Rhb1* allele.

These data indicate that Bhd and *Tsc1/Tsc2* have opposing roles in the regulation of amino acid homeostasis in *S. pombe*, and point toward a role for the mammalian BHD protein, folliculin, in a pathway functionally opposing that of TSC1/TSC2/Rheb. If this relationship is conserved in mammalian cells, studies of BHD may substantially alter our understanding of the role of mTOR activation in cystic lung disease in LAM.

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Activation of the mTOR Pathway in Sporadic Angiomyolipomas and Other Perivascular Epithelioid Cell Neoplasms

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Angiomyolipomata (AML) and lymphangiomyomatosis (LAM) belong to a family of tumors known as perivascular epithelioid cell tumors (PEComas) that share a common immunophenotypic profile of muscle and melanocytic differentiation. These tumors are clonal in nature and are strongly associated with tuberous sclerosis and genetic analyses have reported allelic imbalance at the TSC2 locus on 16p13. The latter is an autosomal dominant disease that is characterized by the multi-system development of benign tumors stemming from underlying mutations of either the TSC1 or TSC2 tumor suppressor gene. AML of the kidney develops in greater than 50% of TSC patients and is a major source of morbidity secondary to hemorrhage and destruction of renal parenchyma. Recent studies suggest that up to one-third of adult TSC females also have manifestations of LAM based on radiologic characteristics of their lungs.

In the context of non-TSC, non-LAM associated AMLs and non-renal PEComas, the functional status of the TSC2 signaling pathway has not been reported. Studies over the last several years have uncovered a critical role of the *TSC1/2* genes in negatively regulating the Rheb/mTOR/p70S6K cascade. Here, we examined the activity of this pathway in sporadic AMLs and PEComas using immunohistochemical and biochemical analyses. We found increased levels of phospho-p70S6K, a marker of mTOR activity, in 15 of 15 non-TSC AMLs. This was accompanied by reduced phospho-AKT expression, a pattern that is consistent with the disruption of TSC1/2 function. Western blot analysis confirmed mTOR activation concurrent with the loss of TSC2 and not TSC1 in sporadic AMLs. Similarly, elevated phospho-p70S6K and reduced phospho-AKT expression was detected in 14/15 cases of extra-renal PEComas. These observations provide the first functional evidence that mTOR activation is common to sporadic, non-TSC-related AMLs and PEComas. This suggests the possibility that mTOR inhibitors such as rapamycin may be therapeutic for this class of disease.

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TSC1 and Rheb specify cell fate and control orientation of asymmetric cell division in *Drosophila*.

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Background: The cellular origin of LAM is not well understood. Melanocyte and melanoma-associated antigens are expressed by angiomyolipoma and LAM cells, leading us to hypothesize that melanocytes and LAM might share a common cellular origin. Melanocytes are derived from the neural crest, which is a transient structure of the vertebrate embryo formed by cells with multipotential and self-renewing capacity, with remarkable migratory behavior and diversity. Therefore, we analyzed the role of TSC1/TSC2 in asymmetric cell division of neural progenitor cells during *Drosophila* peripheral nervous system development. Asymmetric cell division is a conserved mechanism for generating cellular diversity during this process, and is highly dependent on the regulation of Notch activity by Numb, an inhibitor of Notch signaling.

Results: We found that loss of TSC1 or ectopic Rheb expression within the sensory organ precursor (SOP) lineage resulted in supernumerary bristle and socket cells in about 40% of flies and balding in 40-70% of flies. We next used *in vivo* confocal microscopy to image TSC1 mutant and Rheb transgenic pupae to ask whether and how TSC1 regulates cell fate specification within the SOP lineage. TSC1 does not appear to be required for proper Numb and Partner of Numb (PON) localization during interphase in SOP lineage. However, cell division was significantly delayed in both TSC1 mutants and Rheb transgenic pupae, with delays of 50 min for pIIb, 80 min for pIIa, and 58 min for pIIIb cell division in the Rheb transgenics. 17% of SOPs lacking TSC1 underwent apoptosis after complete division, and an additional 17% of SOPs were arrested after mitosis and did not undergo subsequent divisions. About 30% of SOP cells showed defects in planar cell polarity during mitosis as measured by the position of the asymmetrically-localized Numb crescent relative to the anterior-posterior axis of the fly.

Conclusions: We conclude that TSC1 participates in establishing planar cell polarity of the SOP cell and determining cell fate specification during *Drosophila* sensory organ development. Our data point toward a possible role of TSC1 and TSC2 in establishing of tissue polarity and cell fate decision in higher organisms. We speculate that Notch-mediated cell fate decision lead to aberrant cellular differentiation in LAM and angiomyolipomas, and that LAM cells may be derived from a neural crest progenitor cell.

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Malignant Nerve Sheath Tumor (MPNST) is a highly chemo-resistant sarcoma with devastating 21% five-year survival within the context of Neurofibromatosis type 1, and 42% in sporadic MPNST (Evans DGR et al J. Med.Genet. 2002). Upregulated mTOR signaling was linked to MPNST and inhibition with rapamycin decreased growth of two MPNST cell lines *in vitro* (Johannessen CM et al., PNAS, 2006). mTOR inhibitors can enhance the effect of chemotherapeutics (Beuvink I et. al. Cell 2005). We tested the effect of mTOR inhibitors in combination with the chemotherapeutic agent Doxorubicin and/or an EGFR tyrosine kinase inhibitor previously used in a clinical trial in patients with MPNSTs, in 5 MPNST cell lines. The mTOR inhibitor decreased growth 19-60% after four days of treatment. Combination with the EGFR inhibitor decreased growth by 54%-70%. Inhibition of mTOR also increased the cytotoxic effect of Doxorubicin, correlating with a two-fold increase in apoptosis. To test the effect of mTOR inhibition *in vivo* we used a Xenograft model in which the MPNST cell line STS26T is injected subcutaneously into athymic nude mice. We treated *nu/nu* mice with gavage starting 3 or 16 days after tumor cell injection. Tumors in the placebo group reach 10% body weight within 4 weeks. Treatment with the mTOR inhibitor prevented further growth of the tumors in the early treatment; in the late treatment growth was reduced 75% up until day 30 post-injection. Combinations with other agents are in progress. This study shows that targeting the mTOR pathway slows down growth of MPNST cells *in vitro* and *in vivo* and might be considered as MPNST treatment strategy, alone or in combination with chemotherapeutic agents or targeted therapies. Supported by NIH-RO1-NS28840 and the Translational Research Initiative of Cincinnati Children's Hospital.

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Psychosocial Condition in Patients with Lymphangiomyomatosis

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Lymphangiomyomatosis (LAM) is a rare multi-systemic disorder that affects women primarily of reproductive young age who are carrying social roles. Progressive cystic changes of the lungs cause crucial physiological dysfunction leading to respiratory failure, which might make the patients change their life style, and affect psychosocial conditions.

Aims: To understand the psychosocial condition of the patients with LAM, especially focusing to depression and anxiety of patients.

Methods: 59 patients with LAM were studied. We analyzed dyspnea index, pulmonary function tests, the hospital anxiety and depression scale (HADS), MOS-Short Form 36, St. George's respiratory questionnaire (SGRQ), and, Kupperman Menopausal Index. Moreover we analyzed the free described texts about their anxiety and questions (n=total 279) using the methods of qualitative study by the independent researchers. Every response for the open questions was categorized by the contents and the order of frequency.

Results:(1) The psychosocial subscales, "Mental Health" and "Impact" strongly correlated with dyspnea index, HADS and Kupperman's index. (2) 62% of LAM patients suffered mild or severe depression or anxiety.

(3) 78% of LAM pt. had "climacteric symptoms" which required care or therapy.

(4) Patients who had worse pulmonary function received more hormone therapy than mild diseases. LAM patients under hormone therapy had worse depression and anxiety scores (HADS) than no hormone therapy.

(5) HADS and Kupperman's index did not significantly correlated with %FEV1.0, PaO₂, and %DLco, however significantly correlated with dyspnea index.

(6) The patients had worried about "Relationship with family and surrounding others", "Diagnosis & treatment", and "Employment & economic burden (expenses)", and "Childbirth", etc.

Conclusion: Not only the treatment of the physical disorder, but also the understanding and managements of psychosocial conditions, especially anxiety and depressions, are necessary for the patients with LAM.

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Cell Signaling Pathways that Target Harmatin

Tuberous sclerosis (TSC) is an autosomal-dominant disease characterized by tumors, which can grow in any organ of the body and by numerous abnormal blood vessels around tumors. Two tumor suppressor genes, *TSC1* and *TSC2*, are known to be associated with the development of TSC, and mutations in either gene are responsible for both the familial and sporadic forms of TSC. Harmatin, the TSC1 protein product, stabilizes TSC2 through binding with it, thereby preventing TSC2 from ubiquitination and degradation. Tuberin, the protein product of TSC2, acts as a GTPase-activating protein to regulate RHEB (GTPase) function through decreasing the ratio of GTP to GDP bound on RHEB. GTP-bound RHEB is known to activate mTOR, whereas GDP-bound RHEB inhibits mTOR activity, suggesting that the TSC1/TSC2 complex can downregulate the mTOR pathway through suppressing RHEB function. The tuberous sclerosis 1 (TSC1)/TSC2 tumor suppressor complex serves as a repressor of the mammalian target of rapamycin (mTOR) pathway, and disruption of TSC1/TSC2 complex function may contribute to tumorigenesis.

Here, we show that I κ B kinase β (IKK β), a major downstream kinase in the tumor necrosis factor α signaling pathway, physically interacts with and phosphorylates TSC1 at specific serine residues, resulting in suppression of TSC1. The IKK β -mediated TSC1 suppression activates the mTOR pathway, enhances angiogenesis, and culminates in tumor development. pTSC1 is associated with the expression of pIKK β (S181), pS6K1 (T389), and vascular endothelial growth factor in multiple tumor types and correlates with poor clinical outcome of breast cancer patients. Our findings identify a pathway that is critical for inflammation-mediated tumor angiogenesis and may provide a target for clinical intervention in human cancer including TSC.

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Loss of Heterozygosity of the *TSC* Genes and Overexpression of mTOR-related Proteins in Multifocal Micronodular Pneumocyte Hyperplasia (MMPH) of the Lung

[Introduction] Tuberous sclerosis complex (TSC) is an autosomal-dominant disorder characterized by central nervous system and visceral hamartomatosis. Pulmonary lymphangiomyomatosis is well known as a pulmonary manifestation in the patients with TSC. Recently, multifocal micronodular pneumocyte hyperplasia (MMPH) has been reported as another, rare, pulmonary manifestation of TSC. MMPH is characterized by proliferation of large plumped type II pneumocytes although other TSC-associated hamartomas are mesenchymal tumors. However, it is unclear whether MMPH is neoplastic or reactive. The objective of this study is to determine whether MMPH is a neoplasm or not by examining abnormalities of *TSC* genes and mTOR pathway.

[Methods] Three female patients with TSC and one without TSC were analyzed. For analysis of LOH, two or three lesions of MMPH and normal lung tissue in the lung specimens were selected. To extract DNA from the lesions of MMPH, the lesions were microdissected from the specimens that were immunostained with cytokeratin. LOH in both six microsatellite loci on 9q34 and 16p13.3 was analyzed including the *TSC1* and *TSC2* gene-associated region, respectively. Immunohistochemical examinations were also carried out with the EnVision+System (Dako Cytomation, Glostrup, Denmark) using Phospho-mTOR Antibody and Phospho-S6 (pS6) Antibody.

[Results] All nine lesions except one lesion of MMPH had *TSC2* LOH, whereas no lesion of MMPH did *TSC1* LOH. To compare LOH pattern in two or three lesions of MMPH selected in each case, the seven lesions in three cases demonstrated that *TSC2* LOH pattern differed from one another. Immunohistochemically, MMPH cells have an overexpression of pS6 and mTOR proteins in all cases.

[Conclusion] Results of LOH and immunohistochemical analysis suggest that loss of function in the *TSC2* gene causes MMPH. Therefore, MMPH may be neoplastic. Furthermore, having different LOH pattern among different lesions in each patient

indicates that multifocal lesions of MMPH do not have a common genetic origin. Aberration of *TSC1* occupies an important position of the neoplastic process in pneumocytes including precancerous epithelial hyperplasia and adenocarcinoma. Abnormalities of *TSC* genes may play a critical role in tumorigenesis of the lung.

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The mutation analysis of the *Birt-Hogg-Dubé* gene in patients with pulmonary cysts and recurrent spontaneous pneumothorax

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Rationale: It is speculated that there exist some predisposing factors or genetic defects in patients with multiple pulmonary cysts and recurrent spontaneous pneumothorax. Birt-Hogg-Dubé (BHD) syndrome, a rare inherited autosomal genodermatosis recognized first in 1977, is characterized by clinical manifestations including fibrofolliculomas of the skin, renal tumors, pulmonary cysts with spontaneous pneumothorax. The *BHD* gene, a tumor suppressor gene located in chromosome 17p11.2, has recently been identified to be defective in patients with BHD syndrome. We investigated whether the *BHD* gene is mutated in patients with pulmonary cysts with spontaneous pneumothorax, but with neither renal tumors nor fibrofolliculomas of skin.

Methods: We studied 7 patients with pulmonary cysts: 6 patients had complicated with spontaneous pneumothorax and 5 had a family history of pneumothorax. The genomic DNA was isolated from peripheral blood leukocytes and then the *BHD* gene was examined by polymerase chain reaction, denaturing high-performance liquid chromatography and direct sequencing.

Results: Germline mutation of the *BHD* gene was identified in 5 patients. All mutations were unique and 4 of them were novel, including 3 different deletions or insertions detected in exon 6, 12, and 13, respectively, and one splice acceptor site mutation in intron 5 resulting in in-frame deletion of exon 6. The remaining is a cytosine insertion in exon 11, a common mutation in so-called “hot spot” of a mononucleotide (C)₈ tract. There was no missense mutation detected in our study group.

Conclusions: Germline mutation of the *BHD* gene is involved in some patients with pulmonary cysts and pneumothorax even though they were not complicated with kidney and skin abnormalities. Accordingly, clinical phenotype of the BHD syndrome may be variable and further studies is needed to evaluate the role of the *BHD* gene in pathogenesis of pulmonary cysts and pneumothorax.

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Expanding the Differential Diagnosis of Cystic Lung Disease at HRCT

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OBJECTIVES: To describe the uncommon causes of cystic lung disease that may mimic lymphangiomyomatosis (LAM) or Langerhan's cell histiocytosis and review the pertinent clinical and pathologic features of these rare lung diseases.

METHODS: A review of patient case files referred to the Rare Lung Disease Consortium with a suspected diagnosis of LAM resulted in a series of cases with alternative cystic lung disease diagnoses. The CT findings in patients with documented "LAM mimics" will be displayed and the disease processes reviewed.

RESULTS: Multiple cystic lung diseases may mimic LAM on chest CT. These include uncommon entities such as lymphoid interstitial pneumonia (LIP), follicular bronchiolitis associated with Sjogren's disease, amyloidosis, light chain deposition disease, Birt Hogg Dube, bronchopulmonary dysplasia and metastatic endometrial cell sarcoma. When applicable, clinical or radiologic findings that may assist in differentiating these entities will be reviewed.

CONCLUSION: Although cystic lung disease is most often secondary to LAM or Langerhan's cell histiocytosis, other entities may result in a similar radiographic appearance and should be considered in the differential diagnosis.

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Chylous Effusion Following Lung Transplantation for Lymphangiomyomatosis: Utilization of a Pleurovenous Shunt

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Chylous effusions are a well described complication of lymphangiomyomatosis (LAM) in both the pre-lung transplant and post-lung transplant patient. Chylous effusions can cause significant morbidity to patients and most treatment modalities have limitations to complete success. We describe the use of a pleurovenous shunt to treat a refractory chylous effusion in an 18 year old patient following lung transplantation for progressive LAM. The shunt was placed as a salvage attempt after multiple conventional therapies failed. Following shunt placement, the patient had complete resolution of the chylous effusion and subsequent discharge home from a prolonged hospitalization. The patient has continued to do well on follow-up visits and remains effusion free, eight months after the procedure. The use of a pleurovenous shunt for refractory chylous effusions is a viable alternative to conventional therapy.

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A Study Of The Efficacy And Safety Of Sirolimus (Rapamycin) Therapy For Renal Angiomyolipomas In Patients With Tuberous Sclerosis Complex And Sporadic Lymphangiomyomatosis

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Introduction/rationale

In cells lacking TSC1/2 the mTOR pathway is activated. Animal and cell culture models suggest that the mTOR inhibitor sirolimus may reduce the size of and induce apoptosis in TSC related tumours.

Methods

We are conducting a phase II trial of the safety and efficacy of sirolimus in patients with tuberous sclerosis and sporadic LAM. The primary endpoint is the response of renal angiomyolipomas (AMLs) as assessed by MRI scan. We are also assessing lung function, cognition, seizure frequency and adverse events.

Results

Twelve patients have been recruited to date. One withdrew after two months of therapy. In all other patients a reduction in the size of AMLs has been observed. All but one of the patients has experienced adverse events, the majority of which have been low grade.

Conclusion

Sirolimus has a biological effect in TSC and LAM related renal angiomyolipomas. The TESSTAL trial establishes the case for larger scale randomised control trials of mTOR inhibition in TSC and LAM and is an important paradigm for the translation of genetic knowledge to novel therapy.

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Identification of the Benign Mesenchymal Tumor Gene, HMGA2, in Lymphangiomyomatosis (LAM)

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The normal expression pattern of HMGA2, an architectural transcription factor, is primarily restricted to cells of the developing mesenchyme, prior to their overt differentiation during organogenesis. A detailed in situ hybridization analysis demonstrated that the undifferentiated mesoderm of the embryonic lung expressed *Hmga2* but it was not expressed in the newborn or adult lung. Previously, HMGA2 was demonstrated to be misexpressed in a number of benign, differentiated mesenchymal tumors including lipomas, uterine leiomyomas and pulmonary chondroid hamartomas. Here we show that HMGA2 is misexpressed in pulmonary lymphangiomyomatosis (LAM), a severe disorder of unknown etiology consisting of lymphatic smooth muscle cell proliferation that results in the obstruction of airways, lymphatics and vessels. Immunohistochemistry was performed using antibodies to HMGA2 and revealed expression in lung tissue samples obtained from 21 patients with LAM. In contrast, HMGA2 was not expressed in sections of normal adult lung or other proliferative interstitial lung diseases, indicating that the expression of HMGA2 in LAM represents aberrant gene activation and is not due solely to an increase in cellular proliferation. A subset of patients the study were examined for chromosomal disruption at the HMGA2 locus using FISH analysis. HMGA2 misexpression in lam cells does not arise due to translocations at the locus and is similar to the situation observed in uterine leiomyomatosis. In vivo studies in transgenic mice demonstrate that misexpression of HMGA2 in smooth muscle cells resulted in increased proliferation of these cells in the lung surrounding the epithelial cells. Here we have demonstrated for the first time that the disorder develops with the coordinate misexpression of HMGA2 in an adult cell population. The fact that all LAM samples were identified with significant HMGA2 expression strongly suggests that the gene is playing a critical role in the pathogenesis of this disease. The results suggest an additional target towards which treatment strategies for LAM can be directed. Additionally, the involvement of HMGA2 in LAM allows for the classification of the disease with the other mesenchymal tumors known to be associated with HMGA2. Therefore,

therapeutic efforts directed at these more common tumors by targeting HMGA2 can additionally be expected to aid in the treatment of LAM.

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Essential Role of Rheb in Estrogen-induced Growth of TSC2 Deficient Cells

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Lymphangi leiomyomatosis (LAM) is a systemic disease characterized by proliferation of atypical muscle-like cells within the lung parenchyma. LAM usually affects women during their childbearing years, suggesting that female hormones such as estrogen (E2) are involved in tumor growth. Inactivating mutations in one of the two Tuberous Sclerosis Complex (TSC) genes, *Tsc1* and *Tsc2*, and loss of heterozygosity (LOH) in the *Tsc2* gene are linked to LAM. In agreement with a role of E2 in LAM, E2 induces the growth of TSC2-deficient cells and tumor cells derived from LAM patients. TSC2 acts as a GTPase-activating protein that inhibits the activity of Rheb, an upstream activator of mTOR, which promotes cell growth. This led us to hypothesize that Rheb is important for the E2-induced growth of TSC2 deficient cells. TSC2-null cells from the Eker rat (ELT3 cells) that are responsive to E2 (kindly provided by Dr. C. Walker) were used as model in which to test this hypothesis. E2 elevated the expression of Rheb protein and phosphorylation of the Rheb/mTOR downstream target S6 in ELT3 cells. Real-time PCR demonstrated that Rheb was induced by E2 at transcriptional level. Downregulation of Rheb by small interference RNA abolished E2-induced ELT3 cell growth. This effect correlated with alterations in the levels of important G1/S cell cycle regulators. Downregulation of Rheb increased the expression level of the cell cycle inhibitor p27 and decreased expression of cdk2, cyclin D1 and phospho-Rb. These data demonstrated that E2 regulates Rheb expression, and establishes Rheb as an important component of E2-induced TSC2 deficient cell growth.

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Interaction between Tuberin and Serum Response Factor: Potential Role in LAM

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LAM is a disorder characterized by abnormal abundance of smooth muscle cells (SMC) within various structures of the lung. Mutations in *TSC1* or *TSC2* tumor suppressor genes were found in LAM patients and it was firmly established a key role of these genes in controlling signaling events that regulate protein synthesis and cell growth. Thus, loss of *TSC2* function results in constitutive activation of p70S6K, the downstream effector of mTOR. Less explored however, is the function of these genes on regulation of transcription, another important level of gene expression.

Differentiation of normal SMC and concomitant up-regulation of SM-specific gene expression, including SM- α -actin, SM-myosin heavy chain, and SM22 α , requires the nuclear activity of the transcription factor serum response factor (SRF). Moreover, dysfunction of SRF occurs in several pathological states of the muscle. SRF binds to selective DNA sites within the regulatory region of the majority of SM-promoters. We showed that cells within LAM lesions exhibited high levels of SRF. Using natural as well as artificial promoters in transiently transfected SMC and *Tsc2*-deficient cells, we demonstrated that tuberin, *TSC2* gene product and hamartin, the *TSC1* gene product modulate SM-specific gene expression. While tuberin reduced promoter activity of SRF-dependent promoters, hamartin significantly augmented it. This latter enhancing effect of hamartin was blocked by inhibitors of the RhoA pathway or by overexpression of tuberin.

To elucidate the mechanism by which tuberin inhibits SRF-dependent promoter activity, we hypothesized that tuberin binds to SRF, and this binding interferes with SRF-mediated transcription function. We based this hypothesis on our work on modulation of SRF function by transforming growth factor (TGF) beta, which is a key mediator of lung remodeling, affecting gene expression, cell proliferation, differentiation, and migration. We performed immunoprecipitation (IP) assays of cell lysates prepared from HEK293 cells with anti-SRF antibody. Western analysis reveals that tuberin but not hamartin was able to associate with SRF. Consistently, IP assays using anti-tuberin antibody confirmed binding of tuberin to SRF. No binding of SRF could be detected in lysates immunoprecipitated with anti-hamartin antibody.

This data indicate that the inhibitory effect of tuberin on SM-specific gene expression may stem from tuberin's ability to bind SRF. We speculate that mutations found within tuberin may interfere with its capacity to inhibit SRF-dependent genes in LAM. This in turn may lead to increased gene expression of SM-specific genes that is characteristic of LAM cells.

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Hypoxia regulation of mTORC1

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mTORC1 (mammalian target of rapamycin complex 1) plays a critical role in integrating a variety of signals, both intracellular and extracellular, with the cellular protein translation machinery. We have discovered that the tumor suppressor complex TSC1/TSC2, formed by the tuberous sclerosis complex 1 and 2 proteins and which is inactivated in LAM (lymphangi leiomyomatosis), is necessary for transducing signals from oxygen to mTORC1. In response to hypoxia, mTORC1 is inhibited in a manner that requires the TSC1/TSC2 complex. Failure to downregulate mTORC1 in TSC1/TSC2-deficient cells under conditions of hypoxia is associated with inappropriate cell proliferation and might contribute to tumor growth. We recently identified a critical component of the hypoxia signaling pathway, the protein REDD1 (regulated in development and DNA damage 1). REDD1 is induced by hypoxia and is both necessary and sufficient for mTORC1 inhibition. Using epistasis experiments we have determined that REDD1 functions upstream of the TSC1/TSC2 complex. REDD1 however, does not appear to interact with TSC1/TSC2. REDD1 is an evolutionarily conserved 25 kDa protein with no identifiable structural or functional domains and no homology to other human proteins of known function. To understand how REDD1 functions both biochemical and genetic experiments have been undertaken. Structure-function analyses using both deletion mutants as well as linker substitutions have revealed the minimum REDD1 domain that is necessary for mTORC1 inhibition. Using an inducible cell system, where the expression of epitope-tagged REDD1 can be controlled by exposure to tetracycline, we have characterized the subcellular localization of REDD1 and have determined that REDD1 forms part of a larger complex. Validation experiments with endogenous REDD1 have been conducted following the generation of anti-REDD1 antibodies. To evaluate the role of REDD1 in the response to hypoxia in the mouse, a mouse strain has been generated using ES cells (embryonic stem cells) from an exon trap library harboring a beta-geo cassette (a fusion of the beta-galactosidase and neomycin cDNAs) inserted in frame within the *REDD1* locus. Preliminary experiments indicate that this allele (REDD1 beta-geo) reports on the transcriptional activity of the locus and completely disrupts REDD1 function. Since REDD1 links hypoxia with the TSC1/TSC2 complex and mTORC1 and since LAM is characterized by hypoxia and aberrant mTORC1 signaling, understanding how REDD1 functions might have profound implications in unraveling the biology of LAM.

Promotion of elastic fiber formation: towards a synthesis. Mervyn Merrilees, Brent Beaumont, *Kathy Braun, *Stephen Evanko and *Thomas Wight Department of Anatomy with Radiology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, and *Benaroya Research Institute, Seattle, Washington.

Unlike emphysematous lung, LAM lung is not deficient in elastic fibers; the amount of elastin is increased in absolute terms, and the proportion of elastin relative to total tissue volume is similar to normal lung. The arrangement of fibers, however, is markedly changed. As alveolar walls of LAM lung thicken, the open network of elastic fibers is replaced by dense aggregations of elastin. While it is not clear if these fibers are solely the result of *de novo* synthesis or include a rearrangement of existing fibers, restoration of a normal network will likely require the synthesis and assembly of new fibers. Our studies on assembly of elastic fibers, however, indicate that this is unlikely due to the inhibitory effects of the matrix proteoglycans versican and biglycan which are increased in LAM lung. These proteoglycans inhibit fiber assembly by binding to and causing premature release from the cell surface of the tropoelastin chaperone, elastin binding protein (EBP). EBP is an enzymatically inactive β galactosidase that binds both tropoelastin and the galactosugars of the glycosaminoglycan side chains of proteoglycans. A decrease in the galactosugar content around cells promotes EBP-mediated elastogenesis. Several methods of decreasing the proteoglycan content of the cell coat have been identified and each leads to increased elastic fiber formation. Over-expression of the small chainless variant of versican, V3, is especially effective and believed to displace the larger V1 versican isoform from the cell surface by competing for binding to hyaluronan through the hyaluronan binding region (HABR) common to V3 and V1. Over-expression of versican antisense, which reduces total versican production, similarly increases elastogenesis. Mutant biglycan core protein, in which key serine residues have been replaced with alanines to prevent glycosaminoglycan chain initiation, also increases fibrillogenesis.

Recently we have determined that an HABR complex, or Metastatin, purified from bovine nasal cartilage by an HA affinity column, induces an elastogenic phenotype similar to that seen in the V3 and versican antisense over-expressing cells. Our model predicts that occupation of binding sites on HA by hyaluronan binding sequences results in the exclusion of the large versican isoforms and enhancement of elastic fiber assembly. The inhibition of V3-induced elastogenesis by cells over-expressing mutant V3, in which the HABR has been deleted, further supports this model. These findings raise the possibility of controlling elastogenesis in lung and other tissues through manipulation of the levels of the matrix proteoglycans around cells. Whether or not such manipulation will enable remodelling of LAM lung and restoration of a functional elastic fiber network remains to be determined.