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**Pulmonary arterial involvement leading to alveolar hemorrhage in lymphangiomyomatosis**

**Introduction:** Lymphangiomyomatosis (LAM) is a rare disease characterized by proliferation of smooth muscle like-cells (LAM cells) around airways, blood vessels and lymphatics, with cystic destruction of the lung. Clinically, patients may develop progressive dyspnea, cough, wheezing, pneumothoraces, chylothorax, and hemoptysis. Acute respiratory failure secondary to diffuse alveolar hemorrhage is a very rare manifestation in LAM patients, with two previous descriptions in the literature. The mechanism that explains this presentation is not defined. This case report describes a patient with diffuse alveolar hemorrhage leading to acute respiratory failure and death that had the diagnosis of LAM and pulmonary arterial disruption caused by LAM cells confirmed after autopsy.

**Case Report:** A 39-year-old woman presented with a history of progressive dyspnea for seven years, when she was misdiagnosed with asthma. In the last month, dyspnea worsened, she complained about mild hemoptysis and hospital admission was necessary. She progressed to acute respiratory failure and pulmonary sepsis, with a rapid deterioration of hypoxia, when oral intubation and mechanical ventilation were necessary. Thoracic computed tomography demonstrated bilateral and diffuse ground-glass attenuation opacities predominant in lower lung lobes and thin walled cystic lesions diffusely distributed. Bronchoscopic examination revealed a moderate amount of blood from both lungs, consistent with the diagnosis of diffuse alveolar hemorrhage. She developed hemodynamic instability, progressing to death. Histological examination in autopsy showed cystic lesions in pulmonary parenchyma, with atypical smooth muscle cells proliferation around bronchiolar, blood vessels and lymphatics, positive for antibody against melanoma-associated antigen (HMB-45) and  $\alpha$ -smooth muscle actin in immunohistochemical analysis, consistent with LAM. These cells were found within small pulmonary artery walls and disruption of arterial elastic layer was also seen. Diffuse alveolar hemorrhage and hemosiderin-laden macrophages in alveolar spaces were also identified.

**Discussion:** Hemoptysis is a rare manifestation in patients with LAM and the mechanism speculated to explain this presentation is the obstruction of pulmonary venules due to LAM cells, promoting pulmonary venous hypertension. In this case, we demonstrated the pulmonary arterial involvement by LAM cells, with disruption of arterial elastic layer that was probably the main responsible for the pulmonary bleeding, and not only venous obstruction. LAM should be included in the differential diagnosis of diffuse alveolar hemorrhage when a woman of reproductive age presents with acute respiratory failure associated to a significant decrease in hemoglobin, and diffusely distributed ground-glass attenuation opacities combined with bilateral thin walled cystic lesions in thoracic computed tomography.

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**Evaluation of dynamic hyperinflation in patients with lymphangiomyomatosis as a mechanism of exercise limitation**

**Rationale:** Lymphangiomyomatosis (LAM) is a rare disease which affects women at reproductive age, characterized by proliferation of atypical muscle cells around the airways. As many patients have airflow obstruction, the progressive inflation of lung volumes during exercise (dynamic hyperinflation, DH) is a plausible mechanism of exercise limitation and higher dyspnea.

**Objective:** We aimed to define the prevalence and predictors of DH in LAM patients.

**Methods:** Twenty two consecutive patients with LAM (mean age,  $42 \pm 8.3$  years; FEV1,  $77 \pm 28\%$  predicted; DLCO,  $66 \pm 26\%$  predicted; inspiratory capacity – IC,  $99 \pm 20\%$  predicted; RV/TLC, median, interquartiles 1 and 3, 112, 92 and 144% predicted, respectively, where RV is residual volume and TLC is total lung capacity) performed spirometry, body plethysmography, DLCO and an incremental cardiopulmonary exercise testing on cycle (CPET). DH was quantified by IC at rest and every two minutes during CPET. Decrease of 10% or more in the value of IC during CPET was used to define patients with DH. The symptoms were evaluated every two minutes using modified Borg scale (0-10).

**Results:** During CPET, twelve patients (55%) showed DH with a mean IC change ( $-22.6 \pm 9.5\%$  vs  $+0.7 \pm 8.3\%$ ,  $p=0.001$ ) when compared to the non-DH group. They also had lower FEV1 %predicted ( $61 \pm 26\%$  vs  $95 \pm 16\%$ ,  $p=0.002$ ), and greater RV/TLC %predicted (median, interquartiles 1 and 3, 141, 125 and 157%, respectively vs 99, 87 and 109%, respectively,  $p=0.003$ ) and RV %predicted ( $175 \pm 57\%$  vs  $103 \pm 20\%$ ,  $p=0.001$ ). Mean DLCO %predicted was lower in DH patients ( $55 \pm 28\%$  vs  $80 \pm 17\%$ ,  $p=0.02$ ). There were no differences between DH and non-DH groups in the maximum VO2 %predicted ( $80 \pm 28\%$  vs  $86 \pm 17\%$ ,  $p=0.52$ ), however the dyspnea intensity at exercise cessation (median Borg scale) trended to be higher in DH patients ( $5$  vs  $3$ ,  $p=0.21$ ). The decrease in IC was greater in patients with low FEV1 %predicted ( $r= +0.67$ ;  $p=0.001$ ), high RV/TLC %predicted ( $r= -0.62$ ;  $p=0.002$ ), low DLCO %predicted ( $r= +0.49$ ;  $p=0.01$ ) and high values in the modified dyspnea Borg scale ( $r= -0.46$ ;  $p=0.03$ ).

**Conclusion:** The dynamic hyperinflation during exercise is frequent in LAM patients, resulting in augmented dyspnea. The occurrence of airflow obstruction, air trapping and DLCO reduction at rest seem to be good predictors of DH. Future interventions over this exercise-limiting mechanism might contribute to decrease symptoms and even increase exercise tolerance.

**William Chang, MD**  
University of Nottingham

## **Regional and National Variability Suggests Underestimation of Prevalence of Lymphangiomyomatosis**

**Rationale:** The prevalence of LAM was last recorded as one case per 1.1 million of the total population, however, the present true prevalence is not known. The rate of diagnosis may alter between regions due to differing expertise in recognising rare diseases.

**Objectives:** To determine whether the prevalence of LAM varies regionally and nationally, to identify reasons for these differences and to establish whether the incidence of LAM has increased in the last 5 years.

**Methods:** Cases with LAM were obtained from patient groups and national databases from seven countries (total 985 patients). Prevalence estimates were calculated for each region of the seven countries using female population figures from census data. Incidence estimates were also calculated for the USA and UK. Regional variation in prevalence and changes in incidence over time were analysed using Poisson regression and linear regression on STATA.

**Measurements and Main Results:** Prevalence of LAM ranged nationally from 3.4 to 7.8 patients per million women and significant variation exists between all 7 countries ( $p$  value  $< 0.01$ ). In the USA, significant differences seen across all states ( $p < 0.02$ ). No significant correlation exists between prevalence of LAM and number of pulmonary doctors per million women or percentage of population with health insurance. The incidence has not significantly increased over the last 5 years in the USA and UK.

**Conclusion:** The overall prevalence of LAM is higher than previously recorded and regional and national variation suggests that there may still be large numbers of patients with LAM undiagnosed.

Debbie Clements, PhD  
University of Nottingham

## Regulation of Matrilysin/MMP-7 Expression in TSC-related Angiomyolipomata

**Introduction:** The mechanism of destruction of the lung parenchyma in LAM is unknown, although the activity of matrix metalloproteinases (MMPs) may be a contributing factor. We have shown that MMP-7 (matrilysin) expression is elevated in cells derived from angiomyolipomata (AMLs), and in LAM lung nodules, and sought to characterize pathways by which this upregulation occurs.

**Results:** Primary cells from AMLs of patients with tuberous sclerosis express relatively very high levels of MMP-7 mRNA by quantitative RT-PCR, and protein by ELISA, compared to our control smooth muscle cells (airway smooth muscle). Levels of MMP-7 protein, but not mRNA, are increased further by treatment with exogenous VEGF-A. The increase in MMP-7 protein expression in response VEGF is not sensitive to rapamycin, suggesting it is not mediated by mTOR. In addition, unstimulated AML cells express VEGF-A endogenously, raising the possibility that an autocrine VEGF signaling loop contributes to high basal levels of MMP-7 expression in these cells.

**Conclusions:** We hypothesize that elevated MMP-7 expression in primary AML cells is a consequence of autocrine VEGF signaling. It remains to be seen whether this pathway is also relevant to the MMP-7 seen in LAM lung nodules by immunohistochemistry. MMP-7 activity in pulmonary LAM cells might contribute to cyst formation, either directly, or indirectly via activation of other MMPs.

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**A genetic and immunohistochemical study of 10 cases of sporadic Lymphangiomyomatosis**

**Introduction:** Lymphangiomyomatosis (LAM) is a rare and fatal lung disease affecting women predominantly of child-bearing age. Mutation of one *tsc2* (tuberin) allele, preceded or followed by LOH (loss of heterozygosity) of the other allele, is currently considered to cause sporadic LAM. Therefore it is proposed that the lack of functional tuberin, leading to phosphorylation and activation of the mTOR (molecular target of rapamycin)/S6K1 signaling pathway, is essential to the pathogenesis of LAM disease. The purpose of this study is to assess the roles of *tsc2* mutations or LOH and mTOR/S6K1 signaling pathway in the pathogenesis of 10 sporadic LAM cases.

**Method:** DNA samples prepared from LAM lesions laser-microdissected from frozen lung tissue of 10 patients with sporadic LAM were studied for LOH by MLPA (multiplex ligation-dependent probe assay) and 9 of them for mutations in *tsc2*. These 9 samples were subject to whole genome amplification, and analyzed by deep sequencing of all *tsc2* exons using the Roche-454 platform. In addition, serial sections of FFPE lung tissue sampled from 3 of the sequenced cases were immunostained for LAM markers smooth muscle (SM) actin and HMB 45, tuberin, total and phosphorylated mTOR (p-mTOR), and total and p-S6K1.

**Result:** None of the 10 cases showed LOH. Four of the 9 samples sequenced showed one *tsc2* mutation and 1 showed two *tsc2* mutations. The mutation frequency was between 5.6 to 46%. Seven cases were analyzed by immunohistochemistry (IHC) and showed diffuse immunoreactivity for tuberin in LAM lesion, which was similar to that of normal alveoli, reactive epithelial and mesothelial cells. Total and p-mTOR were detected with similar intensity in the LAM lesions in 1 case with *tsc2* mutation and in 1 case without *tsc2* mutation. In the rest of the cases total and p-mTOR were not significantly detected in LAM lesions, while in contrast p-mTOR was strongly positive in bronchial and reactive epithelial cells. S6K1 and pS6K1 were similarly positive in all LAM lesions and strongly positive in bronchial and reactive epithelial cells. In all cases vascular and bronchial SM were negative for tuberin, total and p-mTOR and total and p-S6K1.

**Conclusion:** Sporadic LAM does not require *tsc2* LOH to develop. Furthermore, some LAM cases have no *tsc2* mutations at all (44.4% in this study). IHC analysis seems to indicate that activation of mTOR is not always required in the pathogenesis of sporadic LAM.

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University of Miami

**Doxycycline reverses 17beta-estradiol mediated effects on estrogen receptor alpha and beta via PI3K pathway in LAM**

Our previous studies have shown that the invasion and destruction of lung parenchyma in LAM is, at least partially, an estrogen driven process. Doxycycline (Dox), a nonspecific matrix metalloproteinase inhibitor, reverses 17 beta-estradiol ( $E_2$ )-mediated increases in MMP-2 activity and LAM cell (LAMD-SM) invasiveness. The present study is focused on the mechanism of Dox on estrogen receptor (ER)-dependent and independent activation in LAMD-SM. LAMD-SM (passages 5-8, n=3), isolation and propagation previously described, were treated with Dox in the presence and absence of  $E_2$ . All experiments were performed in phenol red-free DMEM supplemented with 100 $\mu$ g/ml of penicillin/streptomycin and glucosamine containing 20% charcoal-stripped fetal bovine serum to avoid stimulation of the ER. Cells were treated with either vehicle, a physiological concentration of  $E_2$  (0.1nM), Dox (10 $\mu$ g/ml) or a combination of  $E_2$ +Dox. Proteins were extracted from cell lysates and western blot analysis was performed for ERalpha, ERbeta and AKT protein expression. In LAMD-SM, Dox reversed the  $E_2$ -mediated 2 fold increase in AKT phosphorylation (\*p<0.05) back to baseline (p=0.5).  $E_2$  stimulation increased ERalpha protein expression 2.7 fold (\*p<0.05) and decreased ERbeta protein expression 2.5fold (\*p<0.05) compared to vehicle, both of which were also reversed by Dox (ERalpha, \*\*p<0.005 and ERbeta, p=0.05 compared to  $E_2$  treatment). These results suggest that Dox effects on the expression of ERalpha and ERbeta maybe through the AKT pathway.

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Università degli Studi di Milano

### Characterization of LAM/TSC Cells Isolated From Patient Chylous

Lymphangiomyomatosis (LAM) is a rare disease characterized by widespread proliferation of abnormal smooth muscle-like cells that leads to cystic destruction of the lung parenchyma. LAM cells migrate or metastasize to other organs, such as lung, lymph node and kidney. Tuberous sclerosis complex (TSC), an autosomal-dominant disease characterized by hamartoma formation in various organs, is caused by mutations in two tumor suppressor genes *TSC1* and *TSC2*, respectively encoding hamartin and tuberlin. Cells with *TSC2* mutation have been found in angiomyolipomas and lung lesions of LAM patients. We recently isolated and characterized a TSC tuberlin-deficient cell population from the chylous of a LAM/TSC patient with a germline *TSC2* mutation. As previously reported TSC lesions may be caused by *TSC2* gene mutation with loss of heterozygosity caused by methylation of *TSC2* promoter (*TSC2*<sup>-meth</sup> cells). Tuberlin expression in chylous *TSC2* cells can be induced by 5-azacytidine, a DNA demethylating agent, and trichostatin A, a histone deacetylase inhibitor. These cells are positive to alpha-actin antibody, CD44v6 and HMB45 antibodies, markers of TSC and LAM cells, and required the supplementation of epidermal growth factor (EGF) for proliferation. EGF can not be substituted by insulin-like growth factor-1 (IGF1), such as in the case of *TSC2* smooth muscle cellular populations previously isolated (*TSC2*<sup>-/-</sup> and *TSC2*<sup>-meth</sup> ASM cells). The blockade of EGF and IGF-1 receptors by means of specific monoclonal antibodies reduced gradually cell proliferation and caused cell death. Rapamycin efficacy was more effective in chylous *TSC2* cells than in *TSC2*<sup>-/-</sup> and *TSC2*<sup>-meth</sup> ASM cells. It is well known that interleukin-8 (IL-8) and -6 (IL-6) production is regulated by PI3K and MAPK pathways. Chylous *TSC2* cells secrete high amount of IL-8 and IL-6, that are not affected by either rapamycin or anti-EGFR antibody exposure. Thus it is conceivable that the proinflammatory cytokine release may be involved in the pathologic mechanisms activated by these cells. The acquisition of mesenchymal characteristics for cancer cells is a transient event that might be important for migration and tissue invasion. In addition chylous *TSC2* cells express vimentin, a protein used as marker for identification of mesenchymal characteristics, while E-cadherin, usually not expressed in invasive cancer cells, is not detectable. Thus isolated chylous LAM/TSC2 cells present the inhibition of tuberlin synthesis secondary to the methylation of *TSC2* promoter. These cells release inflammatory cytokines and present mesenchymal characteristics, in addition as any *TSC2* cells described by our group they are sensitive to anti-EGFR antibody.

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## Development of a LAM Model by Using Human TSC2 Deficient Smooth Muscle Cells Derived from a Renal Angiomyolipoma

Lymphangiomyomatosis (LAM) is a progressive and often fatal interstitial lung disease characterized by diffuse proliferation and invasion of abnormal smooth muscle cells in involved organs, cystic degeneration of lung parenchyma, infiltration of the axial lymphatics, and renal tumors. LAM affects between 30-40% of women with tuberous sclerosis complex (TSC), a tumor suppressor gene syndrome caused by mutations in the *TSC1* or *TSC2* genes. We developed a procedure for a quick invasion of the respiratory system by endonasally administrating *TSC2*<sup>-/-</sup> ASM cells, derived from a renal AML of a TSC2 patient. We previously showed that alveolar lung walls and lymph nodes were quickly and massively infiltrated. *TSC2*<sup>-/-</sup> ASM cells ( $2 \times 10^5$ ) were administered in immunodeficient (nu/nu Hsd:athymic) female nude mice. After 4 or 26 weeks from endonasal administration anti-EGFR antibody (starting dose of 400 mg/m<sup>2</sup> followed by subsequent dose of 250 mg/m<sup>2</sup>) and rapamycin (4mg/kg) were intraperitoneally injected 2 times a week for 4 weeks. *TSC2*<sup>-/-</sup> ASM cells caused progressive destruction of lung parenchyma with an emphysematous-like picture that was reversed by anti-EGFR treatment, while rapamycin was less effective and caused hemoptysis. *TSC2*<sup>-/-</sup> ASM cells grow and proliferate mainly in lung parenchyma and at a less extent in lymph nodes, as showed by Ki-67 staining. *TSC2*<sup>-/-</sup> ASM cells promoted a significant increase of LYVE-1 reactivity in lungs and in lymph nodes suggesting a correlation between *TSC2*<sup>-/-</sup> ASM cells and lymphangiogenesis. LYVE-1 reactivity decreased following anti-EGFR antibody and rapamycin treatments but, while anti-EGFR antibody suppressed the excessive lymphatic vessel in lungs, rapamycin caused their collapse. In association with the lung destruction, the presence of *TSC2*<sup>-/-</sup> ASM cells caused a marked increase of lung VEGF levels of murine origin, while human VEGF was not detected. Such enhanced local production of VEGF was significantly reduced by anti-EGFR antibody and rapamycin. Our work has shown that *TSC2*<sup>-/-</sup> ASM cells migrate, proliferate and invade lymph nodes and lungs causing LAM-like lesions. *TSC2*<sup>-/-</sup> ASM cells cause an enhanced secretion of VEGF that may be involved in stimulating lymphatic vessel growth in lymph nodes and lungs. Anti-EGFR antibody is more effective than rapamycin in promoting *TSC2*<sup>-/-</sup> ASM cell death and regeneration of lung parenchyma. The massive lung growth of lymphatics is reduced by anti-EGFR antibody. The *in vivo* study confirms the earlier *in vitro* data suggesting a therapeutic potential for anti-EGFR antibody in LAM and TSC treatment.

## Yoshikazu Inoue, MD, PhD

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### Quantitative CT in lymphangiomyomatosis: Comparison with sero-physiological markers and disease severity

**Background and Hypothesis:** Lymphangiomyomatosis (LAM) is a rare systemic disease, pulmonary lesion of which is characterized by progressive multiple cysts. Cystic pulmonary changes are considered to be prognostic in LAM patients. We have reported that quantification of lung attenuation by multi-detector-row CT (MDCT) at inspiration well reflected abnormal results of pulmonary function tests in patients with chronic obstructive pulmonary disease (COPD) (AJR, 192: 267-272, 2009). We hypothesized that low attenuation volumes (LAV), quantified by MDCT reflect the severity of LAM.

**Subjects and Methods:** To clarify the hypothesis, we performed volumetric MDCT with 3D post-processing at full inspiration and full expiration in 32 patients with LAM. We quantified total lung volumes (TLV), and LAV (attenuation values less than  $-950$  HU) at inspiration and expiration (TLVi, TLVe, LAVi, LVAe). The data was compared with serum biomarkers such as vascular endothelial growth factor (VEGF)-D, and physiological data such as TLC, FEV<sub>1.0</sub>, VC, PaO<sub>2</sub>, etc. and disease severity by “LAM handbook”, Japanese respiratory society, stages of GOLD for COPD.

**Results and Discussion:** There were significant correlation between TLVi and TLC, (TLVi-TLVe) and vital capacity, LAVi and FEV<sub>1.0</sub>, LAVi and PaO<sub>2</sub>, LAVE and FEV<sub>1.0</sub>, FEVe and RV, LAVE and PaO<sub>2</sub>, LAVi/TLVi and FEV<sub>1.0</sub>%, LAVE and RV/TLC ( $p < 0.05$ ).

**Conclusion:** Quantitative CT is useful method to determine disease severity or stage of pulmonary LAM. Serum VEGF-D reflects more systemic abnormality.

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### Serial CT Density in Lymphangioleiomyomatosis (LAM)

**Introduction:** Lymphangioleiomyomatosis (LAM) is a difficult disease to study because of its rarity and typically slow disease progression.

**Methods:** We evaluated paired computed tomography (CT), spirometry and lung volumes over 1 year from the first 11 patients analyzed for the MILES (Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus Trial) study. We determined CT density and lung function testing correlation and variability. Participants' baseline demographics included female sex 100%, age  $50.6 \pm 2.9$  (Mean  $\pm$  SEM for all analyses), and race (Caucasian 82%, Asian 18%). Baseline FEV<sub>1</sub> was  $1.28 \pm 0.11$  L (48.8% predicted). CT density was measured on the Siemens syngo In Space 4D Lung Parenchyma Analysis module (Siemens AG Inc, Erlangen, Germany).

**Results:** A high correlation was found between TLC and CT determined whole lung volume ( $R^2=0.89$ ,  $p<0.0001$ ). We found that variability between baseline and 12 month CT density was extremely small, regardless of the method used. Percentile density at 15% of the Hounsfield unit histogram (PD15) changed  $-2 \pm 0.7\%$  over one year in the cohort ( $P=0.03$ ). The percentage of lung  $< -1000$  HU,  $-950$  HU and  $-910$  HU increased  $0.26 \pm 0.13\%$ ,  $1.36 \pm 0.7\%$ , and  $1.83 \pm 1.16\%$ , respectively. Best signal to noise ratios were seen for the PD15 and indexed percentage of lung  $< -1000$  HU to CT scan derived TLC.

**Conclusion:** We suggest that future analyses use the PD 15 method or methods that directly measure cyst volume percent.

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**Thoracoscopic Covering Technique for diffused cystic lung diseases to prevent recurrent pneumothorax; study of LAM and BHD syndrome**

**Introduction:** Almost physicians have considered there is nothing but pleurodesis to prevent pneumothorax for LAM and BHD patients. Since 2003, we have conducted TPC (total pleural covering technique) to prevent recurrent pneumothorax in 25 LAM patients and have succeeded in 90% of the cases, up to 48 months after surgery. Through this experience, we studied the effectiveness of the covering technique for BHD.

**Patients and Methods:** Pleural covering was performed thoracoscopically in 24 cases of diagnosed BHD, and 10 cases of suspicious BHD through histopathology and genomic DNA analysis. Multiple lung cysts in BHD are characteristically distributed over middle and lower lobes, especially mediastinal and basal area of the lung, very few in the upper lobe. We covered the middle and lower lobes of the lung with absorbable mesh by thoracoscopy. The covered area is the middle and lower lobe at right lung, the lower lobe at left lung including interlobular space. Mesh material used the regenerated oxidized cellulose mesh which never caused adhesion to the thoracic wall but thickened visceral pleura. We reported about this detailed mechanism at LAMposium 2007 and 2008.

**Result:** There is no recurrent pneumothorax in 34 cases of BHD syndrome after surgery.

**Discussion:** We have reported in previous years that total pleural covering (TPC) has prevented recurrent pneumothorax without adhesion in LAM. We have named LPC (Lower pleural covering) for BHD, in comparison with TPC for LAM as well. Lung transplant is not necessary for patients with BHD, because it does not result in respiratory failure. Significance of LPC for BHD is as follows: It is impossible to remove all the bullas for diffused cystic lung diseases in either VATS or open surgery. Removing the bulla will likely to lead to worsening the already affected respiratory function. Chemical pleurodesis is generally recommended for diffused cystic lung diseases with recurrent pneumothorax, but we, as surgeons, are not sure that it has proved to be effective enough to prevent recurrent pneumothorax, especially in patients with underlining disease, and will also decrease the respiratory function. In addition, terrible adhesion from pleurodesis will become adversely very inconvenient when another operation is necessary for recurrent pneumothorax. We hope to avoid pleurodesis for diffused cystic lung disease from the point of surgeons' view.

**Conclusion:** As with the case in LAM with TPC, LPC for BHD is effective to prevent the recurrent pneumothorax without decreasing respiratory function and adhesion compared with pleurodesis.

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**Placental Alkaline Phosphatase (PLAP) and Podoplanin (D2-40) Define Two Subtypes of Cells in Lymphangiomyomatosis**

**Background:** Lymphangiomyomatosis (LAM) is a poorly understood rare fatal lung disease of premenopausal women. LAM lesions show infiltrating smooth muscle (SM)-like cells causing cystic destruction of lungs, eventually mandating lung transplant. LAM cells often metastasize to regional lymph nodes, circulate in blood, and occasionally produce distal metastases, including, to transplanted lungs. Occasionally LAM occurs in association with tuberous sclerosis disease and *tsc2* mutations were found in a few sporadic LAM cases. Since we noticed that vascular SM immunoreact for PLAP (Placental Alkaline Phosphatase) we evaluated LAM lesions for its expression. Moreover, since PLAP circulates patients with PLAP-positive tumors, we sought to evaluate the integrity of the vasculo-lympatic channels in/around LAM lesions.

**Design:** Serial sections of FFPE lung tissue sampled from 21 patients with LAM were immunostained for LAM markers SM  $\alpha$ actin (SMA) and HMB45, PLAP, vascular endothelial marker CD31 and lymphatic endothelial marker D2-40 (podoplanin).

**Result:** SMA was detected in most LAM cells followed in frequency by D2-40. PLAP and HMB45 were detected in a lower number of cells. Predominantly D2-40-positive lesions also had abundant HMB45-positive cells, but only rare PLAP-positive cells. Predominantly PLAP-positive lesions were negative or occasionally weakly positive for D2-40 and had a few HMB45-positive cells. Furthermore, cells that expressed PLAP did not express D2-40 or HMB45. Blood vessels stained weakly for PLAP but were negative for D2-40 or HMB45. D2-40 stained only lymphatic channels in normal or other disease lung controls. CD31 stained most of the epitheloid cells lining the LAM cysts.

**Conclusions:**

- PLAP is a novel marker for a subpopulation of LAM cells, immunoreacting significantly stronger than vascular or airway SM cells.
- Since PLAP, a GPI-anchored membrane protein is extruded on exosomes and circulating LAM cells can also carry PLAP, it should be cleaved from both by serum Phosphatidyl Inositol Phospholipase D (PIPLD). In such a case serum PLAP level could potentially be a surveillance marker for LAM patients.
- Podoplanin (D2-40) is a novel marker for a large subpopulation of LAM cells. Since a larger number of LAM cells stain for D2-40 than HMB45, the former may be a better diagnostic marker for LAM.
- Since podoplanin is involved in cell invasion, its presence in LAM lesions may have functional implications in progression of the disease.
- There are either PLAP-predominant or podoplanin-predominant LAM lesions/cases.

**Doug Medvetz, PhD**  
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**Folliculin, the *BHD* gene product, is phosphorylated under nutrient deprivation conditions and may be a positive regulator of AMPK and mTORC1**

**Introduction:** Birt-Hogg-Dube (BHD) is a tumor suppressor gene syndrome associated with facial skin tumors (folliculomas), renal cell carcinoma, and cystic lung disease. Folliculin (FLCN), encoded by the BHD gene, has been linked to the TOR (target of rapamycin) and AMPK (AMP-activated protein kinase) signaling pathways, and FLCN has been shown to be modified by phosphorylation, however its function is not well understood. The clinical similarities between BHD and TSC have led us to hypothesize that the proteins responsible may reside in similar pathways.

**Methods:** U251 (human glioblastoma), UOK257-2 (BHD re-expressing cells derived from a human BHD renal tumor, a gift from Laura Schmidt and Marston Linehan at the NIH), and HEK293 cells were used. The activity of mTORC1 (phospho-S6 Ser235/36 and phospho-S6K Thr389), and AMPK (phospho-ACC Ser79) was monitored by immunoblot.

For mass spectrometric identification of phosphorylated residues, HEK293 cells were transfected with myc-FLCN, serum starved for 24 hours and the myc immunoprecipitates separated using SDS-PAGE. The gel was Coomassie stained, and the myc-FLCN band excised, digested with trypsin, and subjected to reversed-phase microcapillary/tandem MS (LC/MS/MS).

**Results:** In U251 and UOK257-2 cells, serum starvation for 24 hours or nutrient deprivation for 2 hours led to an upshift in the FLCN band. Stimulation with 20% serum for 15 minutes after 24 serum starvation reduced and downshifted the FLCN signal. A series of potential FLCN phospho bands was observed under serum starvation in UOK257-2 cells on a low percentage gel. Treatment of immunoprecipitated FLCN or whole cell lysate under serum starvation with lambda phosphatase also resulted in a downshift of these bands. Taken together these data indicate that FLCN is phosphorylated under nutrient deprived conditions. In a preliminary study using mass spectrometry to identify phospho-sites, FLCN was phosphorylated at serines 62 and 73, consensus sites for casein kinase 2 (CK2)/AKT and CDK5 respectively.

To determine the functional consequences of BHD loss, BHD was down-regulated using siRNA in U251 cells. Under serum starvation conditions phospho-ACC levels were lower and phospho-S6 levels were higher compared to control siRNA. Serum stimulation of BHD siRNA treated cells led to higher levels of phospho-ACC, along with lower levels of phospho-S6 and phospho-S6K compared to control, consistent with prior findings from our group (van Slegtenhorst, JBC 2007; Hartman, Oncogene 2009).

**Conclusions:** Our data indicate that FLCN phosphorylation is increased under low nutrient conditions, and that FLCN is a nutrient-dependent, positive regulator of mTORC1 and AMPK activity.

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## The Presence of P311 in Lymphangiomyomatosis Lesions May Link TGF beta 1 Expression with the mTOR Pathway

**Introduction:** Lymphangiomyomatosis (LAM) is a rare fatal lung disease effecting women predominantly of child-bearing age. LAM is characterized by the infiltration of the lung by smooth muscle (SM)-like cells leading to cystic destruction and pulmonary insufficiency. LAM had been associated with mutations in the tuberous sclerosis complex gene 2 (TSC2 or tuberin) leading to activation of the mTOR (molecular target of rapamycin)/S6K1 signaling pathway. Active mTOR binds to eIF3 (eukaryotic initiation factor 3), displacing and activating S6K1, which promotes translation of cell growth-regulation genes.

P311 is an 8-kDa protein found in SM cells and neurons. We recently found that *p311* null mice have a severe decrease in SM TGFβ1 translation. Since LAM cells exhibit SM-like differentiation, we evaluated LAM lesions for expression of P311 and TGF beta 1.

**Design:** Serial sections of FFPE lung tissue sampled from 21 patients with LAM were immunostained for LAM markers SM actin and HMB 45, P311 and TGFβ1. Additional LAM lesions were laser-microdissected for P311 co-immunoprecipitation studies, followed by mass spectrometry to identify P311 binding partners.

**Results:** P311 was focally positive in LAM lesions with intensity similar to or higher than that of neighboring vascular SM. LAM cells expressing P311 also showed strong staining for TGF beta 1, while neighboring blood vessel SM did not express TGF beta 1. Co-immunoprecipitation and mass spectrometry demonstrated that P311 interacts with eIF3.

**Discussion:** LAM lesions are focally and concomitantly positive for P311 and TGF beta 1. Since the lack of P311 in *p311* null mice results in a decrease in TGF beta level, we postulate that the expression of TGF beta in LAM cells is related to the production of P311 by these cells. Since TGF beta 1 promotes tumor tolerance and stimulates the production of extracellular matrix degrading proteases, the production of TGF beta 1 by LAM cells is likely to contribute to the invasive nature the disease.

Based on the fact that P311 interacts with eIF3, we propose that by binding to eIF3, P311 facilitates the binding of active mTOR in the tuberin-deficient LAM cells. However, since vascular SM has normal tuberin function, the mTOR pathway is inactive and is not recruited to promote translation of TGF beta 1.

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## Clinical Predictors of Survival in Lymphangioleiomyomatosis

**Background:** Lymphangioleiomyomatosis (LAM) is a rare, progressive, frequently lethal cystic lung disease that almost exclusively affects women. Prognostic information in LAM has been limited by small numbers and heterogeneous study methodology. Early retrospective cohorts cited 5 and 10 year mortality of 40% and 80%, respectively. More recently, mortality at 10 years has been estimated to be approximately 10 to 20% from the onset of symptoms and 30% at 10 years from the time of lung biopsy but varies widely in individual patients. Given the heterogeneous disease course, it would be useful to establish which clinical characteristics are associated with survival in order to develop prediction models for disease outcome.

**Methods:** The LAM Foundation maintains a population based registry of 1149 registered self-identified LAM patients. Of these, 590 have completed a "General Information/Clinical History Questionnaire" with limited demographic and clinical data, 410 of whom were identified as US residents and provided DOB. Vital status was obtained on all 410 participants through December 31, 2007 by linking patient identifiers and the National Death Index. Survival time was calculated as the time since first lung related symptom or physician diagnosis until censoring (still alive, received lung transplant or died). Cox proportional hazard analysis evaluated the association of demographic and clinical features with survival.

**Results:** Among the 410 subjects, there were 25 deaths and 55 lung transplantations during a median of 10.4 years of observation time (range 240 days to 48 years). Of those that died, the median survival time from symptom onset was 13.3 years. The median time to transplantation was 10 years from symptom onset. The estimated 10 year survival transplant free was 87%. Age at disease onset, smoking status, race, presence of tuberous sclerosis, occurrence of pneumothorax, and pregnancy did not demonstrate an association with survival or transplant. Treatment with hormonal therapy was associated with an increased risk of death/transplant (HR 2.93, CI 1.54 - 5.58, p=0.001), particularly progesterone therapy (HR 2.17, CI 1.26 - 3.75, p=0.005). Patients that required oxygen therapy had a worse outcome (HR 4.53, CI 2.76 - 7.42, p<0.001).

**Conclusions:** Estimates of 10 year survival for patients with LAM in the United States are similar to recently published estimates in Europe and Asia. Treatment with hormonal therapy is associated with a worse outcome. Future studies should attempt to identify novel biomarkers to improve upon clinical prediction tools.

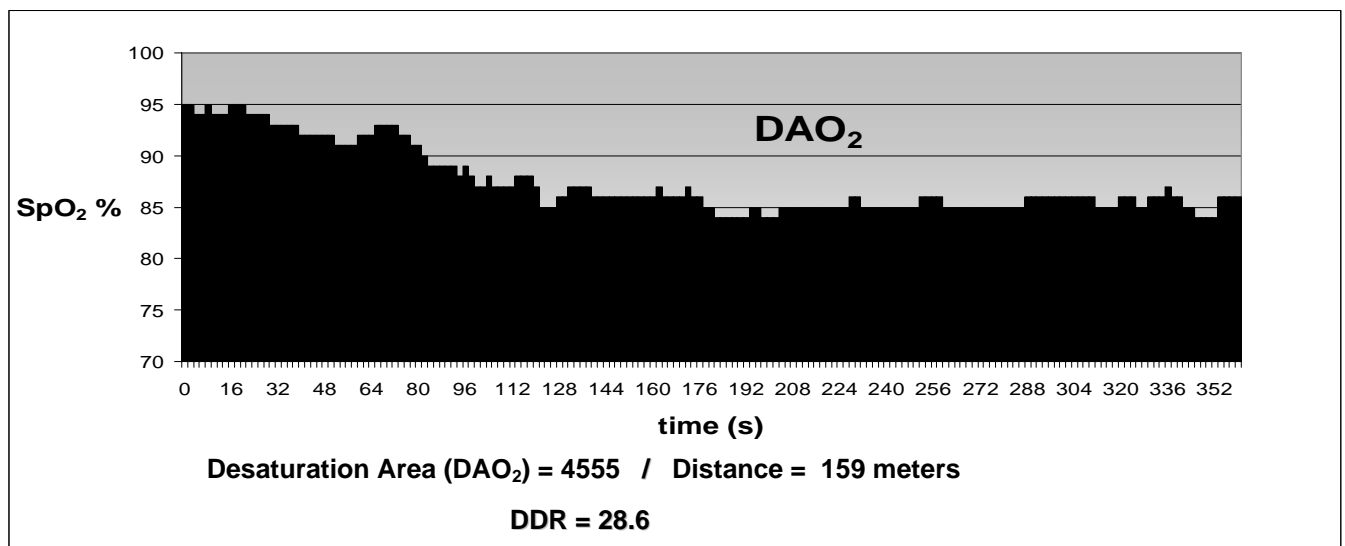
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**Desaturation – Distance Ratio, a novel tool for functional assessment in lymphangioliomyomatosis: correlation between tomographic disease severity analysis and lung function parameters**

**Rationale:** Lymphangioliomyomatosis (LAM) is a rare disease, usually evolving to chronic respiratory failure, characterized by the substitution in normal lung parenchyma for cystic lesions, leading to impairment in gas exchange. Functional variables as carbon monoxide diffusion ( $DLC_O$ ) and forced expiratory volume in the first second ( $FEV_1$ ) are already related to extension of lung cysts in chest computed tomography (CT) scan. We propose a new functional assessment tool to correlate with disease severity in lung CT.

**Methods:** Cross sectional study with 33 LAM patients followed in University of Sao Paulo Medical School Outpatient care. All underwent lung volumes measurements,  $DLC_O$ , chest CT scan (analyzed by two independent pulmonologists, experts in interstitial lung diseases), and six-minute walk test (6MWT) with oxymeter holter. During 6MWT assessment, we acquired a composite index, the desaturation - distance ratio (DDR), calculated using the ratio between  $DAO_2$  (light area) and the walked distance.  $DAO_2$  was obtained subtracting maximal oxygen saturation ( $SpO_2$ ) possible - 100% - of patient's  $SpO_2$  in every 2 seconds (Figure). Severity of pulmonary cystic involvement was graded using a qualitative score (adapted from Avila, NA *et al.* Radiology 2007; 242:277) ranging from 0 to 9, obtaining three grades: mild (0 – 3), moderate (4 – 6) and severe (7 – 9). Correlations were calculated using Spearman coefficient and a p value < 0.05 was considered significant. **RESULTS:** Qualitative CT analysis had excellent agreement between observers ( $k = 0.95$ ), with 12 patients (36%) presenting mild extent of lung cysts, 10 (30%) moderate and 11 (34%) severe. Correlation between DDR and tomographic score was significant ( $r = 0.86$ ,  $p < 0.001$ ) and superior to percent predicted  $DLC_O$  ( $r = -0.76$ ,  $p < 0.001$ ), percent predicted  $FEV_1$  ( $r = -0.55$ ,  $p = 0.001$ ) and walked distance in the 6MWT ( $r = -0.48$ ,  $p = 0.005$ ).

**Conclusion:** The combination between these two main variables in the 6MWT, desaturation area and the walked distance, in a unique index to evaluate the gas exchange, is a promising concept, being a more reliable physiologic tool to assess LAM patients.



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### **Doxycycline in the treatment of lymphangiomyomatosis**

**Rationale:** Lymphangiomyomatosis (LAM) is a rare disease that affects women, characterized by progressive chronic respiratory failure. Studies had shown an increase in metalloproteinases (MMP) 2 and 9 in LAM, leading to collagen degeneration and lung fibrosis. Doxycycline decreases MMP levels justifying its use.

**Methods:** Prospective clinical trial evaluating doxycycline effect (100mg/day) in 26 patients with LAM during a 12-month period. They underwent spirometry, lung volumes, DL<sub>CO</sub> (diffusion carbon monoxide), six-minute walk test (6MWT), lung CT, blood and urine testing for MMP-2 and -9. Severity of pulmonary cystic involvement in lung CT was graded using a qualitative score (adapted from Avila, NA *et al.* Radiology 2007; 242:277), ranging from 0 to 9 (increasing degree of severity). Endpoint was FEV<sub>1</sub> (forced expiratory volume at first second) variation after doxycycline. We stratified patients in two groups, those who increased or stabilized FEV<sub>1</sub> (G<sub>1</sub>) and those who decreased FEV<sub>1</sub> (G<sub>2</sub>). Continuous variables are presented as mean (SD) or median (IQ). Comparisons were performed by Wilcoxon or *t*-test.

**Results:** Thirteen patients improved or stabilized the FEV<sub>1</sub>, mean pre and post doxycycline 2.31 L (0.4) and 2.39L (0.4) p=0.003. The other 13 patients decreased the FEV<sub>1</sub>, mean 2.18 L (0.9) and 1.9L (0.9) respectively, p=0.031. Comparing the 2 groups, TLC (total lung capacity) pre doxycycline was 4.6 L (0.5) in G<sub>1</sub>, and 5.3 L (0.8) in G<sub>2</sub> (p=0.01). After doxycycline, TLC was 4.76 L (0.6) in G<sub>1</sub> and 5.39 L (0.8) in G<sub>2</sub> (p=0.04). Residual volume and TLC ratio (RV/TLC) was 0.37 (0.09) in G<sub>2</sub> and 0.34 (0.08) in G<sub>1</sub> (p=0.34), but after treatment there was evident hyperinflation in patients from G<sub>2</sub> 0.41 (0.12), comparing with G<sub>1</sub> 0.33 (0.08) where RV/TLC reduced (p=0.04); DL<sub>CO</sub> pre doxycycline was 18.5 (5.5) in G<sub>1</sub> and 15.9 (7.6) in G<sub>2</sub> (p=0.33), and post doxycycline 18 (5) in G<sub>1</sub> and 15.1 (7) in G<sub>2</sub> (p=0.25). Distance walked before treatment was higher in G<sub>1</sub> 515 m (66) than in G<sub>2</sub> 432 m (120) (p=0.04); after doxy, distance improved in both groups, 523 m (52) and 473 m (94) respectively (p=0.12). Lung CT score before doxycycline was higher in G<sub>2</sub>, 4 (3-9), comparing with G<sub>1</sub>, 3 (3-5) (p=0.19), increasing mainly in G<sub>2</sub> after doxy. Metalloproteinase blockage was effective in serum MMP-2 and urinary MMP-9 after doxycycline.

**Conclusion:** Based on initial data, doxycycline seems to be an effective medication to treat a subgroup of patients with LAM, probably those with less severe disease.

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**Targeting cytostatic vs. cytotoxic effects on angiomyolipoma cell viability**

Of patients with tuberous sclerosis complex (TSC), 80% develop renal angiomyolipomata and 30% of women develop lymphangiomyomatosis (LAM), a fatal lung disease associated with pulmonary infiltration of spindle-like cells. Recent work has demonstrated that mammalian target of rapamycin complex 1 (mTORC1) inhibition with rapamycin caused angiomyolipoma shrinkage and improvement in LAM. However, angiomyolipomas and pulmonary endpoints trended toward baseline measurements when the drug was discontinued, indicating a greater cytostatic rather than cytotoxic effect.

Endoplasmic reticulum (ER) stress can develop in TSC cells/lesions due to increased protein translation stemming from constitutive mTORC1 activity. We hypothesized that renal angiomyolipoma cells experience ER stress that can be leveraged to result in targeted cytotoxicity. We used a human angiomyolipoma cell line immortalized with HPV E6/7 and telomerase that was stably transfected with empty vector, or the *TSC2* gene (encoding tuberin). Using western blot analyses, we found that the angiomyolipoma cells lacked tuberin and greatly over-expressed phospho-S6 ribosomal protein compared to the tuberin-rescued cell line. In cell viability assays, we found that everolimus (20 nM), an mTORC1 inhibitor, had no effect at 24 hours, but after 72 hours significantly suppressed proliferation of angiomyolipoma cells under serum-free conditions, while tuberin-rescued cells were not affected; supporting a cytostatic effect. Similar studies were performed with the proteasome inhibitor MG-132 (500 nM), which has been shown to induce ER stress. Western blot analyses showed induction of C/EBP-homologous protein (CHOP), a marker of ER stress, by MG-132 in both cell lines. By 8 hours of MG-132 treatment, the viability of angiomyolipoma cells was substantially reduced, while tuberin-rescued cells were much less affected. These results suggest that angiomyolipoma cells may be vulnerable to treatments that exacerbate ER stress. Development of such cytostatic and cytotoxic strategies may provide additional therapeutic opportunities for TSC and LAM patients.

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**Pulmonary Lymphangioliomyomatosis at Ho Chi Minh City, Vietnam: A Case Report**

We report a case of Lymphangioliomyomatosis (LAM) diagnosed in the first time at Pham Ngoc Thach (PNT) hospital, Ho Chi Minh (HCM) city in 2002.

A 31 year-old housewife female patient was admitted to the Lung Department of PNT hospital with a dry cough, left chest pain, left shoulder pain and fatigue in August 2002. She is non smoker and has no children. The past history of this patient included resection of left renal tumor confirmed in histopathology as angiomyolipoma. In 2000, she had been admitted with bilateral pneumothorax and diagnosed as tuberculosis with positive result in PCR for tuberculosis in sputum specimen and she had been treated with anti TB drugs for 8 months. She had been also undergone pleural tube insertion and pleurodesis with Talc in the right pleural cavity.

Chest X ray film revealed a pneumothorax of the left lung. Lung CT scan showed numerous parenchymal thin wall cysts that were distributed in both lungs. These cysts varied from a few millimeters to one centimeter in diameter with a 5 cm bleb in the right lower lung zone and left pneumothorax.

Results of pulmonary function test were abnormal in this patient as FEV1 was significantly lower than the predicted value (FEV1 52% pred), FVC 71% Pred, FEV1/FVC 0,87 (73% Pred).

Arterial blood gas showed the partial pressure of oxygen was 62 mmHg, the partial pressure of carbon dioxide 62 mmHg and the pH 7,407 and the oxygen saturation was 92% while the patient was breathing ambient air. Another blood tests including blood cell count, glucose, creatinine, electrolytes, liver function test were normal.

Histological examination of transbronchially and surgically obtained pulmonary biopsy specimens are essential to confirm the diagnosis.

She was treated with bronchodilators, oxygen therapy, pleural tube insertion...and she was discharged from the hospital with confirmed diagnosis as LAM.

Since 2002, she has been treated with Depo-provera 300 mg every 2 weeks IM and she had to admit the hospital 4 times with pneumothorax and respiratory failure. Chest radiography and CT scan of the chest becomes worse with many cysts in both lungs and pneumothorax. Currently, she feels unwell with cough, shortness of breath, fatigue even at rest and she needs oxygen therapy at home.

In summary, this is the first case with LAM diagnosed at PNT hospital, HCM city, Vietnam. The treatment seems to be unsuccessful and we need a new treatment for this patient.

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**Rapamycin disrupts hamartoma formation induced by TSC2-null skin tumor cells in a xenograft model**

Hamartomas are comprised of cells normally found in an organ but in abnormal numbers and arrangement. The complicated composition of these tumors makes it difficult to identify the inciting cell(s) as well as the cellular targets of therapy. Earlier we showed that skin hamartomas in patients with tuberous sclerosis complex (TSC) contain TSC2-null fibroblast-like cells with increased vessels, macrophages, and keratinocyte proliferation compared to normal skin. Several investigators report that rapamycin decreases the size and redness of TSC skin hamartomas, but it is unclear whether rapamycin eliminates TSC2-null cells in the skin or disrupts interactions between TSC2-null cells and other cells. To study changes induced by TSC2-null cells and the mechanisms of action of rapamycin in the treatment of TSC skin tumors, we administered rapamycin to nude mice grafted with either TSC2-null skin tumor cells or fibroblasts from patient normal-appearing skin. Cells were incorporated into collagen gels overlaid with normal human neonatal keratinocytes, and these constructs were grafted to the backs of nude mice. Mice grafted with TSC tumor cells (n=27) or TSC normal fibroblasts (n=23) received either rapamycin (2 mg/kg) (n=24) or an equal volume of vehicle (n=26) by intraperitoneal injection on alternate days for 12 weeks beginning 5 weeks after grafting. Mice were sacrificed 24 hours after the last injection for analysis of the grafts by immunohistochemistry. Changes in vascularity, number of tumor-associated macrophages, and proliferating keratinocytes were measured by digital analysis of sections stained for CD31, F4/80, and Ki-67, respectively. Grafts containing tumor cells had increased numbers of cells staining for phosphorylated ribosomal protein S6 ( $P<0.0001$ ), vessel size and density ( $P<0.0001$ ), and numbers of macrophages ( $P<0.0001$ ) indicating that TSC2-null cells were sufficient to induce hamartomatous features of TSC skin tumors. Rapamycin decreased tumor cell number in xenografts, as determined by staining with human-specific antibodies against HLA class I or COX IV, but tumor cells persisted throughout treatment. Rapamycin decreased vessel size and density and numbers of macrophages ( $p<0.0001$ ,  $p<0.0001$ , and  $p<0.0001$ , respectively) in tumor grafts but not in normal grafts. Moreover, rapamycin decreased the number of Ki-67-positive epidermal cells in grafts containing TSC tumor cells ( $p=0.047$ ) but not in grafts with TSC normal fibroblasts. In addition to a cytostatic effect on TSC2-null cells, major mechanisms of action for rapamycin may be through anti-angiogenic effects, possibly by decreasing the number of pro-angiogenic macrophages, and by decreasing epidermal proliferation.

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