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## Molecular pathophysiology of lymphatic and vascular anomalies

Vascular malformations are *localized* errors of vascular development. They are often identified on the skin as “birthmarks” of various sizes and shapes. They usually slowly grow with the growth of the child. They may also be encountered in other organs, such as the liver, intestine and the brain. The lesions are consisted of tortuous vascular channels of various types, with continuous endothelium surrounded by various numbers of support cells. Most of these lesions occur sporadically, but also as part of a syndrome or as an inherited disorder.

During the past years, we have identified 1) TIE2/TEK mutations in hereditary mucocutaneous venous malformations (VMCM) (“cavernous hemangioma”), 2) loss-of-function mutations in the VEGFR3 gene, responsible for congenital hereditary lymphedema, 3) KRIT1 mutations in cutaneous capillary-venous malformations associated with cerebral cavernous malformations, 4) glomulin mutations in hereditary glomuvenous malformations (“glomangiomas”), 5) SOX18 mutations in lymphedema-hypotrichosis-telangiectasia, and 6) RASA1 mutations in a newly recognized disorder, which associates atypical hereditary capillary malformations with fast-flow anomalies. We named this entity CM-AVM, for capillary malformation– arteriovenous malformation.

In 1994, when we mapped the 9p21 locus for VMCM, we hypothesized that the variation in size, number and localization of the multifocal lesions may follow Knudson’s double-hit hypothesis for retinoblastoma. Proof for this has started to pile up supporting the idea of paradominant inheritance, the need for a combination of an inherited change with a somatic second-hit in the same gene, i.e. the inherited mutations have only recessive effects at tissular level. Moreover, we have identified local, somatic genetic defects that cause one of the more common sporadic forms of these malformations. This highlights the importance of assessing for tissue-based genetic changes, especially acquired genetic changes, as possible pathophysiological causes, which have been largely overlooked in developmental disorders. Large-scale somatic screens are likely essential in uncovering the nature and prevalence of such changes, and their downstream effects.

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**Stanley G. Rockson, MD, FACP, FACC**  
Stanford University School of Medicine, Stanford

### **Lymphangiogenesis in health and disease**

Substantial advances have accrued over the last decade in the identification of the processes that contribute to lymphatic vascular development in health and disease. Identification of distinct regulatory milestones, from a variety of genetic models, has led to a stepwise chronology of lymphatic development. Several molecular species have been identified as important tissue biomarkers of lymphatic development and function. At present, vascular endothelial growth-factor receptor (VEGFR)-3/VEGF-C/VEGF-D signaling has proven useful in the identification of a broad variety of disease states, including, most notably, the clinically relevant lymphatic metastatic potential and, thus, the assessment of cancer prognosis. Post-natal lymphangiogenesis is increasingly understood to play a vital role in the clinical expression of a broad array of diseases. Therapeutic manipulation of lymphangiogenic potential is likely to play an important role in the control both of those diseases that alter lymphatic function and those that elicit a functional response from the lymphatic system.

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**Eva Sevick-Muracca, PhD**

University of Texas Health Science Center Institute of Molecular Imaging

### **Imaging lymphatic function and dysfunction in humans**

Recently, our group has developed near-infrared (NIR) fluorescence imaging techniques that enable non-invasively imaging of dynamic lymphatic function in normal human subjects as well as patients with lymphatic and lymphovascular dysfunction. Our techniques are also applied to unique mouse models of lymphatic disease and aberrant lymphangiogenesis enabling opportunities to assess molecular causes of dysfunction that may be responsible for human disease. Herein we provide a summary of first Phase I trial of 44 human subjects and describe common lymph imaging phenotypes observed in the mouse models investigated thus far.

Thomas W. Glover, PhD  
University of Michigan

**Lymphedema-distichiasis (LD) syndrome and the hereditary lymphedemas**

The lymphatic system is an essential component of the vascular system and plays a significant role in human health. Disruption of the lymphatic vasculature leads to lymphedema, a chronic and often debilitating condition characterized by edema of affected tissues, most often the arms and legs, resulting from impaired drainage of lymph fluids and tissue damage. Acquired lymphedema resulting from parasitic filarial infection, surgery, trauma, or other infections is a major global health problem that affects millions of people world-wide. Primary or congenital lymphedema resulting from developmental abnormalities of the lymphatic system is associated with a number of genetic disorders including Turner syndrome, Down syndrome, Noonan syndrome and a group of disorders referred to as the hereditary lymphedemas that are inherited in a Mendelian fashion.

The genes responsible for hereditary lymphedema are of great interest to the field due to the potential for providing insights into mechanisms of lymphatic development and the diagnosis, prevention and treatment of both primary lymphedema and secondary lymphatic disorders and cancer. Despite this, only three genes, *FOXC2* (lymphedema distichiasis), *VEGFR3* (Milroy disease) and *SOX18* (hypotrichosis-lymphedema-telangiectasia syndrome) have been identified that, when mutated, lead to hereditary lymphedema. I will review the mechanism by which mutation leads to these disorders, with emphasis on the *FOXC2* gene in hereditary lymphedema distichiasis (LD syndrome). LD syndrome is characterized by lymphedema with variable age of onset and is caused by mutations that inactivate one copy of the *FOXC2* transcription factor. Virtually all LD patients also have congenital distichiasis, or “double rows” of extra eyelashes. Additional clinical manifestations are seen in ~20% of affected individuals and include hydrops fetalis, cystic hygroma, cleft palate, ptosis, congenital heart defects, varicose veins, and occasional kidney and skeletal defects. Our work and that of others has implicated *FOXC2* not only in LD syndrome, but as a key gene in lymphatic and blood vascular development and a picture of how it functions in these processes is beginning to emerge.

**Takuo Hayashi, MD**  
Juntendo University School of Medicine

## Female reproductive system as a target of lymphatic spread of LAM

### Introduction/Rationale

Lymphangi leiomyomatosis (LAM) is a rare benign neoplasm in women of child-bearing age associated with or without tuberous sclerosis complex. An enigma in regard to this disease is how LAM cells metastasize to lungs and lymph nodes, although they are benign neoplastic cells. We previously proposed the hypothesis of the dissemination of the LAM cell cluster (LCC), a unique fragmented cell cluster of LAM covered by monolayer of lymphatic endothelial cells, in the lymph-thoracic-venous system. This hypothesis may explain the mechanism of LAM cells spreading to the lungs and lymph nodes. Although several articles indicated uterine LAM in the patients with pulmonary LAM associated with TSC and these indicate that LAM cells may also be able to disseminate to the genital organs as well as the lungs and lymph nodes, little has been studied regarding the histopathology of the genital organs of patients with LAM. In the current study, to elucidate whether LAM is found in the genital organs of patients with pulmonary LAM, a histopathological analysis in seven patients with pulmonary LAM was performed.

### Methods

The genital organs and retroperitoneal lymph nodes (RLNs) in seven cases, including four surgical and six autopsy materials, were analyzed. Immunohistochemistry for HMB45, alpha-smooth muscle actin (alpha-SMA), CD10, and VEGFR-3 was performed.

### Results

The results showed that six of seven cases had LAM lesions in the genital organs (85.7%). The uterus or adnexal regions had an average of 5.0 or 3.6 lesions and their lesions had an average size of 13mm or 2.8mm, respectively. The size of those lesions was smaller than that of RLNs affected by LAM. Many of the LAM lesions with the dilated lymphatic vessels containing LAM cell clusters (LCCs) were mainly located at the subserosal area of the organs. Uterine LAM showed two histologic types: nodular LAM having usual immunophenotype (HMB45+,  $\alpha$ -SMA+, CD10-); diffuse LAM including a subset of LAM cells having unusual immunophenotype (HMB45+,  $\alpha$ -SMA-, and CD10+).

### Conclusions

LAM was frequently found in the genital organs of the patients with pulmonary LAM. These findings suggest that the spreading mechanism of the genital organs may occur due to the migration of LCC from the axial lymphatics.

Caroline A. Heckman, PhD  
University of Helsinki

## Lymphangiogenic potential of cells isolated from chylous pleural effusions of LAM patients

Lymphangiomyomatosis affects the lungs and axial lymphatics, and is often complicated by chylous effusions including chylothorax and chylous ascites. Recent investigations have shown that LAM cells may invade the lymphatic system as LAM cell clusters (LCC), which consist of a cluster of LAM cells enveloped by lymphatic endothelial cells (LECs) (Kumasaka et al, 2005). For these studies, cells were collected from pleural effusions of LAM patients suffering from chylothorax. Cultured cells were largely positive for smooth muscle cell markers including alpha-smooth muscle cell actin ( $\alpha$ -SMA) and desmin, while only a few cells were positive for lymphatic endothelial cell markers such as vascular endothelial growth factor 3 (VEGFR-3), LYVE-1 and podoplanin. Conditioned medium from the cultured cells stimulated the growth of BaF3 cells engineered to be dependent on the activity of the primary lymphangiogenic inducer VEGFR-3, indicating that the pleural effusion cell isolates expressed VEGFR-3 ligands such as VEGF-C and -D. To assay for lymphangiogenic potential *in vivo*, cells were intradermally injected into the ears of severe combined immunodeficient (SCID) female mice, which were humanely sacrificed 12 days later. The ears were dissected and prepared for whole mount staining. Analysis of the lymphatic and blood vasculatures of the injected ears showed areas of lymphatic vessel sprouting along with large dilated lymphatic vessels, while the blood vasculature was largely unchanged. For additional *in vivo* analysis, cells were labeled using a retrovirus expressing a fusion protein composed of renilla luciferase and the green fluorescent protein (GFP). These were intraperitoneally injected into the lower abdomen of female SCID mice. Once a week for 10 weeks the mice were anesthetized and injected with the renilla luciferase substrate coelenterazine then imaged with an IVIS imaging station. Imaging for luciferase activity indicated the continued growth of the injected cells primarily in the abdominal cavity. By week 10, the overall health of the mice was severely diminished and the mice were sacrificed. Whole mount staining of the abdominal mesentery revealed clusters of cells in the mesenteric lymph nodes further supporting the ability of these cells to invade the lymphatic system. These results not only confirm by experimental means the lymphangiogenic potential of LAM cells, but also show that cells isolated from the chylous effusions of LAM patients are useful tools for modeling the disease both *in vitro* and *in vivo*.

Lisa R. Young, MD  
University of Cincinnati

## Serum VEGF-D prospectively discriminates 'Lone' Lymphangioliomyomatosis from other causes of cystic lung disease

### Background

The diagnosis of sporadic Lymphangioliomyomatosis (S-LAM) can be made on clinical and radiographic grounds if typical cystic lung disease is associated with chyloous complications or angiomyolipomas (AMLs). However, for the majority of women with S-LAM without AMLs or chyloous disease (i.e. "lone S-LAM,") lung biopsy is often required to distinguish LAM from other mimics. We and others have previously reported that S-LAM patients have elevated serum levels of Vascular Endothelial Growth Factor-D (VEGF-D), a lymphangiogenic growth factor.

### Objectives

Our objectives were to determine (1) if serum VEGF-D levels are elevated in patients with S-LAM in the absence of AMLs or chyloous disease (lone S-LAM), (2) whether VEGF-D could be used prospectively to discriminate between LAM and other etiologies of cystic lung disease presenting as diagnostic unknowns, (3) whether VEGF-D levels are stable over time, and (4) if VEGF-D would predict the existence of LAM in women with TSC.

### Methods

We evaluated serum VEGF-D levels by ELISA in 41 women with definite S-LAM, defined either by lung biopsy or typical cystic lung disease in the presence of an AML or proven chyloous disease. Results were compared to VEGF-D levels from 19 women with other LAM mimics, including pulmonary Langerhans cell histiocytosis, emphysema, Sjogrens, or Birt-Hogg-Dube. Samples were also obtained from women with TSC-LAM and TSC with normal chest CT scans. Prospective VEGF-D testing was performed in 33 women presenting for clinical evaluation. Serial VEGF-D levels were also obtained in 4 women on six occasions over eight weeks.

### Results

The mean serum VEGF-D level was elevated in S-LAM patients without AMLs and not significantly different from S-LAM patients with AMLs (1525±268pg/mL vs. 2174±700pg/mL, p=.33). For "lone S-LAM," the mean serum VEGF-D level was significantly greater than for cystic lung disease controls (1023±176pg/mL, n=13 vs. 268±23pg/mL, n=19, respectively, p<0.0001). Receiver operating characteristic (ROC) curves demonstrated that VEGF-D effectively discriminated "lone S-LAM" from cystic lung disease controls, with an area under the curve of 0.935 (95%CI 0.84-1.0). At a cut-off VEGF-D value of 485pg/mL, test sensitivity for "lone S-LAM" was 85% and specificity 100%. For the prospective testing group, six individuals were later diagnosed with S-LAM, 13 were diagnosed with other diseases, and 14 have not pursued definitive diagnostic evaluations and remain undiagnosed. Five of the six patients who proved to have S-LAM (by biopsy) had VEGF-D levels of 500pg/mL or greater; four had a level above 800pg/mL. None of the individuals confirmed to have other cystic lung diseases (based on biopsy, genetic, or serologic testing) had VEGF-D levels above 500pg/mL. Based on inter-assay variability and serum VEGF-D levels from healthy female volunteers (mean 355±29pg/mL, 95%CI 296-415pg/mL, range 150-689pg/mL, n=25), we have proposed a conservative threshold of 800pg/mL for "diagnostic certainty" of S-LAM. Intra-subject variation in VEGF-D levels over time is an important consideration if VEGF-D is to be used as a biomarker for trials. Serial VEGF-D levels fluctuated from 90-108%, 80-124%, 73-131%, and 90-113% in four individual subjects who provided 6 blood samples over a 6-8 week time period. Finally, VEGF-D has potential utility as a screening tool for development of LAM in women with TSC. Serum VEGF-D levels were significantly

elevated in women with TSC-LAM in comparison to women with TSC only (6096±1251pg/mL, n=20 vs. 409±48pg/mL, n=15, p<0.0001).

### Conclusions

A VEGF-D level of 800pg/ml in the setting of a compatible presentation and HRCT is diagnostic for LAM. The prospective evaluation of cystic lung disease patients supports the clinical utility of VEGF-D as a diagnostic test. Serum VEGF-D appears to be relatively stable in individual subjects over a two month period and effectively identifies LAM in women with TSC.

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## Yoshikazu Inoue, MD, PhD

National Hospital Organization Kinki-Chuo Chest Medical Center

### Serum lymphangiogenic/angiogenic factors in lymphangioleiomyomatosis, as diagnostic biomarkers

#### Background

Lymphangioleiomyomatosis (LAM) is a progressive systemic disease in reproductive women primarily, which is characterized by proliferation of LAM cells, causing multiple pulmonary cysts, angiomyolipomas, lymphadenopathy, etc. Recent reports suggested lymphangiogenic/angiogenic factors are involved in pathogenesis of LAM, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factors (bFGF), and angiotensin-converting enzyme activity (ACE) (Seyama *K Lymphat Res Biol.* 2006, Inoue Y *AJRCCM* 2002, Valencia JC *AJRCCM* 2006). We recently reported that serum VEGF-D measurement had diagnostic potential for LAM (Young LR *N Engl J Med.* 2008), so we hypothesized that other serum lymphangiogenic/angiogenic factors might be diagnostic and useful biomarkers.

#### Subjects and methods

To clarify the hypothesis, we measured VEGF-D, VEGF-A, bFGF, and ACE activity in sera from Japanese patients with LAM (n=55, all female, age: 40±10 years old), (n=16, all female, age: 56±21 years old) including Sjogren's syndrome, Langerhans cell histiocytosis (LCH), interstitial pneumonias, and lung cancer, etc) and healthy control (n=26, all female, age: 49±6 years old).

#### Results & Discussion

Serum lymphangiogenic/angiogenic factors elevated in LAM, especially VEGF-D levels were significantly elevated in LAM compared in healthy control or other lung diseases (1191.96 pg/ml (293.71-9765.31), and 309.11 pg/ml (225.01-643.41) (IQR), p<0.0001). Serum VEGF-D was the best diagnostic serum biomarker compared with serum VEGF-A, bFGF, or ACE activity by ROC curves. Using cut-off values of serum VEGF-D as 500, 700, and 800 pg/ml, the sensitivity of the diagnosis of LAM were 86.96, 93.02 and 100 %, respectively. We concluded that measurement of serum VEGF-D is useful for diagnosis of LAM, and the cut-off level of VEGF-D 800 pg/ml would be appropriate level for the diagnosis of LAM.

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**Rishi Kulkarni**  
University of Cincinnati

## NFATc1 Regulates Lymphatic Endothelial Development

The lymphatic vasculature supports interstitial fluid balance, a function critical for efficient gas exchange in the lung. Lymphatic malformations are the most common form of congenital vascular defects. During embryogenesis lymphatic endothelial cells (LEC) sprout from the cardinal vein blood endothelial cells (BEC), a process requiring transcription factor Prox-1. However, the molecular mechanisms of lymphatic endothelial cell lineage selection and patterning are poorly understood. Microarray analysis comparing BEC and LEC isolated by flow from human microvascular endothelial cells from lung (HMVEC-L) identified transcription factors differentially expressed by LEC, including Prox-1 and NFATc1. NFATc1 is critical for cell lineage selection in cardiac valve morphogenesis and osteoclastogenesis. We found NFATc1 was colocalized with LEC markers Prox-1, VEGFR-3 and podoplanin on cardinal vein of mouse as LEC are specified and with these markers as LEC segregate into lymph sacs and on embryonic as well as adult lymphatics of lung and dermis, but not on blood vessels. In *NFATc1* null mice, Prox-1, VEGFR-3 and podoplanin positive endothelial cells sprouted from the cardinal vein at E11.5, but failed to coalesce into lymph sacs. NFATc1 nuclear translocation and activation requires the phosphatase calcineurin. Embryos treated *in utero* with calcineurin inhibitor cyclosporine-A, had cytoplasmic NFATc1 and diminished podoplanin expression on the lymphatics. *In vivo*, injury induced by lung-specific VEGF-A overexpression induces lymphangiogenesis (Mallory et al, *Microvascular Research*, 2006) and NFATc1 was expressed by the VEGF-A-induced lymphatics. However, mice lacking the regulatory subunit of calcineurin A, with diminished nuclear NFAT, failed to respond to VEGF-A with increased lymphangiogenesis. *In vitro*, VEGFR-3 and podoplanin expression by HMVEC-L was reduced by siRNA to NFATc1. This reduction was comparable to that seen with siRNA to Prox-1. In reporter assays, NFATc1 activated lymphatic specific gene promoters for FGFR3 and podoplanin. These results demonstrate a role for the calcineurin-NFAT pathway in lymphangiogenesis and suggest that NFATc1 is the principle NFAT involved.

**Aristotelis Astrinidis, PhD**  
Drexel University College of Medicine

**Inhibition of polo-like kinase 1 (PLK1) decreases the survival of TSC1 and TSC2 null cells**

Pulmonary Lymphangiomyomatosis (LAM) and Tuberous Sclerosis Complex (TSC) are caused by loss-of-function mutations in the *TSC1* and *TSC2* tumor suppressors, encoding hamartin and tuberlin respectively. The hamartin/tuberlin complex negatively regulates the rapamycin-sensitive mTOR/raptor complex (mTORC1), a kinase involved in translation, autophagy, cell cycle and hypoxia. LAM/TSC-derived cells and tumors have mTORC1 hyper-activation, suggesting that mTORC1 is a potential target for therapeutic intervention. Indeed, rapamycin (and rapalogues) are currently under clinical trials for LAM/TSC.

Previously we reported that hamartin interacts with PLK1, a kinase regulating multiple facets of cell division, and that PLK1 expression is increased in cells lacking hamartin or tuberlin. Conversely, PLK1 expression is decreased by reintroduction of hamartin in *Tsc1*<sup>-/-</sup> MEFs. Additionally, we found that PLK1 expression is increased in LAM-derived lung lesions. PLK1 over-expression has been reported in various cancer types, and PLK1 inhibition sensitizes cancer cells to apoptosis. We hypothesize that PLK1 over-expression increases the survival of hamartin and tuberlin null cells, and we predict that PLK1 inhibition sensitizes preferentially the hamartin and tuberlin null cells to apoptosis.

*Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup>/*Tp53*<sup>-/-</sup> MEFs (and controls), and isogenic *Tsc1*<sup>-/-</sup> MEFs retrovirally transduced with hTSC1 (208-T3) or vector (208-P2), were treated with PLK1 inhibitors (GlaxoSmithKline Compound 1, C1, or Boehringer Ingelheim BI-2536), and assayed for viability. The EC<sub>50</sub> values for both drugs were within the published range (5μM for C1 and 10nM for BI2536). The viability of hamartin and tuberlin deficient cells (*Tsc1*<sup>-/-</sup>, *Tsc2*<sup>-/-</sup>/*Tp53*<sup>-/-</sup>, and 208-P2) treated with 10-30μM C1 or 10-30nM BI-2536 for two and four days was significantly decreased, compared to controls (*Tsc1*<sup>+/-</sup>, *Tsc2*<sup>+/-</sup>/*Tp53*<sup>-/-</sup>, and 208-T3). Hamartin and tuberlin null cells treated with PLK1 inhibitors in combination with 0.2 or 2nM rapamycin had significantly lower viability, compared to cells treated with either of the compounds alone.

To determine whether the survival of hamartin and tuberlin deficient cells is decreased by PLK1 inhibition, cells were treated for three days with C1 or BI-2536 alone or in combination with 2nM rapamycin, 300 living cells were plated in 100mm dishes, and allowed to form colonies over 7 days. Inhibition of PLK1 significantly decreased the survival of hamartin or tuberlin null cells, with minimal reduction in the survival of control cells. Dual inhibition of PLK1 and mTORC1 further decreased the survival of hamartin and tuberlin deficient cells.

These data suggest that PLK1 inhibition preferentially decreases the viability and survival of hamartin and tuberlin null cells, compared to control cells, and that PLK1 inhibitors may act synergistically with rapamycin, and provide a first insight for the role of PLK1 in regulating survival mechanisms related to LAM/TSC pathogenesis. We are currently investigating the signaling events downstream of PLK1 inhibition in hamartin and tuberlin null cells.

**Stephen R. Hammes, MD, PhD**  
University of Rochester School of Medicine

### **Steroid production and signaling in women: Everything in moderation**

The classic approach toward explaining sexual dimorphisms in normal physiology or pathologic disease is to incriminate estrogens and androgens as the regulators in women and men, respectively. In fact, normal physiologic levels of estrogens are important for many biological functions in men, including fertility, bone development, and possibly neuronal development and function. Likewise, physiologic levels androgens are important for normal ovarian development, bone formation, and other processes in women. Where problems arise is when the ratio of androgens to estrogen is markedly altered. A classic example is polycystic ovarian syndrome (PCOS), where androgen levels or signaling increase in women, leading to virilization and problems with fertility. In fact, nearly 10% of young women in the United States have PCOS, making it the leading cause of infertility. In addition, PCOS is often associated with obesity and insulin resistance; thus, as the obesity epidemic continues to rage, the incidence of PCOS is on the rise as well. Here we will discuss the mechanisms regulating androgen production and PCOS, focusing on novel potential treatment options. We will then use this background to discuss how both estrogens and androgens may be capable of regulating the progression of LAM.

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**Ken Inoki, MD, PhD**  
Life Sciences Institute

### **Role of TSC-mTORC1 pathway for podocyte injury in the development of diabetic nephropathy**

Diabetic nephropathy (DN) is among the most lethal complications that occur in patients with both type 1 and type 2 diabetes. It is characterized as a major glomerulopathy that develops to glomerulosclerosis, leading ultimately to end-stage renal disease (ESRD). Despite considerable attention from both clinicians and basic scientists, the prevalence of ESRD in diabetic patients is increasing dramatically. Thus, understanding the pathogenesis of DN is crucial to developing new approaches for its prevention and treatment. Recent investigations have revealed that injuries to podocytes play a critical role in the development of diabetic nephropathy. These highly differentiated glomerular epithelial cells and their foot processes comprise the slit diaphragm, a barrier for repelling serum proteins on the surface of glomerular capillaries. Podocyte injury may produce microalbuminuria, an early feature of DN. The molecular mechanisms by which diabetes causes podocyte injury remain unclear. Furthermore, whether podocyte injury is a cause or a consequence of DN also continues to be uncertain. The TSC-mTORC1 pathway is an evolutionarily conserved signaling pathway that regulates growth and survival. This pathway responds to nutrients such as glucose and growth factors, and in turn controls a wide array of cellular processes such as translation, transcription, and autophagy. We have shown that activation of the mTORC1 pathway plays a critical role in diabetes-dependent podocyte injury. Our studies indicate that all pathological alterations present in a mouse model of DN, including podocyte morphological changes, glomerular basement membrane (GBM) thickening, proteinuria, glomerular hypertrophy, and mesangial expansion, can be prevented by treatment with rapamycin, a specific mTOR inhibitor. Moreover, podocyte-specific mTORC1 activation by TSC1 knockout in a non-diabetic mouse recapitulated

podocyte injury and other features of DN in a rapamycin-sensitive manner. These observations indicate a critical role for the site-specific activation of mTORC1 in podocytes during the development of DN, and the attenuation of the mTORC1 pathway in podocytes may be potential approach to the treatment of this debilitating disease.

**Thomas N. Darling, MD, PhD**  
Uniformed Services University

### **Tumor microenvironment in tuberous sclerosis complex**

TSC skin tumors are hamartomas containing increased numbers of fibroblast-like cells, endothelial cells, macrophages, and epithelial cells. The “two-hit” cells in TSC skin tumors are fibroblast-like cells residing in the tumor stroma; we have grown clonal populations of TSC2-null fibroblast-like cells from TSC skin tumors that show biallelic mutations in TSC2 and constitutive activation of mTORC1. No evidence for second-hit mutations were obtained in neighboring cells, suggesting that fibroblast-like cells induce changes in the other cell populations through the release of paracrine factors. Loss of TSC2 function in TSC skin tumor cells is associated with overexpression of MCP-1 and epiregulin. MCP-1 appears to play important roles in TSC tumorigenesis by stimulating macrophage recruitment and angiogenesis. Epiregulin is an EGF family member that acts as a mitogen for multiple cell types. To determine whether TSC tumor cells modify the cellular microenvironment, we developed a xenograft model for TSC skin tumors. TSC2-null skin tumor cells (or control patient fibroblasts from normal-appearing skin) were incorporated into collagen gels and overlaid with normal human keratinocytes, and then grafted onto immunodeficient mice. At 16 weeks after grafting, the histological appearance mimics that of TSC skin tumors. Samples stained with a pan-human HLA class I monoclonal antibody show human cells in the graft epidermis and dermis, and analysis of DNA extracted from laser-microdissected dermis of tumor cells confirms the presence of the second-hit mutation. Grafts containing TSC tumor cells replicate features of TSC skin tumors, including increased phospho-S6 positive cells, CD31 positive vessels, and numbers of tumor-associated macrophages, as compared to grafts made with patient normal fibroblasts. Since the macrophages and vessels are mouse derived, these results suggest that TSC2-null cells orchestrate the changes in the tumor microenvironment without requiring haploinsufficiency of the surrounding cells.

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**Stephen Michnick PhD, FRSC**  
Université de Montreal

### **Cross-talk, integration and specificity in cell fate-determining signal transduction networks**

Systematic analyses of protein interaction networks in human cells and yeast reveals unforeseen mechanisms by which signaling networks may regulate feedback and feed-forward control of for example GPCR, TOR and Akt signaling in the cytosol and the nucleus. For example, genome-wide in vivo studies of protein complexes modulated the TOR inhibitor rapamycin suggest that TOR

controls a broad range of cellular processes including cell polarization and epigenetic control of gene expression. This and other examples will be discussed to illustrate how the systematic analysis of dynamics protein complexes can reveal details of signaling regulatory networks never seen before.

David Kwiatkowski, MD  
Brigham & Women's Hospital

Atorvastatin therapy in *Tsc* mouse models; MMPs in a LAM cell line; and a novel hypomorphic allele of *Tsc2*

Three research findings are presented briefly, two of which are described in greater detail in abstracts by G. Finlay and P. Lee.

We performed two trials of long-term atorvastatin as prevention of tumors occurring in *Tsc2*<sup>+/-</sup> mice. ENU-treated 129/B6 *Tsc2*<sup>+/-</sup> mice develop renal tumors, and were given atorvastatin chow (wt/wt 0.1%) for 1 or 3 months, prior to sacrifice at 6 mo; 129Sv/Jae *Tsc2*<sup>+/-</sup> mice develop liver hemangioma, and were given atorvastatin for 6 months prior to sacrifice at 12 mo. In both cohorts, serum cholesterol levels and levels of phosphorylated S6 (pS6) and GTP-RhoA in healthy tissue were significantly (> 50%) reduced in atorvastatin treated mice as compared to controls. However, no significant reduction in tumor size, morphology or pS6 levels was observed for in either the renal cystadenoma or the liver hemangioma, as compared to the untreated groups. These observations indicate that atorvastatin was effective as an HMG-CoA reductase inhibitor, but not did inhibit the growth of tumors that develop in these *Tsc2*<sup>+/-</sup> models, suggesting that it is unlikely to have benefit as a single agent therapy for TSC-associated tumors.

Increased matrix metalloproteinase (MMP) activity has been implicated in the pathogenesis of lymphangioliomyomatosis (LAM). To examine this in greater detail, we studied immortalized cells that lack TSC2 derived from an angiomyolipoma (AML) of a LAM patient, and a TSC2 addback derivative; as well as MEF lines lacking *Tsc1* or *Tsc2*. We found increased MMP-2 secretion in cells lacking TSC1/TSC2 compared to their respective controls by zymography, which was not affected by rapamycin treatment. Expression profiling confirmed increased MMP-2 gene expression that was not affected by rapamycin. Furthermore, multiple other genes were found to be over-expressed in rapamycin-treated TSC2-deficient cells compared to TSC2<sup>+</sup> cells.

We have generated a novel hypomorphic allele of *Tsc2* (del3), in which exon 3, encoding 37 amino acids near the N-terminus of tuberlin, is deleted. Embryos homozygous for the del3 allele survive until E13.5, two days longer than *Tsc2* null embryos. Embryos die from underdevelopment of the liver, deficient hematopoiesis, aberrant vascular development, and hemorrhage. Mice that are heterozygous for the del3 allele have a markedly reduced kidney tumor burden in comparison to conventional *Tsc2*<sup>+/-</sup> mice. Murine embryo fibroblast (MEF) cultures that are homozygous for the del3 allele express mutant tuberlin, and show enhanced activation of mTORC1, similar to but less pronounced than *Tsc2* null MEFs. Furthermore, the mutant cells show prominent reduction in the activation of AKT. Similar findings were made in the analysis of homozygous del3 embryo lysates. *Tsc2*-del3 demonstrates reduced but not absent GAP activity in a functional assay. The findings indicate that the del3 allele is a hypomorphic allele of *Tsc2* with partial function, and highlight the consistency of AKT downregulation when *Tsc1*/*Tsc2* function is impaired. *Tsc2*-del3 mice also serve as a model for hypomorphic TSC2 missense mutations reported in TSC patients.

Raymond Yeung, MD  
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## The Role of TSC2 in EMT

The mechanism by which the TSC2/mTOR pathway contributes to the infiltration of smooth muscle actin (SMA)-expressing cells in the lung, and the reason why this process is restricted to females in their childbearing age are two of the most intriguing questions in the study of LAM. Accumulating circumstantial evidence suggests a 'metastatic' model for LAM pathogenesis in which the TSC-mutant cells travel in the circulation and eventually populate the lungs in a unique growth pattern that involves the lymphatic vessels. This led us to speculate that the TSC/mTOR pathway may be directly involved in the process of metastasis. Here, we present evidence that certain 'epithelial' cells can undergo epithelial-mesenchymal transition (EMT) upon loss of TSC2 leading to cell detachment and invasion. However, once detached, these cells are prone to anoikis, and estrogen promotes resistance to anoikis, thus allow the cells to reach their destination and to proliferate in the lung. Together, we propose a 'two-hit' model of LAM in which the loss of TSC2 predisposes cells to detach and undergo EMT (i.e. 1<sup>st</sup> hit). The presence of sex-steroid hormones further enhances cell survival of the detached cells (i.e., 2<sup>nd</sup> hit) resulting in 'metastasis' with a strong female predilection.

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## Amino acid-induced mTOR complex1 signaling is regulated by Ca<sup>2+</sup>/Calmodulin through hVps34

Abnormalities in amino acid (AA) metabolism or their excess levels are thought to play causative roles in specific human pathologies, including obesity, diabetes, and cancer. Importantly, this set of diseases has been implicated in a recently recognized pathogenic link, potentially mediated by increased mammalian Target Of Rapamycin (mTOR) Complex1 signaling. mTOR Complex1 is made up of mTOR and three associated proteins; regulatory associated protein of mTOR (raptor), the G protein beta-subunit-like protein (GβL), and the proline-rich Akt/PKB substrate 40 kDa (PRAS40). Unlike growth factors and hormones, which use the canonical insulin signaling cascade triggered by class I phosphatidylinositol 3 kinase (PI3K) to mediate mTOR Complex1 signaling, nutrients, such as AAs, appear to mediate this response through a class 3 PI3K, human vacuolar protein sorting 34 (hVps34). However, the mechanism by which AAs mediate this response is unknown. Here we show that AAs induce a Ca<sup>2+</sup> influx that triggers mTOR Complex1 signaling, whereas blocking the rise in intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) ablates this response. Next, pull-down studies in combination with pharmacological antagonists and small-interfering RNAs (siRNAs) are used to demonstrate that the effects of Ca<sup>2+</sup> are mediated through calmodulin (CaM). Finally, in situ cross linking, far-western blotting, mutagenesis, and biochemical studies demonstrate that the rise in [Ca<sup>2+</sup>]<sub>i</sub> increases the direct binding of Ca<sup>2+</sup>/CaM to an evolutionarily conserved motif in hVps34, which is required for lipid kinase activity and increased mTOR Complex1 signaling. Thus, the link between AAs and Ca<sup>2+</sup>/CaM-regulating hVps34 activity unravels an integral mechanism of AA/hVps34-mediated mTORC1 signaling. Moreover these findings have important implications for elucidating the basic signaling mechanisms linking metabolic disorders with cancer progression.

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*Tsc2*<sup>+/-</sup> mice develop tumors in multiple sites due to misexpression of developmental genes *Hmga2* and *Imp2*

**Introduction/Rationale**

Lymphangiomyomatosis (LAM) is a rare, progressive lung disease that primarily occurs in women of childbearing age. It is caused both spontaneously and by mutations in either the tuberous sclerosis complex genes, *TSC1* or the *TSC2*, both classical tumor suppressor genes. Loss of *TSC2* in Eker Rats leads to misexpression of high mobility group A2 (*Hmga2*). *HMGA* genes are primarily expressed during embryonal development and silenced in adult tissues. Our research group has previously demonstrated that *HMGA2* is misexpressed in pulmonary LAM. Although the recent literature suggests that *HMGA2* is critical in the development and progression of malignant tumors, the exact role of *HMGA2* expression on tumor frequency is unknown. Here we examine *Tsc2*<sup>+/-</sup> mice, analyzing for *Hmga2* gene expression and tumor frequency.

**Methods used**

*Tsc2*<sup>+/-</sup> mice were bred on to the *Hmga2*<sup>-/-</sup> genetic background (both 129Sv/J background) follow simple Mendelian rules since *Hmga2* and *Tsc2* are on separate mouse chromosomes, chromosomes 10 and 17, respectively. *Tsc2*<sup>+/-</sup>, *Hmga2*<sup>+/-</sup> mice were used as positive controls. Mice were monitored for tumors and the tumors accessed for size and grade. *Hmga2* expression was examined in the tumors, by RT-PCR and immunohistochemistry, along with important *Hmga2* induced genes such as the Insulin-like growth factor II mRNA-binding protein 2 (*Imp2*).

**Results**

The *Tsc2*<sup>+/-</sup> mice in this study suffered from tumors/cysts in kidneys, liver, lungs, foot and eye. *Hmga2* expression was only observed in tumor tissue and was absent from normal tissue in these mice. *Tsc2*<sup>+/-</sup>, *Hmga2*<sup>-/-</sup> did not develop tumors in the liver, lung, eyes, foot and had significant reduction in the frequency of renal tumors.

**Conclusions**

This data shows that *TSC2* activation is important for the repression of *HMGA2* and *IMP2* and suggests *HMGA2* has a major role in *TSC2* associated disease progression.