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Loss of the TSC Tumor Suppressors Reprograms Cellular Metabolism to Promote Proliferation

Aberrant regulation of the mammalian target of rapamycin complex 1 (mTORC1) is a common molecular event in a large variety of pathological settings, including genetic tumor syndromes and cancer, obesity and type-2 diabetes, and both childhood and aging-related neurological disorders. Many of the genetic and environmental factors contributing to these diseases affect a signaling network that converges on the TSC1-TSC2 complex. However, the downstream consequences of mTORC1 activation remain poorly defined. Here, we take advantage of genetic settings with loss of the TSC1-TSC2 complex to constitutively activate mTORC1 signaling in a manner that isolates it from upstream pathways. Through a combination of unbiased genomic, metabolomic, and bioinformatic approaches, we demonstrate that mTORC1 activation is sufficient to stimulate specific metabolic pathways, including glycolysis, the pentose phosphate pathway, and *de novo* lipid biosynthesis. This is achieved through the activation of a transcriptional program affecting metabolic gene targets of hypoxia-inducible factor 1 (HIF1) and sterol regulatory element-binding protein 1 and 2 (SREBP1 and SREBP2). While HIF1 stimulates glycolysis, SREBP1 and 2 induce both the pentose phosphate pathway and lipid biosynthesis and are required for the growth factor-independent proliferation of TSC gene-deficient cells downstream of mTORC1. Therefore, we demonstrate that, in addition to promoting protein synthesis, mTORC1 activation is sufficient to promote specific bioenergetic and anabolic processes that are likely to contribute to the pathophysiological properties of a diverse array of diseases.

Channing J. Der, PhD
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Role of Rheb in TCS pathogenesis and as a therapeutic target

Tuberous Sclerosis Complex (TSC) is caused by mutation of either the TSC1 or TSC2 gene and is associated with lymphangioliomyomatosis (LAM). Loss of TSC function leads to chronic activation of the Ras-related Rheb small GTPase. Chronic Rheb activation in turn causes persistent activation of mTOR. Aberrant TSC-Rheb-mTOR signaling in LAM suggests possible therapeutic approaches. These include inhibition of Rheb or mTOR function. Our studies have focused on inhibition of Rheb function, in particular, by preventing Rheb membrane association and signaling to activate mTOR. This may be achieved through inhibition of the farnesyltransferase enzyme that catalyses the lipid modification of Rheb essential for its function. A second approach involves utilization of farnesyl-containing small molecules that may prevent proper Rheb membrane interactions and subcellular localization (e.g., salirasib). Our studies reveal the unexpected complications that arise in target-based drug discovery. For example, we find that salirasib can block mTOR signaling independent of its ability to block the function of farnesylated Rheb function. Finally, we draw parallels between the efforts to develop pharmacologic approaches to target the related Ras oncoprotein and Rheb. What has seemed like a logical and straightforward approach, to target Ras downstream signaling, has revealed complicating issues that may also arise as inhibitors of mTOR are being evaluated for therapeutic treatment of LAM.

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Mammalian Target of Rapamycin Controls the Nuclear Trafficking of Signal Transducer and Activator of Transcription-1 (STAT1)

In patients with lymphangioliomyomatosis (LAM), mutations in the *TSC2* gene cause enhanced activity of ‘mammalian target of rapamycin’ (mTOR), and the growth of hamartomatous tumors. mTOR regulates cell growth by controlling the phosphorylation of downstream effectors involved in protein synthesis, cell proliferation, and cytokinesis. Our recent studies reveal that mTOR controls the nuclear localization of the transcription factor STAT1, a key regulator of genes involved in cell death and cell cycle arrest. mTOR suppresses constitutive STAT1 nuclear content via its associated phosphatases $\Pi 4$ and protein phosphatase 2A. Inactivation of mTOR with rapamycin promotes its interaction with STAT1, and the STAT1 nuclear importin ‘karyopherin alpha’ (KPNA1). The resulting increase in STAT1 nuclear content enhances the induction of STAT1-dependent apoptosis genes. Our results suggest that mTOR might lower the sensitivity of cells to pro-apoptotic stimuli in part by suppressing the transcription of genes involved in cell death. Studies investigating the biochemical mechanism by which mTOR regulates nuclear trafficking represent an additional strategy by which to promote the death of LAM cells.

Marilyn Glassberg, MD
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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 μ 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 μ 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.

Elena Goncharova, PhD
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Combination of rapamycin and simvastatin abrogates TSC2-null tumor growth and prevents tumor recurrence

In lymphangioleiomyomatosis (LAM), mutational inactivation of *TSC1/TSC2* leads to constitutive activation of mTORC1 and abnormal cell proliferation. mTORC1 inhibitor rapamycin and its analogs act predominantly as cytostatic agents, and therapy withdrawal may induce tumor re-growth. Combinational therapy with pro-apoptotic agents may be beneficial for LAM treatment. Statins promote cell apoptosis by inhibiting prenylation of Rho GTPases. Natural and synthetic statins may have differential effect depending on cell type. Thus, synthetic statin atorvastatin has little effect on renal and liver tumor growth in *TSC2*^{-/-} mice and on *TSC2*^{-/-}*p53*^{-/-} MEFs subcutaneous tumor growth. However, as we reported previously, natural statin simvastatin inhibits RhoA and proliferation of LAM-derived and TSC2-null ELT3 cells. Thus, we examined effects of simvastatin and rapamycin combination on subcutaneous xenographic TSC2-null ELT3 cell tumor growth in female nude mice. Untreated group showed 3.5-fold increase in volume at day 40. Single rapamycin (1 mg/kg) and simvastatin (250 mg/kg/day) treatments inhibited tumor growth (53.1±21.3mm³ and 57.7±43.1mm³, respectively, vs. 201±41mm³ in control tumors; p<0.01, n=18 for each group). Importantly, combined treatment markedly reduced tumor volumes compared with initial tumor size (3.6±1.9mm³ at day 40 vs. 56.8±3.4mm³ at day 0; p<0.001, n=18) and with single simvastatin and rapamycin treatment (p<0.05). Untreated tumor-bearing mice had 15% survival by day 50. Simvastatin and rapamycin alone markedly improved survival by 47.4% and 50.0%, respectively; combined treatment further improved survival by 67.0%. Immunohistochemistry with anti-Ki67 antibody demonstrated that either simvastatin or rapamycin alone significantly inhibited DNA synthesis (8.5±1.8% and 11.9±0.05%, respectively, vs. control 13.3±3.3% at day 10; 8.8±1.6% and 8.5±1.0% vs. control 10.6±0.01% at day 20). Marked reduction in phospho-S6, the hallmark of mTORC1 activity, was observed only in rapamycin-treated tumors. In contrast, simvastatin-treated tumors had increased levels of apoptosis assessed by TUNEL. Importantly, simvastatin+rapamycin treatment abrogated DNA synthesis (4.4±2.7% and 0.8±0.6% at days 10 and 20, respectively), promoted apoptosis, and suppressed S6 phosphorylation. Furthermore, tumor recurrence was observed in rapamycin-treated mice at days 5-11 after treatment withdrawal; and tumors reached 10% of mice weight by day 41. Importantly, no tumor recurrence was detected in simvastatin- or simvastatin+rapamycin-treated mice during four months of post-treatment. Collectively, our data demonstrate that simvastatin cooperates with rapamycin in reducing TSC2-null tumor growth and improving animal survival due to simultaneous induction of apoptosis, suppression of mTORC1 activity and DNA synthesis; and suggest that co-treatment with simvastatin prevents post-treatment tumor re-growth compared to single rapamycin treatment.

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Phenotypic Characterization of Disseminated Cells with *TSC2* Loss of Heterozygosity in Patients with Lymphangiomyomatosis (LAM)

Lymphangiomyomatosis (LAM) is an uncommon disease, found primarily in women of childbearing age, and characterized by abnormal proliferation of smooth muscle-like cells (LAM cells). LAM occurs as a sporadic disease (S-LAM) or in association with Tuberous Sclerosis Complex (TSC), an autosomal dominant genetic disorder characterized by multiorgan hamartomata, resulting from mutations or loss of heterozygosity (LOH) in one of two *TSC* tumor suppressor genes, encoding hamartin (*TSC1*) and tuberin (*TSC2*). LAM cells, which appear to contain mutations in one of the tuberous sclerosis genes, cause cystic lung destruction and/or angiomyolipoma, and infiltrate the axial lymphatics. In the present study, we identified molecular markers useful for isolating LAM cells from body fluids and determined the frequency of *TSC1* and *TSC2* LOH. Candidate cell surface markers (e.g., CD9) were selected by gene microarray analysis of human *TSC2*^{-/-} cells. Other markers, e.g., CD235a, CD44v6, had already been shown to recognize LAM cells in lung nodules. Cells from bronchoalveolar lavage fluid (BALF), urine, chylous effusions, and blood were subjected to fluorescence-activated cell sorting (FACS) based on reactivity with antibodies against these proteins and analyzed for LOH using *TSC1*- and *TSC2*-related microsatellite markers and single nucleotide polymorphisms (SNPs) in the *TSC2* gene. For blood samples, a density gradient centrifugation step preceded FACS and LAM cells with *TSC2* LOH were detected in over 90% of cases. The percentage of patients with *TSC2* LOH positivity was greatest among those informative for Kg8, the biomarker located closest the *TSC2* gene. CD44v6⁺CD9⁺ LAM cells from BALF, urine, and chylous fluids with *TSC2* LOH were found in 80%, 69% and 50% of patient samples, respectively. In most cases, LOH patterns were identical in LAM cells from different body fluids; in a minority of cases, however, LOH patterns differed in LAM cells from different body fluids of S-LAM patients, consistent with losses of different regions of chromosome 16 among LAM cells at different sites. These data suggest that different clonal populations of LAM cells might be responsible for lesions in some patients with S-LAM. Patients with *TSC1* mutations were shown earlier to have cystic lung lesions, consistent with LAM. In the current study, no S-LAM patients with *TSC1* LOH were identified, suggesting that *TSC2* gene abnormalities may be responsible for the majority of the cases of S-LAM, and that *TSC1*-mediated S-LAM may be, for the most part, subclinical. These findings are consistent with a common genetic origin of LAM cells in different sites in most cases of S-LAM, in agreement with a metastatic model, but also suggest that LAM cells in S-LAM patients could also arise from different clones. S-LAM appears to be primarily a *TSC2*-mediated disease.

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Regulation and function of the TSC-mTOR pathway

The mammalian target of rapamycin (mTOR) is a central cell growth regulator and forms two distinct functional complexes, TORC1 and TORC2. The TSC1 and TSC2 tumor suppressor genes are key upstream regulators of TORC1. TSC2 functions as a GTPase activating protein, GAP, to inhibit the Rheb small GTPase, which is a potent director activator of TORC1. In TSC mutant cells, TORC1 is highly activated and the uncontrolled TORC1 activity likely contributes to the pathogenesis of TSC and related diseases. To understand the physiological functions, we used mouse models with TSC1 knockout in various tissues. Deletion TSC1 in hypothalamus neurons caused hyperphagia and obesity. Deletion of TSC1 in kidney generated phenotypes mimicking diabetic nephropathy. Reducing TORC1 activity by either rapamycin treatment or reduce raptor level rescued the TSC1 knockout phenotypes. Progresses of these studies will be presented.

Andrey Parkhitko, PhD

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Role of Autophagy in the Pathogenesis of Tuberous Sclerosis Complex and LAM

Introduction: Autophagy is a cellular degradative pathway involving the delivery of cytoplasmic content to the lysosome. Autophagy appears to play a complex and context-dependent role in tumor development. Although mTOR is a known inhibitor of autophagy, the impact of autophagy on TSC and LAM pathogenesis is not well understood.

Methods: TSC2-null mouse embryonic fibroblasts (MEFs), sporadic LAM angiomyolipoma-derived 621-101 cells carrying inactivating mutations of both alleles of TSC2 and Eker rat uterine leiomyoma-derived ELT3 cells were used. Autophagy levels were examined using p62 and LC3-II immunoblotting and by electron microscopy. For *in vivo* studies, the autophagy inhibitor Chloroquine was administered 60 mg/kg/day intraperitoneally when tumors reached 100 mm².

Results: TSC2-null patient-derived cells and TSC2-null MEFs have a 10-fold decrease in levels of LC3 cleavage, a 3-fold increase in p62 accumulation, and fewer autophagosomes, compared to TSC2-reexpressing cells, indicating that loss of TSC2 leads to decreased autophagy. Surprisingly, autophagy could be induced in TSC2-null cells by hypoxia or nutrient deprivation but autophagy levels remained lower than controls. Treatment of TSC2-null cells with the mTOR inhibitor Rapamycin reduced p62 levels and increased LC3 cleavage. These data demonstrate that TSC2 loss impairs autophagy under basal and metabolic stress conditions, while mTOR inhibition induces autophagy in TSC2-null cells. To examine the impact of autophagy dysregulation *in vivo*, TSC2-null ELT3 cells were injected subcutaneously into SCID mice. Chloroquine treatment of these mice carrying ELT3 cell tumors suppressed tumor growth by 2 fold after 7 days of treatment. Furthermore, treatment of ovariectomized SCID mice carrying ELT3 cell xenograft tumors with estrogen upregulated autophagy relative to placebo control, as judged by LC3-II and p62 levels.

Conclusions: Taken together, our data indicate that TSC2-null cells have lower autophagy levels and are highly sensitive to autophagy inhibition, representing a potential "Achilles' heel" which can be targeted therapeutically. We speculate that Rapamycin treatment of TSC and LAM patients enhances cell survival because of autophagy induction, contributing to the fact that angiomyolipomas only partially regress and then regrow after Rapamycin withdrawal. Based on these data, we propose that Hydroxychloroquine, which is a well-tolerated drug widely used for malaria and rheumatoid arthritis, is a potential therapeutic agent for LAM and TSC.

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Pharmacologic inhibition of polo-like kinase 1 (PLK1) decreases the viability and survival of TSC1 and TSC2 null cells via induction of autophagy

Loss-of-function mutations in *TSC1* (hamartin) or *TSC2* (tuberin) cause sporadic pulmonary Lymphangiomyomatosis (LAM) and Tuberous Sclerosis Complex (TSC). The hamartin/tuberin heterodimer negatively regulates the rapamycin-sensitive mTOR/raptor kinase complex, which is involved in translation, autophagy, cell cycle and hypoxia. Rapamycin is currently under clinical trials for LAM/TSC.

We previously reported that hamartin interacts with the mitotic kinase PLK1, and that cells lacking hamartin or tuberin have increased PLK1 expression, which is rescued by reintroduction of hamartin in *Tsc1*^{-/-} MEFs and tuberin in ELT3 cells. Additionally, we found that PLK1 expression is increased in LAM-derived lung lesions. PLK1 expression is increased in various cancers, and PLK1 small-molecule inhibitors are in phase I/II clinical trials for oncology. We hypothesize that PLK1 over-expression increases the survival of hamartin and tuberin null cells.

To test this hypothesis, *Tsc1*^{-/-} and *Tsc2*^{-/-}/*Trp53*^{-/-} MEFs (and controls), and isogenic *Tsc1*^{-/-} MEFs transduced with vector (208-P2) or hTSC1 (208-T3) were treated with the PLK1 inhibitors C1 (GlaxoSmithKline) and BI-2536 (Boehringer Ingelheim), and cell viability was measured by MTT conversion. The viability of hamartin- and tuberin-deficient cells treated with 10-30 μM C1 or 10-30 nM BI-2536 was significantly decreased compared to controls. Hamartin and tuberin null cells treated with PLK1 inhibitors in combination with 0.2 or 2 nM rapamycin had significantly lower viability, compared to cells treated with either compound alone. Additionally, PLK1 inhibitors decreased the clonogenic survival of hamartin- and tuberin-deficient MEFs, and HeLa cells, after shRNA-mediated silencing of TSC1 or TSC2, compared to controls.

PLK1 inhibition causes apoptotic cell death, and PLK1 binds to the caspase inhibitor survivin (BIRC5). Recently, survivin has been implicated in inhibition of autophagy, and increased survivin expression levels have been demonstrated in LAM-derived cells. PLK1 inhibition in hamartin and tuberin null MEFs did not induce apoptosis, measured by PARP and caspase 3 cleavage; however, it significantly decreased the expression of the long-lived protein p62 (SQSTM1) and induced the formation of the 14 kDa LC3B-II, indicative of autophagy. Additionally, rapamycin significantly decreased PLK1 and survivin protein levels, possibly through a mechanism involving translation of the corresponding mRNAs. The role of survivin inhibition in viability and survival of hamartin- and tuberin-null cells is currently under investigation.

These data demonstrate that PLK1 inhibition preferentially decreases the viability and survival of hamartin and tuberin null cells via a mechanism involving autophagy, and provide further insight for the role of PLK1 and survivin in regulating cell survival processes related to LAM/TSC pathogenesis.

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Lymphangi leiomyomatosis (LAM): Insights from PEComa and Mutational Analyses

The effectiveness of rapamycin and related drugs in the treatment of LAM continues to be a subject of clinical investigation. Recently we have had the opportunity to explore the molecular pathogenesis and response to treatment of 3 patients with malignant PEComas (Wagner et al. Clinical Activity of mTOR Inhibition With Sirolimus in Malignant Perivascular Epithelioid Cell Tumors: Targeting the Pathogenic Activation of mTORC1 in Tumors. *J Clin Oncol.* 2010 28:835-40). All 3 patients showed a clear response to rapamycin treatment, which in one case persisted for over a year. One PEComa showed complete genetic loss of TSC1 by both MLPA and FISH studies. All three showed no expression of TSC2 by IHC.

In collaboration with Lucia Schuger, we have used deep sequencing to search for TSC2 mutations in LAM samples. Laser capture microdissection was performed on lung samples from 9 sporadic LAM patients, and DNA was used for amplification and analysis of all coding exons of TSC2. Results are shown in the table. Five of 9 samples analyzed had mutations in TSC2 found by deep sequencing analysis. One sample had two different mutations identified, at different frequencies. These results confirm that the TSC2 gene is commonly involved in the development of sporadic LAM, but also suggest that other genetic mechanisms occur in some cases.

sample	TSC2 findings	TSC1 findings
BBI 9020	781C>T p.261R>W 50%; 3610+1G>A 19%	ND
BBI 9022	789-806del 11%	ND
BBI 9023	none	None
BBI 9030	none	none
BBI 9034	5127delC 43%	ND
BBI 9036	1947-4-2030del 50%	ND
BBI 9038	1837 C>T p.613 Q>X 5%	ND
BBI 9043	none*	ND
BBI 9059	none*	ND
	*these two samples had lower read coverage	
	ND, not done	

Bruce Korf, MD, PhD
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Developing a National Collaborative Network for Clinical Research in Neurofibromatosis.

The Neurofibromatosis Consortium was established with funding by the Department of Defense Congressionally Directed Medical Research Program (CDMRP) in Neurofibromatosis to conduct clinical trials and other clinical studies related to neurofibromatosis. The genesis of the consortium was the recognition that setting up a clinical research study for a relatively rare disorder such as NF poses many challenges. These include recruiting a sufficiently large number of participants to effectively test a hypothesis; maintaining compliance with variance regulatory requirements; developing a tightly designed protocol; and monitoring of data quality and protocol compliance. The NF Consortium consists of nine patient recruitment sites and an Operations Center, which provides support for study logistics, protocol development, data collection, and statistical design and analysis. The study sites and Operations Center were selected through a request for proposals and peer review process administered by the CDMRP. The site PIs serve on a Steering Committee, currently chaired by election of one of the site PIs (Dr. Roger Packer, Children's National Medical Center). There are numerous committees charged to develop study protocols or to manage policy issues such as publication policy and oversight of study site performance. The PIs meet monthly by teleconference and semi-annually face-to-face. Currently there are two active protocols recruiting participants and two that are approved and are in process of being launched. The targets of these treatments are plexiform neurofibromas, learning disabilities, low-grade gliomas, and malignant peripheral nerve sheath tumors (MPNST). The MPNST protocol will be done in collaboration with the SARC consortium. Additional protocols for treatment of NF2 and bone dysplasias are in preparation, both in collaboration with other groups outside the NF Consortium. Furthermore, ancillary studies to existing protocols are being explored as well as engagement of consortium sites in the recruitment of participants for non-treatment trial clinical studies. Finally, analysis of new clinical trials that will test other candidate drugs for various of the manifestations of NF1 are in process, with close ties to the Children's Tumor Foundation Preclinical Consortium and CTF-funded phase I clinical trials.

Professor Alfredo Gorio
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*^{/meth} 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*^{-/-} and *TSC2*^{/meth} 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*^{-/-} and *TSC2*^{/meth} 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.

Alfredo Gorio, PhD
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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 administering TSC2^{-/-} 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^{-/-} ASM cells (2×10^5) 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/m² followed by subsequent dose of 250 mg/m²) and rapamycin (4mg/kg) were intraperitoneally injected 2 times a week for 4 weeks. TSC2^{-/-} 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^{-/-} ASM cells grow and proliferate mainly in lung parenchyma and at a less extent in lymph nodes, as showed by Ki-67 staining. TSC2^{-/-} ASM cells promoted a significant increase of LYVE-1 reactivity in lungs and in lymph nodes suggesting a correlation between TSC2^{-/-} 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^{-/-} 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^{-/-} ASM cells migrate, proliferate and invade lymph nodes and lungs causing LAM-like lesions. TSC2^{-/-} 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^{-/-} 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.

Carlyne D. Cool, MD

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Translational Research from a Lung Tissue Repository

Tissue banking has become increasingly important in modern approaches to disease research including genomics, proteomics, metabolomic and lipidomics. While early tissue banks tended to be small tissue repositories in individual laboratories, there has been an increasing trend towards institution-wide as well as national-level tissue banking of certain materials. Expert panels charged with assessing the state of the science in human lung disease research have consistently identified a lack of adequately preserved human tissue samples as an obstacle to the application and validation of promising molecular technology. As one solution to the lack of adequate human lung tissue, the NIH NHLBI created the Lung Tissue Research Consortium (LTRC) to collect, store and distribute biologic specimens from subjects with chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). The LTRC has shown that multi-institutional, multi-format collection and central banking of biologic specimens from non-neoplastic lung disease can be successfully performed. Our lab, as the current tissue repository for the LTRC, has distributed over 40,000 unique biologic specimens to over 115 investigators in the past three years. Human tissue is essential in forwarding the translational research in lung disease.

Using human lung tissue, we have performed morphometric, immunohistochemical, gene expression and DNA studies on interstitial lung diseases. More recently, we have examined lymphangiogenesis in fibrotic lungs. Lymphangiogenesis occurs in response to inflammation and injury in an attempt to reestablish tissue homeostasis. Growth factors, VEGF-C and VEGF-D, which stimulate lymphangiogenesis, can be measured in the serum and may indicate active lymphangiogenesis. Recently, levels of systemic VEGF-D have been associated with severity of pulmonary involvement in patients with lymphangioleiomyomatosis (LAM). We hypothesize that evidence of lymphangiogenesis occurs in interstitial lung disease and can be found in greater density and length when compared to control lungs.

Using lung tissue from the LTRC tissue repository, we performed immunohistochemistry for lymphatic vessels (D2-40) and blood vessels (CD31) on isotropically oriented tissue sections. Stereology of lymphatic vessels was performed using VisioPharm® Imaging software. The results showed that lymphatic vessels are significantly longer in fibrotic disease as compared to control lung, suggesting that lymphangiogenesis and persistence of lymphatic vessels occurs in interstitial lung diseases, and further implicating lymphatic vessels in the disease process. We are in the process of using stereology to analyze lungs from patients with lymphangioleiomyomatosis.

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An exquisite cross-control mechanism among endothelial cell fate regulators directs the plasticity and heterogeneity of lymphatic endothelial cells

Arteriovenous-lymphatic endothelial cell fates are specified by master regulatory genes, Notch, COUP-TFII and Prox1, respectively. While Notch is selectively expressed in the arteries and COUP-TFII in the veins, the lymphatics, interestingly, express all three cell fate regulators. Studies show that lymphatic endothelial cell (LEC) fate is highly plastic and forward a new concept that all three endothelial cell fates may co-reside in LECs and that a subtle alteration can result in a reprogramming of LEC-fate. Here, we provide a molecular basis to verify this concept by establishing a cross-control mechanism among these regulators. We found that activated Notch or Notch ligands downregulates Prox1 and COUP-TFII through Hey1/2 and that ectopic expression of Notch suppress the lymphatic phenotypes and induces the arterial cell fate. On the contrary, Prox1 and COUP-TFII attenuate VEGF signaling, which is known to induce Notch, by repressing VEGFR-2 and neuropilin-1. We also show that previously reported podoplanin-based LEC-heterogeneity is strongly associated with differential expression of Notch1 in human cutaneous lymphatics. Together, we demonstrate that the three endothelial fate regulators are under an exquisite feedback regulatory network in LECs and that their regulatory “equilibrium” may play an important role in the arteriovenous-lymphatic cell fate specification and LEC-plasticity.

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Vascular Anomalies and mTOR inhibition

Patients with vascular anomalies (VA) have a spectrum of diseases that can be broadly classified into vascular tumors and malformations. Complicated vascular anomalies can cause disfigurement, chronic pain, and organ dysfunction with significant morbidity and mortality. Despite the severity of potential complications, we lack uniform guidelines for the treatment and response to treatment of children and young adults with these diseases. To this date, there are no FDA approved treatment regimens. There are pre-clinical and clinical data supporting the essential regulatory function of the PI3K/Akt/mTOR pathway in vascular growth and organization, and suggest a therapeutic target for patients with complicated vascular anomalies. Our institution has used the mTOR inhibitor, rapamycin for compassionate use in 4 patients with excellent results. We presently have an open FDA funded Phase 2 trial of Rapamycin in the treatment of children and young adults with complicated vascular anomalies. The overall goal of this trial is to objectively determine the effectiveness and safety of the mTOR inhibitor Rapamycin in this patient population.

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Sleep hypoxemia in patients with LAM: relationship to pulmonary function and 6MWT

Rationale: Lymphangiomyomatosis is a progressive obstructive lung disease that occurs in young women. Besides obstruction, impairments on diffusion capacity corroborate the rest dyspnea and hypoxemia. Exercise is an important stressor that causes hypoxemia and correlates with functional impairment. Sleep is another important physiologic moment when cardio respiratory system may be stressed and nocturnal hypoxemia may occur.

Methods: To study oxygen hemoglobin saturation (SpO_2) during sleep and its correlation to pulmonary function and exercise, 25 consecutive outpatients with LAM were evaluated by lung function test (LFT), six minute walk test (6MWT), polysomnography (PSG) and echocardiography. SpO_2 lower than 90% on PSG was considered abnormal.

Results: Mean age was 45 ± 10 years. All patients presented mild dyspnea, airway obstruction and moderate diffusion capacity impairment (median $FEV_1 = 77\%$, $FEV_1/FVC = 0.7$ and $DL_{CO} = 48\%$). On PSG, 14 patients presented SpO_2 lower than 90% for more than 30 min (median 136 min), while median apnea-hypopnea index was 2 (1 – 5). Correlation analysis yielded that SpO_2 decline (initial SpO_2 – final SpO_2) on 6MWT ($r_s = 0.6$), FEV_1 ($r_s = -0.6$), and DL_{CO} ($r_s = -0.7$) were the main variables related to sleep oxygen desaturation ($p < 0.01$).

Conclusion: We conclude that, for the first time, nocturnal desaturation was demonstrated in an outpatient LAM population and was present in more than half of patients, especially among those with reduced FEV_1 and DL_{CO} . Desaturation was more intense during sleep than during 6MWT and patients with nocturnal desaturation did not appear to have impaired sleep efficiency. Concerns about sleep hypoxemia screening must be raised in outpatient LAM populations.

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Insights into therapeutic strategies from TSC animal models

Mutations in either the *TSC1* or *TSC2* gene are causally linked to the development of tuberous sclerosis complex (TSC), a tumor suppressor gene syndrome characterized by multiple tumors of the brain, kidney, heart and skin. Lung involvement, lymphangioleiomyomatosis (LAM), is less common in TSC but LAM also occurs sporadically in the non-TSC population. LAM is a multi-system disorder that primarily affects women of reproductive age. The dominant manifestation of pulmonary LAM is the uncontrolled growth of smooth muscle-like cells within the interstitium of the lung. These cells grow to replace the normal architecture of the lung by 1) expanding the lung interstitium and 2) destroying normal alveoli resulting in characteristic lung cysts. It is likely that the relentless cell growth and the associated destroyed lung lead to the progressive nature of LAM over a variable period. Thus, understanding the pathogenesis of both cell growth and cyst production in LAM lung is critical to discovery of a therapy that will have significant impact clinically. Strong evidence now links loss of functioning *TSC2* with the pathogenesis of LAM that has led to the use of cellular and animal models where *TSC1/TSC2* complex function is disrupted to test potential therapeutic agents. A decade of research has led to improved understanding of the tumor suppressor functions of hamartin and tuberin, the protein products of *TSC1* and *TSC2*, respectively. The hamartin-tuberin complex suppresses the activation of GTPase Rheb (ras homolog enriched in brain). Rheb is a major regulator of mTORC1 (mammalian target of rapamycin complex 1). Thus, in cells lacking functional hamartin or tuberin, elevated levels of active Rheb (GTP-Rheb) lead to constitutive activation of mTORC1, resulting in phosphorylation of p70 S6 kinase (S6K), S6 and 4E-BP1, to regulate protein translation and cell growth. In the past therapeutic options for LAM have focused on inhibiting estrogen with relatively poor outcomes which has dampened enthusiasm for hormonal manipulation in LAM patients. More recently, studies have focused on targeting mTORC1, such as sirolimus and everolimus which has been very successful in animal models and early studies in humans suggest a similar pattern of success. However, these drugs have many potential significant side-effects, including immunosuppression and pneumonitis. Thus, there is a continuing need for alternative and combinatorial treatment approaches for LAM, such as inhibition of G protein function, Rheb and Rho, and inhibition the external forces that drive cell metastasis, cell growth and cyst production.

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Targeting mTOR-induced endoplasmic reticulum stress: a rational treatment for sporadic AML/LAM?

Angiomyolipomas (AMLs) are benign kidney tumors composed of muscle and fat. These tumors are common in both men and women with tuberous sclerosis complex (TSC) and in women with lymphangioliomyomatosis (LAM). AMLs in these patients result from mutation or loss of the TSC1 or TSC2 genes that leads to constitutive activation of the mammalian target of rapamycin (mTOR). These tumors typically follow a benign course in the general population; however, lung leiomyomas associated with LAM may represent a benign metastasis of AMLs. Currently, the cell of origin for LAM leiomyomas is unknown, and there is no treatment to eliminate these growths. Our research focuses on finding drug combinations that result in the selective death of TSC2-null AMLs over cells expressing wild-type TSC2. Recently, it was shown that MEFs and neurons lacking TSC2 show higher levels of basal endoplasmic reticulum (ER) stress. Due to the increased stress, these cells are more sensitive to cell death from treatment with pharmacological ER stressors. Our findings show that AML cells lacking TSC2 have higher basal levels of ER stress compared to rapamycin treated cells by measuring the stress markers eIF2 phosphorylation, spliced XBP1 mRNA and CHOP mRNA. Furthermore, we observed both cleavage of poly-ADP ribose polymerase (PARP) and caspase-3 and increased cell death by treatment with bortezomib/Velcade, a clinically approved proteasome inhibitor that induces ER stress. This bortezomib-induced PARP/caspase-3 cleavage and cell death is blocked by treatment with rapamycin. Combining the bortezomib treatment with Sal003, a compound shown to enhance ER stressor efficacy, lead to improved cell killing and increased PARP/caspase-3 cleavage that was blocked by rapamycin. Interestingly, the bortezomib/Sal003 combination results in heavy cytoplasmic vacuolation that is similarly blocked by rapamycin treatment. In conclusion, loss of TSC2 in AML results in increased basal ER stress which sensitizes these cells to bortezomib/Sal003 treatment in an mTOR pathway/rapamycin-sensitive manner. Bortezomib is FDA approved for cancer treatment and Sal003 has been administered successfully to rats, therefore this drug combination may represent a rational treatment particularly for sporadic AML. Moreover, this cytotoxic drug combination may be superior to rapamycin's cytostatic effects that require chronic drug administration.

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Cystic Fibrosis Foundation's Therapeutics Development Network: Insights for LAM

In 1990, a landmark paper outlined the advantages of a clinical trials network for an orphan disease population. Cystic fibrosis (CF) was ideally suited to benefit from such a network. Moving new discoveries in basic sciences toward the development and clinical testing of new therapeutic agents was very slow, so the CF Foundation (CFF) initiated two programs, one to support biotech companies developing new drugs for CF and a second to accelerate the conduct of clinical trials in patients with CF. Multicenter trials leading to FDA approval of two new drugs in the early 1990's demonstrated the strengths and challenges of conducting large multicenter trials among CF clinical centers. Bonnie Ramsey, MD from the University of Washington working with the CFF pioneered the development of the CF TDN, which following a competitive grant review established 8 Centers as the CF TDN in 1998.

The initial period (1998 to 2002) was characterized by formation of a functional network which included a coordinating center at the University of Washington, committees for governance, protocol review, protocol development, publications, patient advocacy and ethics, and study implementation. Reference laboratories were developed and standard operating procedures were written to ensure standardization across all sites. Expansion to 18 sites occurred in 2002-2005. At the end of seven years, 34 studies had been conducted enrolling approximately 1900 patients and producing 14 publications directly related to TDN trials.

The twin successes of CFF's drug discovery and the TDN's productivity attracted more industry sponsored studies which progressively increased demand on patient enrollment in CF clinical trials necessitating use of CF centers outside the TDN program. The projected number of patients needed in TDN-approved trials based on industry estimates grew from 109 enrolled in 1999 to 2000 patients in 2009. A facilitated expansion of the network occurred from 2006 to 2009 enlarging the TDN to 77 Centers. Thirteen are designated as Translational Centers possessing special capability and experience in CF translational research. In the TDN's current structure the Clinical Research Executive Committee (CREC) is the top level committee which provides scientific leadership for the network. It establishes therapeutic priorities for CFF-sanctioned studies, assigns strategic fit for network protocols, reviews network activities and oversees network structure and governance. The Steering Committee (SC) is the primary governing body for network operations. It establishes network-wide policies and procedures and reviews site performance. Other committees address review of publications, oversight of national resource centers, protocol review and coordination of the special functions of the Translational Centers.

The TDN SC has developed a quality improvement program which tracks the overall activity or performance of TDN sites. Sites provide monthly reports to the coordinating center database which tracks site activity organized by study protocol to facilitate review of various metrics which measure efficiency of start-up, enrollment and overall activity. The TDN website forms a central element of the network. It contains informational items such as site details, contact information, presentations and minutes from meetings and conference calls. Each committee has their own section for placement of relevant documents, rosters, and discussion of topics. All sites can assess the status of clinical trials which are upcoming, under review, or underway. Each site can download the most current data on enrollment by study in which they participate. The network is further linked by communication through quarterly newsletters, biannual webinars and an annual meeting that coincides with the North American Cystic Fibrosis Conference.

The stated mission of the CFF TDN is to facilitate the clinical study of new and existing therapies to cure and control cystic fibrosis. We anticipate that all the participants in the TDN will continue to build on the past success of the program as they work toward this goal utilizing the best practices, high standards and the tireless efforts that characterize their clinical care of patients with CF.