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Conducting clinical trials in rare diseases

Trial designs for rare diseases must meet the same rigorous standards as do designs for trials for diseases that occur with much more frequency. They must ask important scientific questions, minimize bias and have appropriate likelihood of achieving a scientifically acceptable answer. Indeed, there are no designs for rare diseases that are not applicable to any other category of diseases. However, there are many different types of study designs some of which require only a fraction of the number of subjects required to conduct a randomized controlled trial, which is often considered the gold standard by investigators, funding agencies and regulatory authorities.

A randomized controlled trial is considered the gold standard because inherent in its design is the minimization of bias. Thus the results are often considered to provide the strongest evidence in testing a hypothesis. However, randomized controlled trials are not easy to do in that many potential participants object to the concept of randomization and many investigators feel that randomization, in of it itself, is unethical. Randomization requires that the investigator and the subject consider themselves in the state of equipoise in that they truly feel that the treatment received from either arm of randomized trial is equivalent unless proven otherwise. This is difficult for participants who want to believe that their treatment will be based upon what is best for them and not the 'flip of a coin' and difficult for physicians who also think that they are ethically bound to provide the 'best' treatment. Equipoise is made the more difficult since trials are often developed because an investigator feels that an experimental therapy is better and they wish to test that hypothesis in a rigorous fashion. Many subjects object to the trials if they have a likelihood of being assigned a potentially inferior arm (i.e. have a likelihood of not receiving the experimental therapy) or being randomized to a placebo.

Alternate designs can address those issues by the use of external or historical controls or with participants serving as their own control. In the case of external or historical controls, all patients to be recruited on a proposed study would receive the new or experimental therapy and their outcomes would be compared to a population that had already been treated by a standard therapy. This results in considerable savings in terms of the number of patients to be accrued, even though the total number of patients may be a substantial fraction of the total needed in a randomized controlled trial. If historical data are valid and available, this is an efficient design because it requires fewer patients to be accrued. The downside of such a design is that the selection of historical controls must be made with extreme caution so as not to bias the study results. Often it is difficult to know whether bias has been introduced by factors that have not been reported in the historical series or through changes in practice that may affect clinical assessments or outcomes.

A design that avoids this problem is the use of concurrent controls for which participants can serve as their own control. Such designs are desirable if there is less within patient variability in a treatment response than there is between-patient variability. In such cases, outcome estimates will have less variance and the study design will require less accrual. These designs work well for chronic diseases, but there are many settings in which these assumptions can not be justified.

A design that is well suited to rare events and rare diseases is the case-control design. In such a design, individuals in whom a certain outcome has been observed (disease severity or particular event) are matched to controls who did not have such an outcome and then the two groups are compared with respect to a particular intervention or exposure. Such designs can be developed from prospective as well as retrospective data collection perspectives. Retrospective data collection is particularly efficient since one can identify just the cases where the events have occurred and

matched them to a control where a particular event of interest has not occurred. But it suffers because of the reliance on the quality of historical data. Such a design is particularly efficient if there is a long length of time that elapses between exposure and the particular events or outcomes of interest. Such designs can be particularly useful in rare diseases in which there is a long lag time between genotype and phenotypic expression. Again the problem is the same as in the case of historical controls where investigators have to be extremely careful in selecting appropriate controls. Therefore, this design is not ranked as high as the randomized controlled trial in terms of the strength of evidence, because of the potential bias.

Different designs can be used even when treatment arms are prospectively randomized to reduce sample size requirements. Examples include cross-over designs as well as factorial designs. In the former, participants are randomized to a treatment arm for a period at the end of which the outcome is assessed and then 'crossed over' to the other treatment. Factorial designs essentially involved a double randomization in which two questions are asked in the same participant population. This essentially results in a sample size savings of an appropriate 50%, but also assumes that there is no interaction between the two treatments.

Finally, designs for ranking and selection procedures are often helpful and generally require a smaller sample size than randomized controlled trials. In a factorial design, the objective is to maximize the likelihood of selecting the better therapy from a number of therapies as opposed to designing a trial that actually compares therapy directly and measures how much better one is as compared to another. Ranking statistics are often used when information about underlying parametric distributions are unknown. It could be argued that less is learned in such an experimental design and a subsequent experiment is required to measure the actual difference between treatment outcomes. That is because a randomized clinical trial design is to detect a minimal clinical significance between treatments whereas the ranking statistics only seeks to determine which treatment has the better response rate.

There are many approaches to the design of a trial and a number of them can achieve certain economies in terms of the required number of participants. However, the options are not without their drawbacks and require investigators to make a number of assumptions, some of which cannot be verified. It is clear that careful consideration needs to be made regarding those assumptions to identify the study design that best fits the research question.

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Invasion-independent pathway of cancer metastasis: possible implications for the mechanism of LAM cell dissemination

It is generally believed that active invasion by cancer cells is essential to the metastatic process. In contrast, we found an alternative metastatic pathway, named invasion-independent pathway that does not require invasiveness in mouse mammary tumor cell line. This metastasis pathway shares morphological similarities with LAM cell dissemination associated with lymphatic endothelial cells. I present here the process of an invasion-independent pathway in blood-borne metastasis and a possible molecular mechanism of this pathway.

Mouse tumor model revealed the metastatic process involving intravasation of tumor nests surrounded by sinusoidal blood vessels, followed by intravascular tumor growth in the lung, without penetration of the vascular wall during the process. We also examined the relevance of this model in human cancers using archival specimens of 10 common types of cancers. Of all cases, invasion-independent metastasis pathway was particularly prevalent in renal cell carcinomas, hepatocellular carcinomas and follicular thyroid carcinomas.

To clarify the mechanism of this pathway, we established several clonal lines different in metastatic potential. Comparative studies indicated that metastatic potential is strongly correlated with angiogenic activity to form sinusoidal tumor vessels. Differential cDNA analysis identified several gene candidates for promoting metastasis. Of these genes, *secretory leukocyte protease inhibitor (SLPI)* induced sinusoidal angiogenesis and spontaneous lung metastasis to low metastatic cells by transfection method, whereas it inhibited cancer cell invasiveness. These results suggest that this type of metastasis can be regulated by a novel mechanism involving anti-invasive molecules such as SLPI. Further investigations in the experimental system and human cancer cases may present new therapeutic strategies for cancer metastasis.

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Effect of prolactin on tuberin-deficient cells: Activation of signaling pathways in human lymphangioleiomyomatosis lung nodules and cells derived from the Eker rat

Lymphangioleiomyomatosis (LAM) is an uncommon disease, found primarily in women of childbearing age, and characterized by abnormal proliferation of smooth muscle-like cells (LAM cells). These cells possess mutations in the tuberous sclerosis genes, *TSC1* or *TSC2*, cause cystic lung destruction and/or angiomyolipoma, and infiltrate the axial lymphatics. *TSC* genes control cell growth, size, survival, and motility through effects on the mammalian target of rapamycin (mTOR) signaling pathway. Prolactin (PRL), an important reproductive hormone in women, is involved in immunoregulation and cell proliferation, differentiation and survival. Given the particular importance of the hormone, PRL, in women of childbearing age, and the fact that PRL has effects on critical cell functions, we hypothesized that PRL signaling may have an important role in the regulation of *TSC2*^{-/-} cells such as those found in LAM. Sources of prolactin include both pituitary and extrapituitary sites, including cancer cells (e.g., breast). We found that PRL and PRL receptor (PRLr) were present in LAM lung lesions (n=20 patients). Higher levels of PRL and PRLr mRNA were found in the laser-capture microdissected (LCM) LAM lesions from lung than in adjacent smooth muscle cells in vascular walls (n=10 patients). Consistent with activation of PRLr in LAM lung lesions (n=20 patients), we found activated downstream proteins, p-JAK2, p-STAT3 and p-p44/42 MAPK. Eker rat embryonic fibroblasts, which lack *Tsc2* (*Tsc2*^{-/-}), contained more PRLr than did their *Tsc2*^{+/+} counterparts. PRL activated the STAT1, STAT3, p44/42 and p38 MAPK pathways in a time- and concentration-dependent manner. Further, PRL promoted proliferation of *Tsc2*^{-/-} cells, to a greater extent than *Tsc2*^{+/+} cells. A PRLr antagonist, SI79D-PRL, inhibited PRL-induced proliferation of *Tsc2*^{-/-} cells and increased amounts of the short form PRLr. Further, SI79D-PRL enhanced the production of an alternatively spliced short form of the PRLr that has been reported to inhibit the activity of the PRLr long form. Based on these observations, loss of *TSC2* function appeared to result in higher levels of PRLr and activated downstream molecules in LAM lung lesions and PRL-dependent proliferation of *Tsc2*^{-/-} rat embryonic fibroblasts. These findings are consistent with an important role for PRL signaling in the pathogenesis of LAM and as a potential therapeutic target.

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Pathology of PEComa family of tumors

PEComa belongs to a family of related neoplasms, which also includes angiomyolipoma (AML) and lymphangiomyomatosis (LAM), all sharing a morphologically and immunophenotypically distinctive cell type, the so-called “perivascular epithelioid cell” (PEC), which lacks a normal cellular counterpart. This “PEC” characteristically shows evidence of both smooth muscle and melanocytic differentiation. Clinically, PEComas show a marked female predominance (approximately 7:1) and most often affect middle-aged adults. The most common anatomic sites are abdomen and pelvis, retroperitoneum, uterus, and gastrointestinal tract. A minority (~20%) arises in somatic soft tissue and skin. Although AML and LAM are strongly associated with the tuberous sclerosis complex (TSC), most PEComas are sporadic; only a small subset arises in TSC patients.

Histologically, PEComas show a nested architecture, with the nests surrounded by a delicate capillary vasculature. Tumor cells are usually large and epithelioid with sharply-defined cell borders, with a similar proportion of cases composed predominantly of clear cells and cells with granular, eosinophilic cytoplasm; a minor spindle cell component is relatively common. A careful search will often reveal focal areas in which the cells are associated with blood vessel walls, which is a helpful diagnostic clue. Approximately 20% of PEComas show marked stromal hyalinization (“sclerosing PEComa”); this subset has a predilection for the retroperitoneum. A minority of PEComas show striking pleomorphism and resemble pleomorphic sarcomas. By immunohistochemistry, PEComas often show a mixed melanocytic and myogenic phenotype. Nearly all PEComas are positive for HMB-45, which is the most sensitive marker for this tumor type. Most are also positive for microphthalmia transcription factor (MiTF), and a smaller subset for MART1. The most sensitive myogenic marker is smooth muscle actin (SMA); desmin is less commonly positive. We now recognize that co-expression of smooth muscle and melanocytic markers is not required to confirm the diagnosis of PEComa; some cases show HMB-45 reactivity in the absence of SMA or other myogenic markers.

Most PEComas are benign or indolent, although a subset pursues an aggressive clinical course. Pathologic criteria for malignancy in PEComas have recently been proposed. Any combination of the following features likely warrants designation as a malignant PEComa: tumor size >5 cm, mitotic activity >1 per 50 high power fields, coagulative necrosis, high nuclear grade, and infiltrative growth. However, some large PEComas with a low level of mitotic activity but no other malignant pathologic features appear to be clinically benign.

Recent studies indicate that PEComas frequently show deletions of the TSC2 locus at chromosome 16p13, similar to renal AML, which result in activation of the mTOR (mammalian target of rapamycin) signaling pathway. These findings have therapeutic implications for patients with clinically aggressive malignant PEComas, as mTOR inhibitors have shown efficacy in clinical trials of some human malignancies in which this pathway has been implicated.

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Therapeutic targeting PI3K-mTORC1 signaling pathway in pulmonary LAM and hamartoma syndromes

Hamartoma syndromes, dominantly inherited cancer predisposition disorders, affect multiple organs and are manifested by benign tumors consisting of various cell types native to the tissues in which they arise. In the past few years, three inherited hamartoma syndromes, Cowden syndrome (CS), tuberous sclerosis complex (TSC) syndrome, pulmonary lymphangiomyomatosis (LAM) and Peutz-Jeghers syndrome (PJS), have all been linked to a common biochemical pathway: the hyperactivation of the mammalian target of rapamycin complex 1 (mTORC1) intracellular signaling. Three tumor suppressors, PTEN (phosphatases and tensin homolog), tuberous sclerosis complex TSC1/TSC2, and LKB1, are negative regulators of mTORC1 signaling; disease-related inactivation of these tumor suppressors results in the development of PTEN-associated hamartoma syndromes, TSC, and PJS, respectively. The goal of this presentation is to provide a roadmap for navigating the inherently complex regulation of mTORC1 signaling while highlighting the progress that has been made in elucidating the cellular and molecular mechanisms of hamartoma syndromes and identifying potential therapeutic targets for treatment. Importantly, because the mTORC1 pathway is activated in the majority of common human cancers, the identification of novel molecular target(s) for the treatment of hamartoma syndromes may have broader translational implications, and is critically important not only for therapeutic intervention in hamartoma disorders, but also for the potential treatment of cancers.

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Predictors, barriers and motivating factors for clinical trial participation in lymphangioleiomyomatosis

BACKGROUND: Lymphangioleiomyomatosis (LAM) is a rare, progressive, frequently lethal cystic lung disease that almost exclusively affects women. There are no proven therapies for LAM, but an improved understanding of the pathogenesis has identified several promising targets for clinical trials. Barriers, modifiers, and benefits involved in participating in randomized controlled trials of common diseases such as cancer have been well studied. We are unaware of any published studies evaluating barriers, modifiers, and benefits involved in participating in clinical trials in rare diseases.

METHODS: We performed a cross-sectional survey of a population-based registry of approximately 800 LAM subjects in North America to identify predictors of trial participation. Logistic regression analysis evaluated the association of demographic and clinical features with trial participation.

RESULTS: 39 out of 232 (17%) LAM patient respondents in North America have participated in a clinical trial. The most common reasons against trial participation were not meeting enrollment criteria (47%), drug toxicity (25%), and stable disease (19%). Age, disease duration, lack of at least some college education, use of oxygen therapy, and presentation without chest pain, were associated with trial participation in unadjusted analyses. Results of multivariate analyses indicate that patient age was the strongest independent predictor for trial participation (OR= 1.63, $p = 0.026$ for each decade greater in patient age). The most common reason reported for trial participation was to help future patients (85%).

CONCLUSIONS: These findings suggest that study entry criteria and stability of disease are barriers to trial enrollment. Altruism is commonly a motivating factor. Older LAM patients and those with more advanced disease are more likely to have participated in clinical trials.

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Analysis of RAD001 dosing used to treat SEGA in patients with TSC

RATIONALE: Subependymal giant cell astrocytomas (SEGA) occur in 10-15% of patients with tuberous sclerosis complex (TSC) and can cause headaches, nausea, vomiting, ataxia, altered mental status, and death. As an alternative to brain surgery, we are evaluating the safety and effectiveness of the mTORC1 inhibitor RAD001 (everolimus) to treat SEGA. Here we examine treatment response in relation to drug dose, serum trough levels, and the extent of S6K1 activation.

METHODS: This is a phase I/II open-label clinical trial. All patients have a definite diagnosis of TSC, based on clinical and/or genetic criteria, and SEGA identified by serial MRI scanning prior to study enrollment demonstrating interval tumor growth. The targeted RAD001 serum trough levels for this study is 10-15 µg/ml, comparable to that employed in transplantation therapy. Primary endpoint is percent reduction in tumor volume at six months. Daily RAD001 dose and interval serum trough levels were collected and analyzed for relationship to treatment outcome. Effect of RAD001 on S6K1 phosphorylation, a downstream target of mTORC1, was also examined in peripheral blood mononuclear cells of a subset of study participants.

RESULTS: The median daily RAD001 dose at six months was 6.2 mg/m²/day. Corresponding median serum trough levels at the same time point was 5.6 µg/ml. Significant variability of serum levels achieved by a given dose was observed, likely due to concurrent medications and individual pharmacogenetic variation. Despite achieving serum trough levels lower than target, all patients completing the main study phase demonstrated a reduction in SEGA tumor volume of 40%. Response to treatment varied significantly among patients, even when corrected for serum drug level. S6K1 phosphorylation was readily detectible in untreated patients but was decreased in those receiving RAD001.

CONCLUSION: Lower doses of RAD001 than that used in clinical transplantation medicine are effective in reducing SEGA tumor volume and decreasing S6K1 phosphorylation in patients with tuberous sclerosis. The response of SEGA to RAD001 is not clearly related to serum trough levels, suggesting that lower doses may be of benefit in this disorder.

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High expression endothelin receptor variants associate with lymphangioliomyomatosis (LAM)

LAM is an idiopathic disorder marked by abnormal proliferation of smooth muscle in the lung parenchyma of females. Although associated with Tuberous Sclerosis gene abnormalities, a causative mechanism for LAM is not known. Endothelin-1 stimulates vascular smooth muscle contraction, hypertrophy, and proliferation by coupling to endothelin receptor A (ETRA). Emerging data suggests the endothelin axis is involved in tumor progression and metastasis. We have found an association between specific ETRA gene (EDNRA) single nucleotide polymorphisms (SNPs) and severity of lung disease in cystic fibrosis females. This gender-specific finding in CF, coupled with the role of endothelin in smooth muscle proliferation and tumor progression led us to postulate that the same EDNRA SNPs may be overrepresented in LAM patients. This could contribute to the development and/or severity of LAM. Fifty five women, of apparent Caucasian background, with LAM participated. DNA was genotyped at 5 EDNRA SNPs. Two SNPs showed genotype distributions in which the allele associated with severe lung disease in CF females (n=386) is overrepresented in the LAM cohort (n=55) when compared to CF females with mild lung disease and reported SNP frequencies in the general Caucasian population. For each SNP, the percentage of LAM patients carrying the severe genotype exceeds either CF group. The SNP with the strongest association is found in 90% of LAM patients, 70% of severe CF patients, and 51% of mild CF patients, compared to 63% of the general Caucasian population. EDNRA mRNA expression by smooth muscle cells is enhanced by exposure to estrogen and variants associating with severe CF lung disease and LAM exhibit mRNA expression levels 60% higher than the other alleles, potentially explaining the gender association. Data generated by this study may lead to additional screening parameters and treatment modalities, such as endothelin receptor antagonists.

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Structural analysis of mTORC1 and its inhibition by rapamycin

The mammalian target of rapamycin complex 1 (mTORC1) formed by mTOR, raptor, mLST8 and PRAS40 is a pivotal trigger of cell growth in response to the nutrient and energy status of the cell, and its deregulation has recently emerged as the key driver in human cancers including Lymphangi leiomyomatosis. Here we describe three-dimensional reconstructions of the intact complex as well as free raptor at nominal resolutions of 25Å and 30Å, respectively, using cryo-electron microscopy and single-particle analysis. The molecular architecture of the holoenzyme contains a two-fold symmetry, suggesting a dimeric mechanism of activation. Observation of the obligate dimeric complex unveils the mechanisms underlying the kinase function of mTORC1 and its inhibition by FKBP12-rapamycin. Additionally, the structure of mTORC1 provides an adequate framework in which the EM-reconstruction of raptor can be meaningfully fitted. Upon complex assembly, mTOR and raptor form a dimeric interface that is acutely sensitive to the treatment of rapamycin. Dimeric particles accommodate rapamycin at a distant site that is independent of the raptor-binding region. Consequently, conformational modifications via raptor remain to be a critical requirement for the rapamycin sensitivity of mTORC1. Although the role of mLST8 is not fully defined, antibody labeling of mLST8 reveals that exposure of the WD40-repeats at distal ends of the complex could potentially serve as platforms for additional protein-protein interactions and nucleate subsequent signaling processes. Since inhibition of mTOR is thought to be a promising strategy for treating cancers, as suggested by several recent clinical studies, this work will elucidate the biochemical mechanism of mTOR regulation of cell growth and human diseases, as well as contribute valuable information for the effective design of novel mTOR-inhibiting drugs.

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Melanoma associated antigens as T cell targets in LAM

Antibody HMB45, used to diagnose LAM, is reactive with melanoma-associated antigen gp100. Both gp100 and MART-1, likewise expressed in LAM, are frequently targeted by tissue infiltrating T cells in melanoma and vitiligo. The current study describes expression analysis of gp100 and MART-1 and additional melanoma-associated antigens tyrosinase, TRP-1 and TRP-2 in LAM. Also, lung infiltrating immune cells (CD4 and CD8 T cells, macrophages and dendritic cells) were semi-quantified, comparing abundance in LAM to unaffected lung tissue and melanoma metastases. Finally, well characterized HLA-A*0201 restricted T cells reactive with gp100 or tyrosinase-derived peptides were reacted with smooth muscle α -actin⁺, estrogen receptor α ⁺ cells cultured from resected LAM lung tissue of an HLA-A*0201+ transplant recipient, looking for cytotoxicity, T cell clustering and cytokine secretion. Immunohistochemical analysis of lung tissue from 5 LAM patients and 3 controls, and 3 melanoma tumors confirmed expression of gp100 and MART-1 by partially overlapping subsets of LAM cells. Expression of gp100 and some MART-1, as well as tyrosinase, TRP-1 and TRP-2 measured in stained cells/area was detectable in LAM, and expression of TRP-1 was more prominent in LAM tissue than in metastatic melanoma. T cell abundance was comparable in LAM (4% of sorted cells) and normal lung yet reduced compared to melanoma, whereas CD11c+ dendritic cells in LAM were 50% more abundant than in normal lung and 50% less than in melanoma. Macrophages were much more abundant in LAM than in control lung, and comparable to melanoma tumors. Differences in T cell numbers were primarily attributable to elevated CD8+ T cell infiltration in melanoma. Importantly, melanoma derived, gp100 reactive T cells but not tyrosinase reactive T cells were cytotoxic towards cultured LAM cells. Specificity of these responses was confirmed by T cell clustering observed in presence of HLA-matched melanocytes and LAM cells, but not fibroblasts or mismatched melanocytes, and by significant cytokine secretion by gp100 reactive but not tyrosinase reactive T cells in response to HLA-matched LAM cells as measured by IFN- γ and IL-2 ELISAs. These data have important implications for immune targeting of LAM cells. First, the proportion of targetable LAM cells extends beyond those detectable by HMB45 staining. Second, LAM cells functionally process and present gp100 derived peptides to CD8+ cytotoxic T cells. Finally, existing immune responses in LAM leave room for improvement. Taken together, these data support the feasibility of using vaccines anti- melanoma vaccines for the treatment of LAM.

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Estrogen and MEK-dependent pulmonary metastasis of tuberin-deficient cells—a metastatic mouse model of lymphangiomyomatosis

Introduction: LAM is a devastating disease affecting young women. The pathogenesis of LAM is very unusual: histologically-benign smooth muscle cells carrying *TSC2* mutations metastasize to the lungs, where they cause emphysema-like cystic lung degeneration. The reasons that LAM affects exclusively women are not yet clearly defined, and animal models that recapitulate the metastatic behavior of *TSC2*-null cells have not been previously developed. To determine whether estrogen promotes the metastasis of *TSC2*-null cells, we established a xenograft mouse model of LAM.

Methods: Mice were implanted with estrogen or placebo pellets. For xenograft tumor establishment, 2×10^6 ELT3 cells were bilaterally injected into the flanks of the mice. For lung colonization, 2×10^5 ELT3 cells were injected into the lateral tail vein. Lung metastases were scored from five-micron H&E stained sections of each lobe. MEK1/2 inhibitor, CI-1040 (150 mg/kg day by gavage, twice a day), was initiated one day after cell inoculation. To detect circulating ELT3 cells, real-time PCR of rat-specific DNA was performed. Bioluminescent signals were recorded at indicated times post-cell injection using the Xenogen IVIS System. Total photon flux at the chest regions and from the dissected lungs was analyzed. For anoikis studies, ELT3 cells were plated onto poly-hydroxyethyl methacrylate (PolyHEMA) culture dishes. Cell death as a function of cleaved caspase-3 or DNA fragmentation was measured.

Results: Using a xenograft model, we have found that estrogen increases the level of disseminated circulating ELT3 cells and promotes lung metastases of ELT3 cells in both male and female mice. Estrogen-treated primary tumors exhibit higher levels of nuclear phospho-p42/44 MAPK compared with placebo. These results suggest that the MEK/MAPK pathway contributes to the estrogen-induced metastatic potential of *Tsc2*-null ELT3 cells. CI-1040 reduces the number of circulating tumor cells, and decreases the frequency and the number of lung metastases by 100%. When ELT3 cells were injected intravenously, estrogen enhances their survival, and CI-1040 blocks the lung colonization. In vitro, estrogen reduces the anoikis, which is associated with enhanced MAPK phosphorylation and decreased levels of the pro-apoptotic Bim. Estrogen-decreased Bim protein level is restored by the inhibition of MEK and proteasome inhibitor. Both the estrogen-increased survival and resistance to anoikis are reduced by the MEK inhibition. These data indicate that the activation of MEK/MAPK signaling pathway might contribute to the elevated metastatic phenotype of ELT3 cells. This animal model may have relevance to both LAM pathogenesis and to the development of targeted therapeutic strategies for LAM.

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Neural crest origin and notch-dependent cell-fate decision in tuberous sclerosis complex

Unusual cell lineage expression patterns are a hallmark of TSC. For example, LAM and angiomyolipoma cells express smooth muscle and melanocytic markers, and angiomyolipomas contain fat, smooth muscle, and vascular elements. The underlying molecular mechanisms and the cell-of-origin that gives rise to LAM and angiomyolipomas are not yet well defined.

We used gene set enrichment analysis (GSEA) to reveal key developmental processes affected by dysregulation of the TSC pathway. Fifty-one out of 1746 gene sets were significantly ($p \leq 0.01$) regulated by Rheb in a LAM patient angiomyolipoma-derived cell line that carries bi-allelic TSC2 gene inactivation. Among the 51 gene sets, eight were related to cell differentiation, including mesenchymal cell differentiation (which had the lowest p value), neural crest cell migration, and neural crest cell differentiation. These data prompted us to ask whether the cells that give rise to angiomyolipoma and LAM may originate from neural crest-derived precursors. We found that angiomyolipoma-derived cells express a class of neural crest specifier genes, such as Snail1, Twist1, Sox9, and FoxD3, and that LAM cells inappropriately co-express neuronal and glial lineage marker.

The Notch signaling pathway plays a key role in controlling neural crest development in mammals. To address the hypothesis that loss of TSC1 impacts Notch-dependent cell-fate determination, we analyzed the role of TSC1/TSC2 in asymmetric cell division during *Drosophila* external sensory organ development, which is highly dependent on the regulation of Notch activity. We found that *Tsc1* mutations or Rheb overexpression causes a cell-fate switch consistent with Notch activation. To determine whether TSC1/2 impact Notch signaling in mammalian cells, we downregulated Rheb using siRNA in a LAM patient-derived angiomyolipoma cell line. Rheb depletion significantly lowered Notch activity and the transcript level of the Notch 1 downstream effector, HES-1. In both flies and mammals, we observed Rheb-dependent dysregulation of recycling endosomes and Delta trafficking. Because endosomal recycling of Delta results in Delta activation, these data indicate that Rheb activates Notch via the Notch ligand, Delta. Importantly, in both flies and mammals, Rheb-induced Notch activation is insensitive to Rapamycin, suggesting that it may be mTORC1-independent.

We conclude that LAM cells may be derived from a neural crest progenitor cell and that TSC1 participates in determining Notch-mediated cell fate specification, which may lead to aberrant cellular differentiation in LAM and angiomyolipomas. Aberrant cell-fate decisions could be a key event in the formation of TSC tumors and LAM.

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Estrogen receptor signaling - receptor subtypes, cellular locations, and signaling networks

Both the steroid hormone 17 β -estradiol (E2) and growth factors stimulate proliferation of estrogen-dependent breast cancer cells, and interactions between these signaling pathways occur at several levels. E2 exerts its action via estrogen receptor (ER) proteins of two subtypes, ER α and ER β . Both subtypes are present in normal breast cells, but ER α is overexpressed in most ER+ breast tumors, whereas ER β expression is decreased. ERs are classified as ligand-activated transcription factors residing primarily in the nucleus, and binding of the hormone ligand results in ER dimerization, recruitment of coactivator molecules, and activation of target gene transcription. ER α has greater biological activity, and ER β may act to modulate E2 proliferative effects through ER α . ER activity in breast cancer can be suppressed by antiestrogens such as tamoxifen, which bind ERs and prevent the recruitment of coactivator proteins. E2 responses have now also been functionally linked to cytoplasmic signaling pathways, including tyrosine kinase receptors for EGF and IGF-1 and the cytoplasmic tyrosine kinase c-Src; these may influence both biological and therapeutic responses. EGF and IGF-1 stimulate ER activity in the absence of E2 via activated protein kinases that phosphorylate steroid receptors and receptor coregulators. More importantly, E2 binding to cognate cytoplasmic or membrane-associated ERs, primarily ER α , rapidly activates intracellular signaling cascades including c-Src, ERK, and PI3K/Akt. These E2-stimulated phosphorylations ultimately alter tumor cell function by stimulating cellular proliferation and survival, and suppressing apoptosis. In breast cancer cells, ER interacts directly with the intracellular tyrosine kinase c-Src and other cytoplasmic signaling molecules, such as Shc, PI3K, MNAR, and p130 Cas. Although the hierarchy among these associations is not understood, it is clear that c-Src plays a fundamental role in both growth factor and E2-stimulated cell growth, with participation of other tyrosine kinase growth factor receptors such as those for EGF or IGF-1. STAT transcription factors are important pathway to integrate E2 cytoplasmic and nuclear signaling. STAT5 is phosphorylated in the cytoplasm at an activating tyrosine in response to E2 or EGF, then is translocated to the nucleus to stimulate target gene transcription. E2 stimulates recruitment of STAT5 and ER to the promoter of proliferative genes, and STAT5 knockdown prevents recruitment of either protein to these promoters. STAT5 activation by E2 in breast cancer cells requires c-Src and EGF receptor, and inhibition of c-Src or EGFR, or knockdown of STAT5, prevents E2 stimulation of gene transcription and breast cancer cell proliferation. Hyperactivation of the EGF receptor-c-Src STAT5 pathways, either by overexpression or increased cellular activation, renders cells resistant to suppressive actions of tamoxifen, and has been identified in tumors therapeutically resistant to tamoxifen. Tamoxifen-resistant cells arising through multiple pathways, including knockdown of BRCA1 (breast cancer susceptibility gene-1) mRNA, or hyperactivation of c-Src, appear to have significantly higher levels of ER α in the cytoplasm, thus allowing amplified signaling through cytoplasmic pathways. In opposition to this, inhibition of c-Src or growth factor receptor activities can restore sensitivity to antiestrogens, and may form the basis of potential combinatorial therapies. Crosstalk between growth factors and steroids in both the cytoplasm and nucleus may thus have profound impact on complex biological processes such as cell growth, and play a significant role in the treatment of steroid-dependent cancers.

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p27 as mediator of cell motility/migration downstream of PI3K/mTOR: therapeutic implications for cancer and LAM

The PI3K pathway is a master regulator of cell growth, proliferation, survival, motility, and differentiation^{1,3}. PI3K and its inhibitory phosphatase, PTEN, play important roles in the activation of cell polarity and in the coordination of cell migration⁴. Oncogenic PI3K activation is frequent in human cancers^{1,5,6} arising through amplification of the PI3K subunit *P110-alpha*, mutation of the PI3K *p85* subunit⁶ receptor tyrosine kinase (RTK) activation, mutational activation of *Ras* and *PTEN* loss^{7,8}. PI3K activates phosphoinositide-dependent kinase 1 (PDK1), which in turn activates AGC family kinases including PKB (also known as Akt), protein kinases A, G and C, the serum and glucocorticoid-inducible kinase (SGK), the 70 KDa S6 kinase (p70^{SGK}), and 90 KDa ribosomal kinase (p90^{RSK})^{2,3,9}. In addition to phosphorylation by PDK1, AGC kinases require a second activating phosphorylation. PKB/Akt activation requires phosphorylation by mTOR-riCTOR¹⁰, and we showed that activated mTOR recruits raptor to phosphorylate and activate SGK1¹¹. PKB/Akt also stimulates mTOR by phosphorylation of the mTOR inhibitor TSC2 (review). Work from our lab and others demonstrate the interplay between PI3K/mTOR pathways and the cell cycle via effects on the cdk inhibitor, p27. New data below also describe a role for PI3K/TORC1/2 activation in p27-mediated cell motility.

p27 plays a dual role to inhibit cyclin E-Cdk2 and to promote assembly of D-type cyclin-Cdks. While p27 is often reduced in cancers, p27 gene deletion is rare and p27 is rarely completely lost in human cancers. Cytoplasmic p27, observed in many cancers is associated with poor patient prognosis. Our prior work showed that PKB/Akt phosphorylates the cell cycle inhibitor p27 at threonine 157 (T157) to impair its nuclear import leading to cytoplasmic sequestration of p27¹². We recently showed that mTOR:raptor phosphorylates SGK1 to modulate p27 function¹¹. mTOR activation, by refeeding of amino acid deprived cells or by TSC2 shRNA, activated SGK1 and p27 phosphorylation at T157 and both were inhibited by short-term rapamycin and by SGK1 shRNA. mTOR overexpression activated both Akt and SGK1, causing TGF-beta resistance through impaired nuclear import and cytoplasmic accumulation of p27. TORC1 phosphorylated SGK1 *in vitro* and rapamycin or *raptor* shRNA impaired mTOR driven p70 and SGK1 activation, but not that of Akt, and decreased cytoplasmic p27. SGK1 phosphorylated p27 *in vitro* and shRNA to SGK1 reduced cellular p27pT157. Thus, mTOR may promote G1 progression in part through SGK1 activation and deregulate the cell cycle in cancers through both Akt and SGK-mediated p27 T157 phosphorylation and cytoplasmic p27 mislocalization.

Cytoplasmic p27 acquires an oncogenic gain of function, by altering the cytoskeleton to promote cell motility. p27 binds RhoA and inhibits RhoA-ROCK causing actomyosin destabilization^{13,14}. p27CK-knock-in to p27 null mice caused cytoplasmic p27 localization, a gain of cell motility independent of effects on cyclin-Cdks and expansion of progenitor/stem cell like populations and lung tumor formation. Thus, p27 regulates both cell proliferation and migration and cytoplasmic p27 appears to have a pro-oncogenic action to promote cell motility independent of its action on cell cycle, which may explain why p27 is rarely entirely lost in human malignancies.

AGC kinases also stimulate cell motility. AGC family kinases PKB, SGK1 and RSK1 all phosphorylate p27 at T157, T198, or both. T157 phosphorylation impairs nuclear import of p27 and T198 phosphorylation stabilizes p27 in the cytoplasm. Cells overexpressing RSK2, SGK and mTOR are all more motile and have reduced actin stress fibers, reduced RhoA-GTP and p-cofilin and this is reversed by downregulating p27 via shRNAp27. Thus, effects of these kinases on cell motility are p27

dependent. PTEN-delete cells show increased p27-RhoA co-precipitation and increased p27^{pT198}. T198 phosphorylation increased p27:RhoA binding in vitro and p27^{T198A} binds less well to RhoA than WTp27 in cells.

p27 appears to be required for PI3K driven metastasis. MDA-MB-231-derived lines selected for high metastatic to lungs and bone showed activation of PI3K effectors phospho-mTOR, pPKB/Akt, pSGK and pRSK, increased stable cytoplasmic p27 and increased cell motility that was reversed by p27 knockdown. Moreover, the increased formation of lung metastasis by MDA-MB231-4715 was reversed by knockdown of p27 to levels similar to those in parental MDA-MB-231. These data implicate p27 as a key mediator of metastasis. Thus, the action of PI3K to stimulate cell motility and tumor progression may arise in large part through oncogenic effects of cytoplasmic p27. Identifying the interaction sites between p27 and RhoA may permit the design of therapeutic targeting agents that disrupt this interaction to prevent tumor invasion and metastasis. In cancers, cytoplasmic p27 may be a useful predictor of response to PI3K and TORC1/TORC2 inhibitor drugs and help select patients likely to respond to these agents. Inhibition of these pathways may also be of benefit in other hyperproliferative disorders with critical activation of this pathway, such as pulmonary lymphangioleiomyomatosis or TSC.

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Buttons and Zippers – Functionally specialized junctions at sites of fluid and cell entry into lymphatic vessels

Movement of fluid and cells into lymphatic vessels plays a key role in tissue fluid control, immune responses, and inflammation. Despite recent advances in understanding lymphatic function, the cellular features responsible for entry of fluid and cells into lymphatics and factors that regulate lymphatic growth are incompletely understood. We found the presence of highly specialized junctions between endothelial cells of initial lymphatics at likely sites of fluid and cell entry. Overlapping flaps at the border of oak leaf-shaped endothelial cells of initial lymphatics lacked junctions at the tip but were anchored on the sides by discontinuous button-like junctions that differed from conventional, continuous, zipper-like junctions in collecting lymphatics and blood vessels. Button-like junctions were composed of discontinuous adherens junctions and tight junctions. The junctions were disrupted by inhibition of VE-cadherin by a function-blocking antibody but not by deletion of PECAM-1. Growth of lymphatics in the inflammatory airway disease accompanying *Mycoplasma pulmonis* infection was blocked by selective inhibition of integrin $\alpha 5\beta 1$. Our findings indicate that fluid and cells enter initial lymphatics through specialized, discontinuous button-like junctions. The growth of new lymphatics is blocked by inhibition of integrin $\alpha 5\beta 1$, which is overexpressed in inflamed lymphatics and is essential for formation of lymphatic sprouts.

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Tuberous sclerosis and LAM: Pathogenic mechanisms

Tuberous sclerosis complex (TSC) is a multisystem disorder in which the manifestations can include tumors in the brain, heart, kidney, and skin, as well as mental retardation and autism. My laboratory has a particular interest in the renal manifestations of TSC (cysts, angiomyolipomas, and carcinomas) and in the pulmonary manifestation, lymphangiomyomatosis (LAM). About 30% of women with TSC develop LAM, which is an unusual lung disease in which benign-appearing smooth muscle cells proliferate extensively in the lungs, leading to cystic, emphysema-like lung destruction and lung failure. LAM pathogenesis appears to involve one of the most unusual pathogenic mechanisms in human disease: the metastasis of histologically benign cells.

The protein products of the *TSC1* and *TSC2* genes (hamartin and tuberin, respectively) physically interact to inhibit the activity of the kinase, TORC1. This mTOR inhibition is achieved the small GTPase Rheb which is regulated by the GAP (GTPase activating protein) region of *TSC2*. A great deal of attention has been focused on the role of TORC1, which is inhibited by Rapamycin, in the pathogenesis of TSC and LAM. A key area of uncertainty is whether Rheb has TORC1-independent targets that are disease-relevant.

Currently we are examining 1) the mechanisms through which estrogen promotes the metastasis of *TSC2*-null cells, thereby promoting LAM pathogenesis, focusing on the role of the MEK pathway in enhancing the survival of circulating *TSC2*-null cells; 2) the role of the TSC pathway in neural development, using the *Drosophila* external sensory organ (ESO) as a model of asymmetric cell division; and 3) the connections between the TSC proteins and the Birt-Hogg-Dube (BHD) protein. BHD (like TSC) is associated with renal tumors, facial lesions, and cystic lung disease. In *S. Pombe* we have found that the BHD homolog functions in the TOR signaling pathway, but in opposition to the TSC proteins.