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14. ABSTRACT The purposes of this work are three related objectives: to generate human TSC2-/- LAM cell lines; to generate matching inducible isogenic TSC2 knock-in cell lines; and to confirm the absence and rescue of TSC signaling in these LAM lines. We generated 400 immortalized LAM cell lines from two different LAM tissue sources and used immunoblot analysis to determine the loss of the TSC2 protein itself, or function as indicate by phosphorylation of the ribosomal protein S6 in the absence of serum. Despite the fact that the original LAM tissues suggested loss of TSC2 by FISH analysis in a subset of the tissue, none of the newly developed lines appear to be negative for TSC2. We feel, however, that these lines still be valuable to the TSC and LAM communities and we will therefore, disseminate the generated cell lines upon request.					
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Introduction

Tuberous Sclerosis Complex (TSC) and Lymphangioleiomyomatosis (LAM).

TSC is an autosomal dominant disorder characterized by widespread benign tumors (known as hamartomas), epilepsy, mental retardation, and autism. Occurring 1 in 6,000 live births, TSC is linked to mutations in the tumor suppressor genes, TSC1 and TSC2. Mutation in either of these two genes leads to the clinical manifestations of TSC. A common characteristic of the disease is renal dysfunction. Approximately 70-80% of TSC patients develop renal angiomyolipomas (AMLs), which if left untreated, exhibit a high incidence of spontaneous hemorrhage. TSC patients also present with evidence of a devastating form of cystic lung disease called LAM. LAM is a unique and rare cystic pulmonary disease that afflicts predominately premenopausal women. While its prevalence is not precisely known, up to one thousand women may be affected by LAM annually in the United States. The clinical symptoms are dyspnea, chronic cough, wheezing, pneumothorax, and chest pain. These symptoms occur and worsen as LAM cells migrate into the lung, causing cystic parenchymal destruction and progressive respiratory failure. Currently, the only treatment for LAM is lung transplantation. The genetic connection between LAM and TSC is evident in work done by Henske et al., revealing inactivating mutations in the TSC2 gene in both TSC patients with LAM (TSC LAM) and non-TSC LAM patients (sporadic LAM)¹. TSC patients with clinically diagnosed LAM were thought to be quite rare (<4%), but recent studies using High Resolution Computed Tomography (HRCT) scans indicate evidence of LAM in 26-42% of women with TSC².

E6 and E7 immortalization. Normal human cells have a finite life span in culture before they irreversibly withdraw from the cell cycle and die. This process is referred to as either cellular senescence or crisis. The passage at which crisis occurs is cell type specific, although most primary cells will undergo senescence somewhere between 5 and 25 cell divisions. This finite life span presents a major hurdle for studying disease cells *in vitro*. A process referred to as “immortalization” relieves this requirement for senescence and allows cells to grow past crisis. Unlike transformed or cancer cells, immortalized cells will not form tumors in nude mice and cannot be grown in suspension or soft agar, i.e., are anchorage dependent. *This is a critical distinction with regards to TSC cells as they are benign tumors, not malignant in nature.* Therefore, the defining characteristic of immortalized cells is the ability to overcome crisis and continue to proliferate indefinitely, without being tumorigenic. One well established strategy to immortalize human cells is to express the E6 and E7 proteins of the high risk Human Papilloma Virus (HPV-16, HPV-18) that inhibit the cellular tumor suppressor proteins, p53 and pRB, respectively³. This approach has three benefits: E6/E7 expression rarely leads to cellular transformation; E6/E7 are encoded on a single mRNA by alternative reading frame usage and requires only a single transcript be expressed; and this system has a proven track record of success with difficult-to-immortalize cell types. We have successfully used the E6/E7 immortalization strategy to immortalize human TSC2^{-/-} AMLs, subungual fibroma and normal fibroblasts from a TSC patient. These novel human TSC cell lines do not exhibit any signs of transformation and provide a proof of principle for the E6/E7 immortalization process for generating human TSC cell lines.

Rationale/Purpose The clinical manifestations of LAM and TSC are unmet medical needs. No approved therapies exist and only one therapeutic, the mTOR inhibitor rapamycin [Wyeth (Madison, NJ)], is under clinical investigation. We believe that the lack of developed therapeutics to treat TSC and LAM in humans is in part a result of focusing TSC research predominantly on rodent cell lines derived from TSC1 and TSC2 knock-out animals. These rodents do *not* develop either LAM or AMLs and so such lines may not prove accurate for identifying compounds to treat these human-specific lesions⁴. Several of these rodent lines display anchorage-independence, another key difference with TSC manifestations in humans. There are no human TSC^{-/-} LAM cell lines and only one known immortalized human AML cell line⁵. However, this line does not possess mutations in either TSC1 or TSC2 and may not represent the majority of AMLs *in vivo*. *We have successfully used the E6/E7 immortalization strategy detailed in this proposal to generate the first TSC2^{-/-} human AML cell lines*, and we are confident that this process will be successful in generating the first human TSC^{-/-} LAM cell lines.

Proposal Overview This proposal outlines three related objectives: to generate human TSC2^{-/-} LAM cell lines; to generate matching inducible isogenic TSC2 knock-in cell lines; and to confirm the absence and rescue of TSC signaling in these LAM lines. **We anticipate disseminating the generated cell lines to the TSC research community.** They represent a novel and important set of reagents that will help us gain a better understanding of the molecular and genetic mechanisms contributing to TSC and LAM cell pathogenesis so that we may therapeutically target these pathways. Each specific aim is divided into two sections: experimental methods and potential problems/alternative approaches.

Specific Aim 1: To generate human TSC2^{-/-} LAM cell lines.

Strategy: Infect TSC2^{-/-} human LAM cells with a MoMLV retrovirus carrying an E6, E7, and G418- resistance encoding vector.

Specific Aim 2: To generate conditional TSC2 knock-in LAM cell lines.

Strategy: Infect a TSC2^{-/-} LAM cell line with a MoMLV retrovirus carrying a tetracycline-inducible TSC2 encoding vector and a transactivation plasmid.

Specific Aim 3: To confirm aberrant and rescued TSC signaling in TSC2^{-/-} LAM and isogenic TSC knock-in cell lines, respectively.

Strategy: Verify both serum-independent and rescued serum-dependant S6 and S6kinase phosphorylation of the TSC2^{-/-} LAM and TSC2 knock-in cell lines by immunoblotting.

Body

Tissue acquisition A LAM lesion is much more heterogeneous than an AML with only 10-20% at most being negative for TSC2. Therefore it is critical to obtain LAM samples that have verified TSC2-negative cells within the tumor cell mixture. We obtained two LAM samples from Dr. Joel Moss, NHLBI (Bethesda, MD) designated LAM1 and LAM4. By FISH analysis, Dr. Moss was able to determine that ~35% of the cells in the LAM1 sample were LOH for TSC2 and ~20% in LAM4. We used these samples to generate our LAM cell lines.

Immortalization A heterogeneous cell population of LAM1 and LAM4 were infected as a pool with a recombinant deficient Moloney Murine Leukemia Virus (MoMLV) carrying the E6/E7 genes from the Human Papilloma Virus (HPV) and a neomycin-resistance cassette. Cells were then grown in neomycin selection medium for 20 days, and ~200 individual colonies were isolated. The success of gene transfer was deemed to be approximately 90% as determined by co-infection with Green Fluorescent Protein (GFP). The parental primary cells will grow in culture for 8-10 passages, while E6/E7 expressing clones have been growing in culture for 6 months and do not appear to be slowing.

TSC2, S6-P (Ser235/236) immunoblot validation The first step towards confirming the generation of a LAM line is to verify the loss of expression of the TSC2 protein or function indicated by phosphorylation of the ribosomal S6 protein in the absence of serum (Figure 1, 2). LAM4 was analyzed similarly (data not shown). For the S6-P analysis, duplicate 10cm plates of each LAM clone that did not exhibit a strong TSC2 signal by western blot, and TSC2 positive and negative control lines (MEF118, MEF119 from TSC2 knockout mice), were grown in medium lacking fetal bovine serum for 48 h. 20% serum was added to one of the duplicate plates and grown for another hour. Cells were then harvested and 30ug of total protein was analyzed by western blot.

Figure 1.

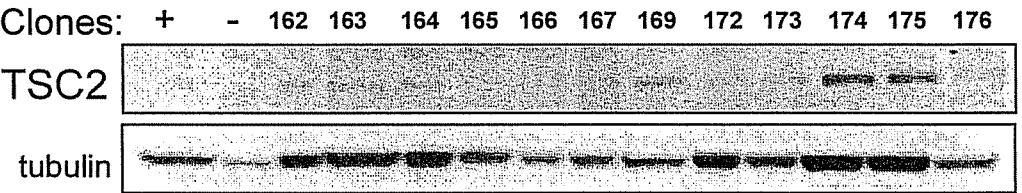
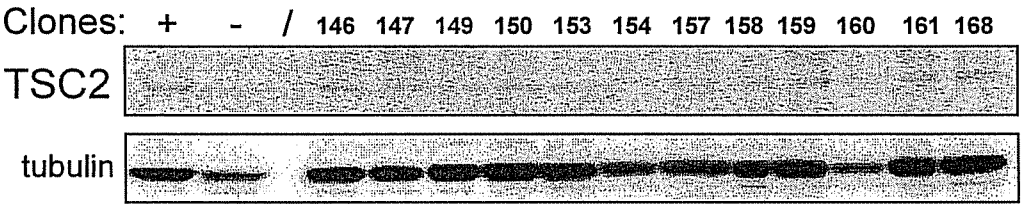
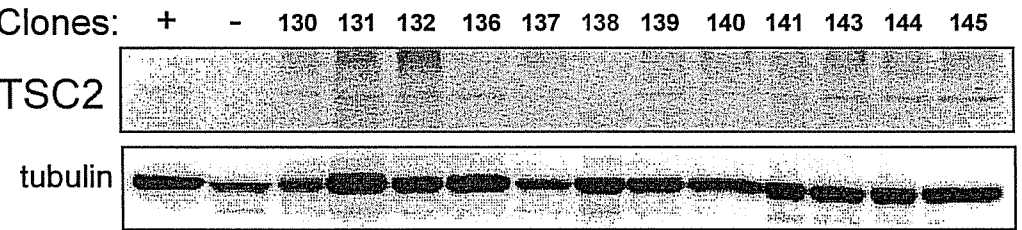
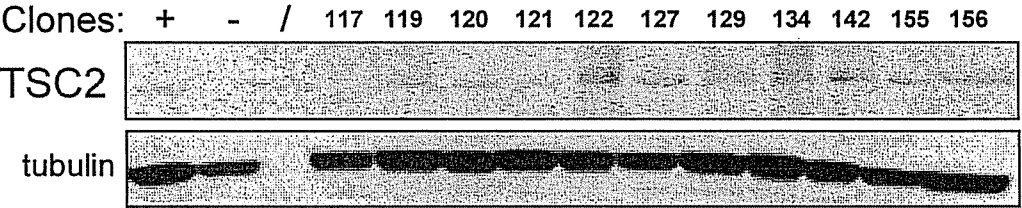
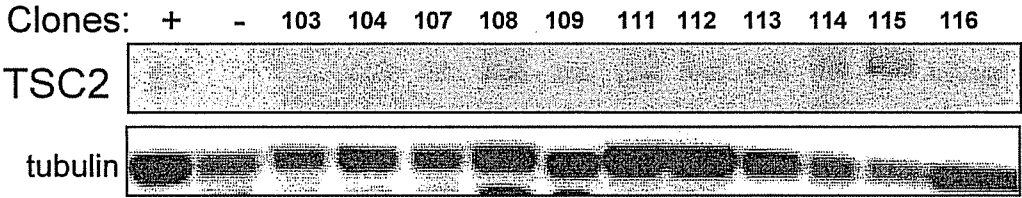


Figure 1

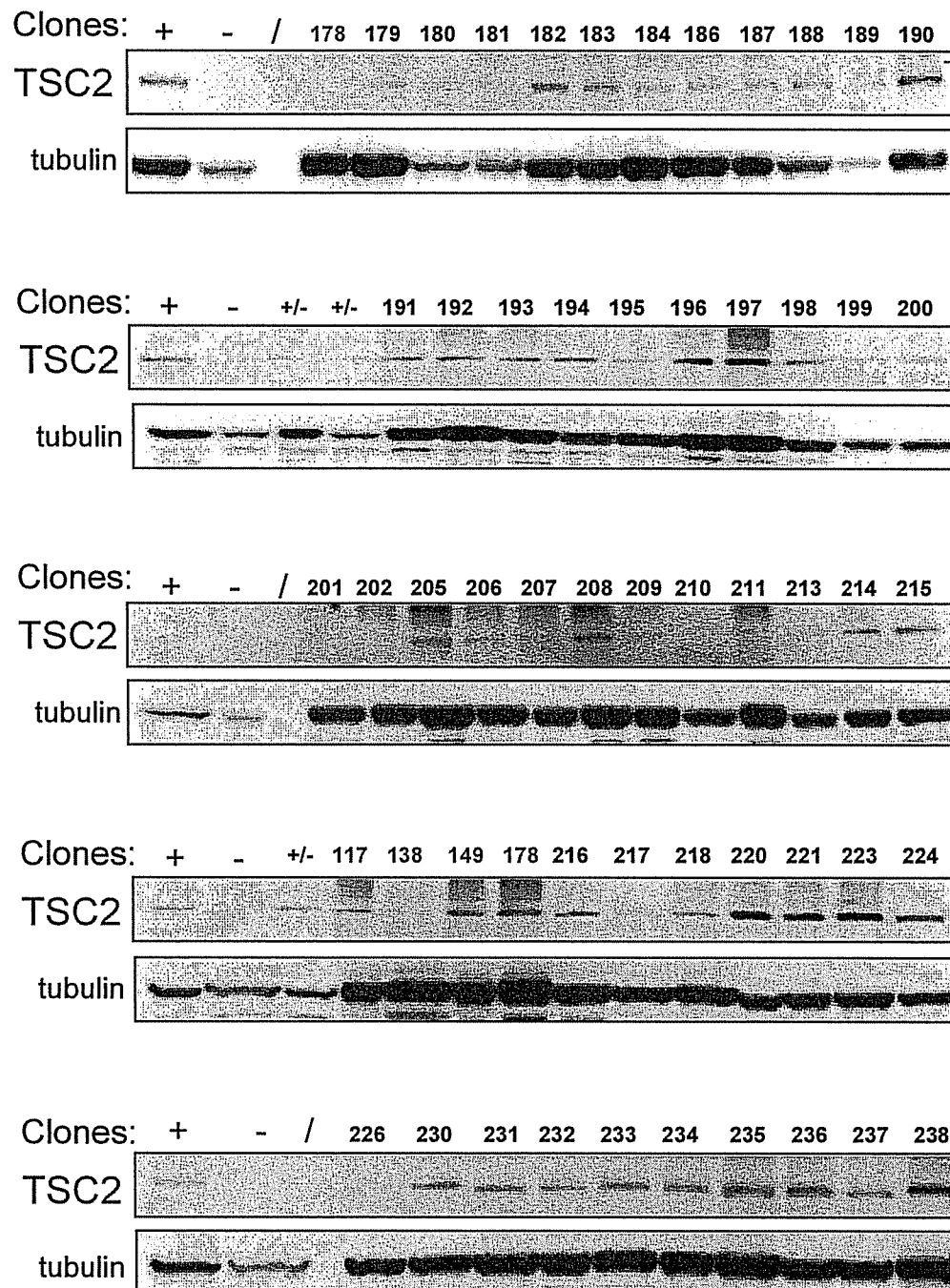


Figure 1. Immunoblot validation of TSC2 expression in LAM1 clones. 30ug of total protein from each LAM1 clone and TSC2 positive and negative control lines (+, -) were separated by 4-20% SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-TSC2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-alpha tubulin (Sigma-Aldrich, St. Louis, MO). Clones listed in red were re-blotted to confirm TSC2 expression.

Figure 2.

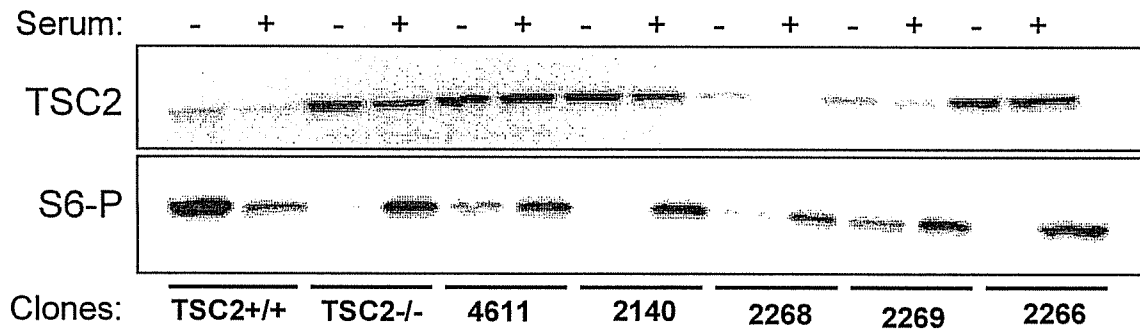


Figure 2. Immunoblot validation of S6-P (Ser235/236) expression in LAM1 clones. Two, 10cm plates of each LAM clone and positive and negative TSC2 controls were grown in medium without serum for 48h, then 20% fetal bovine serum was added to one of the duplicate plates, and cells were harvested 1 h post serum addition. 30ug of total protein from each LAM1 clone was separated by 4-20% SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-TSC2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-S6-P (Cell Signalling Technology, Danvers, MA).

Key Research Accomplishments

- Generation of ~400 LAM cell lines from LAM tissue taken from two different patients
- Established successful methods for immortalization of human LAM primary cells using exogenous expression of the E6/E7 genes from HPV
- Established screening protocol to examine TSC2 expression or function in human LAM cells

Reportable Outcomes

- Successful immortalization of human LAM cells using E6/E7 constitutive expression

Conclusions

We have successfully immortalized ~400 clones from two LAM tissues using the E6/E7 genes from HPV. We subsequently examined the expression of TSC2 in all clones and S6-P in the absence of serum of several clones that had questionable TSC2 expression by blot. We must conclude however that there is loss of TSC2 expression or function in any of the clones examined. The prevalent expression of GFP which was infected simultaneously with E6/E7 suggests the gene delivery system worked well. Either stable incorporation into the genome or subsequent toxicities from the HPV genes or increased cell proliferation is most likely the cause of no TSC2^{-/-} cells surviving. It is possible that the original LAM material was, in fact, not LOH for TSC2, but we don't find this probable. The lack of TSC2^{-/-} LAM or human TSC cell lines available suggests that loss of this protein may in some way prevent immortalization.

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