Molecular Pathogenesis of Genetic and Inherited Diseases

Isolation and Growth of Smooth Muscle-Like Cells Derived from Tuberous Sclerosis Complex-2 Human Renal Angiomyolipoma

Epidermal Growth Factor Is the Required Growth Factor

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Tuberous sclerosis complex (TSC) is an autosomal dominant syndrome characterized by the multiorgan development of benign and occasionally malignant tumors that most frequently affect the central nervous system, kidney, and skin.1 In particular, the kidney tumors include angiomyolipomas and renal cell carcinomas, of which the former may cause renal failure as a result of the replacement of kidney parenchyma with tumoral tissue, possibly leading to life-threatening hemorrhage.2 Angiomyolipomas consist of smooth muscle cells, adipose tissue, and disorganized thick-walled vascular channels.3

TSC is characterized by mutations in the TSC1 or TSC2 tumor suppressor genes, which seem to act together as a complex of the encoded hamartin (TSC1) and tuberin (TSC2) proteins.4 Mutations in TSC1 on chromosome 9q34 and TSC2 on chromosome 16p13 lead to similar clinical phenotypes, which are more severe in the case of the TSC2 subtype.5–7 The loss of heterozygosity (LOH) for the wild-type allele corresponding to the germline TSC1 or TSC2 mutation in the TSC lesion can be explained with the two-hit tumor suppressor gene model.8 LOH of both has been documented in angiomyolipomas, astrocytomas, and rhabdomyomas from TSC patients.9,10 The frequency of LOH varies significantly among tumor types, being high in angiomyolipomas and low in central nervous system lesions.9

TSC can occur in association with pulmonary lymphangioleiomyomatosis (LAM), a progressive and often fatal interstitial lung disease characterized by the diffuse proliferation of abnormal smooth muscle cells and cystic degeneration of lung parenchyma.11,12 The smooth muscle cells in angiomyolipomas are very similar to those of...
pulmonary LAM, and genetic data suggest that LAM may be the result of benign cell metastases, a highly unusual disease mechanism. The same mutation and LOH have been found in the abnormal pulmonary smooth muscle cells and angiomyolipoma of a large percentage of LAM patients with renal angiomyolipomas, which suggests that LAM and TSC may have a common genetic origin.

The TSC1/TSC2 complex negatively regulate cell size and proliferation. TSC2 is a direct target of Akt, a PI3K-regulated effector that promotes cell growth and survival by means of a mammalian target of rapamycin (mTOR)-dependent mechanism. Most of the extracellular and intracellular signal pathways involved in the regulation of growth factor- and nutrient-mediated cell growth are integrated by mTOR, as is suggested by the increased phosphorylation of S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein-1 (4EBP1). Rapamycin is a microbial product that counteracts these effects by inhibiting mTOR, and Akt is a prosurvival and pro-oncogenic protein that is phosphorylated after the activation of growth factor receptors. Akt phosphorylation decreases the ability of TSC2 to inhibit the phosphorylation of the mTOR substrates S6K and 4EBP1, and excessive Akt, mTOR, and S6K activation causes various types of tumor, including hamartomas. TSC1 or TSC2 mutant cells show a high degree of S6K and 4EBP1 phosphorylation, but the overexpression of TSC1 and TSC2 inhibits the phosphorylation of both, thus suggesting that the major cell function of TSC1/TSC2 is to inhibit translation by blocking the phosphorylation of S6K and 4EBP1.

We here describe the isolation and characterization of two cell populations from an angiomyolipoma of a TSC2 patient: actin-positive smooth muscle-like cells and keratin 8/18-positive epithelial-like cells. We identified the mutation (corresponding to a TSC2 alteration) that led to the loss of wild-type alleles in the smooth muscle cell population. The growth and proliferation of the LOH smooth muscle-like cells required epidermal growth factor (EGF) in the culture medium, and the cells released abundant insulin-like growth factor (IGF)-I into the medium. The addition of IGF-I to the culture medium stimulated the proliferation of control smooth muscle cells from human aorta, but not that of the TSC2 LOH smooth muscle-like cells. Our experiments have been repeated several times throughout the last 2 years, thus confirming the reliability of our TSC2 human smooth muscle cells.

Materials and Methods

Establishment of the Angiomyolipoma Culture

The spontaneous renal angiomyolipoma sample (30 cm) was obtained during total nephrectomy from a 42-year-old female with a history of TSC who had given her informed consent according to the Declaration of Helsinki. The study was approved by the Institutional Review Board of Milan’s San Paolo Hospital. The tumor tissue was dissociated using sterile filtered collagenase type II (Sigma, St. Louis, MO) in phosphate-buffered saline after manual dissociation by means of repetitive pipetting. The collagenase was neutralized with a serum-containing medium (50/50 mixture of Dulbecco’s modified Eagle’s medium/Ham F12; Euroclone, Paignton, UK) supplemented with hydrocortisone 2 × 10⁻⁷ mol/L (Sigma), EGF 10 ng/ml (Sigma), sodium selenite 5 × 10⁻⁸ mol/L (Sigma), insulin 25 μg/ml (Sigma), transferrin 10 μg/ml (Sigma), ferrous sulfate 1.6 × 10⁻⁶ mol/L (Sigma), and 15% fetal bovine serum (Euroclone) as indicated by Arbiser and colleagues. The CT/G human aorta vascular smooth muscle cells (VSMCs) were maintained in F12 medium containing 10% fetal bovine serum (American Type Culture Collection, Manassas, VA).

Histology and Immunohistochemistry

The tissue was frozen in liquid nitrogen-cooled isopentane, and stored at −80°C until sectioning. The angiomyolipoma was routinely stained with hematoxylin and eosin (H&E). The slides were deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Endogenous peroxide activity was blocked with 3% hydrogen peroxide for 30 minutes at room temperature, followed by a brief rinse in distilled water and a wash in phosphate-buffered saline. The tissue sections underwent pepsin enzyme digestion before antibody staining. Nonspecific background noise was inhibited by means of incubation in 5% goat serum. The primary α-actin HHH35 (1:100; DAKO, Carpinteria, CA), HMB45 (1:100, DAKO), and RhoA antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) were incubated overnight at 4°C, and localized using the avidin-biotin complex immunoperoxidase method with the immunopure standard ABC staining (Pierce, Rockford, IL) diluted 1:100.

Cell Immunofluorescence Microscopy

The cells were cultured on glass slides, permeabilized with 70% methanol for 10 minutes, and dried in air. The primary antibodies against α-actin (1:100; Sigma), vimentin (1:70; Santa Cruz), HMB45 (1:100; DAKO), S100 (1:8000; DAKO), CD68 (1:100, DAKO), keratin 8/18 (1:100, Santa Cruz), hamartin (1:100, a gift from Dr. Nellist and Dr. Halley, Erasmus University, Rotterdam, The Netherlands), and RhoA (1:100; Santa Cruz) were applied overnight at 4°C. The samples were incubated for 3 hours at room temperature with fluorescein isothiocyanate-conjugated rabbit antibody (Chemicon, Temecula, CA) for α-actin, HMB45, CD68, and keratin 8/18, and fluorescein isothiocyanate-conjugated donkey anti-goat antibody (Chemicon) for vimentin and S100, and rhodamine-conjugated goat anti-rabbit antibody (Chemicon) for hamartin and RhoA. After washing, the slides were mounted in 50% glycerol with 1 μg/ml 4,6-diamidino-2-phenylindole.

Mutation Study

The DNAs were extracted from peripheral lymphocytes and cultured cells using the Wizard Genomic DNA puri-
fication kit (Promega, Madison, WI). All of the exons of TSC1 and TSC2 from the genomic DNAs were amplified by means of standard polymerase chain reaction (PCR) and previously described primers in a 25-μl final volume mix containing 10 to 50 ng of genomic DNA, 200 μmol/L dNTPs, 1.5 mmol/L MgCl2, 10 pmol of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The TSC1 and TSC2 amplimers were divided into those that were successfully amplified at annealing temperatures of, respectively, 55°C, 60°C, and 65°C. Mutations were detected by submitting the PCR products to denaturing high-performance liquid chromatography (DHPLC) (Transgenic, Crewe, UK). To enhance heteroduplex formation, the untreated PCR product was denatured at 94°C for 5 minutes followed by gradual reannealing at 35°C for 1 hour. The samples were analyzed at the melt temperature determined using the DHPLC melt software. The products showing variant DHPLC melt profiles were directly sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems), and the results were analyzed using sequence analysis 3.4.1 software (Applied Biosystem). The sequencing reactions in which mutations were identified were repeated at least twice.

**LOH Analysis**

The panel of microsatellite markers near the TSC2 locus on chromosome 16p13.3 consisted of Kg8, D16S287, D16S291, D16S525, D16S665, D16S3024, and D16S3394. The sense primers were labeled with 6-FAM fluorescent dyes (M-Medical, Cornaredo, Italy). The primer sequences were obtained from the Genome Database (www.gdb.org). LOH was analyzed in a 25-μl final volume mix containing 10 to 50 ng of genomic DNA, 200 μmol/L dNTPs, 1.5 mmol/L MgCl2, 10 pmol of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems). PCR amplification consisted of 94°C for 10 minutes followed by 32 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and final extension at 72°C for 15 minutes. The PCR products were analyzed using a 310 ABI prism (Applied Biosystems). All of the analyses were repeated at least twice.

**Western Blotting**

The tissues were homogenized using an Ultra Turrax polytron in 5 vol of a homogenization buffer consisting of 25 mmol/L Tris-HCl, pH 7.4, 0.4 mmol/L sodium azide, 0.4 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine-HCl, 0.4 mmol/L EDTA, 0.4 mmol/L EGTA, and 0.25 mol/L sucrose. The homogenate was centrifuged at 1500 × g for 10 minutes at 4°C, and the supernatant was used for Western blot analysis. The cells were lysed in lysis buffer (5 mmol/L EDTA, 100 mmol/L deoxycholic acid, 3% sodium dodecyl sulfate). The tissue and cell samples (50 μg) were boiled, electrophoretically run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL). After being blocked at room temperature for 3 hours with 5% dry milk (Merck, Darmstadt, Germany), the membranes were incubated overnight at 4°C with antibodies against tuberin (1:1000; Cell Signaling, Beverly, MA) and tuberin (C-20) (1:100; Santa Cruz), phospho-tuberin (1:1000; Cell Signaling), hamartin, the β subunit of the IGF-1 receptor (1:100; Santa Cruz), EGF receptor (1:200; Santa Cruz), phospho-Akt (1:500; Promega), Akt (1:200; Santa Cruz), phospho-STAT6 (1:1000; Cell Signaling), S6K1 (1:1000; Cell Signaling), phospho-S6 ribosomal protein (Ser235/236) (1:1000; Cell Signaling), phospho-4EBP1 (1:1000; Cell Signaling), 4EBP1 (1:1000; Cell Signaling), phospho-extracellular signal-regulated kinase (ERK) (1:1000; Cell Signaling), and phospho-mTOR (1:1000; Cell Signaling). The membranes were washed and incubated for 1 hour with antimouse antibody (1:10,000; Chemicon) for phospho-S6K and anti-rabbit antibody (1:10,000; Chemicon) for all of the other antibodies. The reaction was revealed using the SuperSignal West Pico chemiluminescent substrate (Pierce).

**Evaluation of Cell Growth and Proliferation**

The growth rates of the smooth muscle-like and epithelial-like cells were compared by counting at least 400 to 500 cells in an improved Neubauer chamber after 4 and 8 days of culture. The proliferation growth factor dependence of the smooth muscle-like cells and VSMCs were assayed by replacing EGF with IGF-1 (50 and 5 ng/ml), by counting the cells after 4, 7, 10, 15, and 21 days of culture in the Neubauer chamber. The action of rapamycin was monitored by adding 1 ng/ml to the A+ cells at plating time, with or without EGF, and measuring cell proliferation after 3, 5, and 10 days. Anti-EGF receptor (clone 225; Calbiochem, Darmstadt, Germany), anti-EGF receptor (clone EGRFR1; Calbiochem), and anti-IGF-1 receptor (clone αIR3; Calbiochem) were added at a concentration of 5 μg/ml to the complete medium and to the medium deprived of EGF, and cell proliferation was evaluated after 2, 5, 10, and 12 days of culture. The action of wortmannin (Sigma) and PD98059 (Sigma) was evaluated by adding them to the culture medium, and evaluating their effect on cell proliferation 48 hours later. Each data point of the proliferation experiments is the mean of four independent experiments.

**IGF-1 Enzyme-Linked Immunosorbent Assay**

The IGF-1 released by the A+ cells was assayed using an IGF-1 enzyme-linked immunosorbent assay kit in accordance with the manufacturer’s instructions (KAPB2010; Biosource Europe S.A., Nivelles, Belgium). The cells were incubated for 24 hours before the medium was collected and IGF-1 determined. These assays were performed 14 and 21 days after A+ plating.
Statistical Analysis

The data are expressed as mean values ± SEM, and were statistically analyzed using Student’s t-test; significance is indicated for P values of *<0.05 and ***<0.001.

Results

Angiomyolipoma Immunohistochemical Staining

The H&E-stained sections of the angiomyolipoma (the source of the isolated cells, see below) contained three typical histological components: adipocytes, smooth muscle cells, and vascular structures (Figure 1a). The cells positive for α-actin were mainly present in the vessel walls and related surroundings (Figure 1b); the HMB-45-labeled cells23,24 were located in the outermost layer of blood vessels and throughout the angiomyolipoma (Figure 1c). The immunohistochemical evaluation of RhoA, a small GTPase that promotes the formation of stress fibers, showed several positively stained cells in the angiomyolipoma (Figure 1d). The activation of RhoA, Rac, and Cdc42 is critical for cell adhesion and motility, and their dysregulation induces cell transformation and metastasis.25

Cellular Characterization

Two cell populations were isolated from the angiomyolipoma and cultured in monolayers before being separated by sequential subcloning to obtain pure homogeneous cultures. Routine H&E staining (Figure 2A) showed that one population had a characteristic flat and elongated appearance (Figure 2A; a to c) and the other had an epithelial-like morphology (Figure 2d). The growth of the flat and elongated cells was much faster than that of the epithelial-like cells (Figure 2B): from a plating density of $3 \times 10^4$ cells, they quadrupled to $12 \times 10^4$ in 8 days. All of these cells were strongly positive for smooth muscle-specific α-actin antibody (Figure 3a), with the stain being diffused throughout the cytoplasm, and are probably smooth muscle cells. They were also positive for HMB-45 antibody (Figure 3e), which is consistent with the previously described angiomyolipoma phenotype. Because they were negative for S100 (Figure 3b), vimentin

![Figure 1. a: Histological and immunohistochemical staining with α-actin-, HMB-45-, and RhoA-specific antibodies on adjacent sections of an angiomyolipoma in a TSC patient. H&E staining. b: Immunolabeling with α-actin antibody shows positive cells surrounding a blood vessel and others spread in the parenchyma. c: The HMB-45-labeled cells distributed in the periphery of a blood vessel and in the surrounding parenchyma. d: The RhoA-positive cells were small and distributed throughout the angiomyolipoma but away from blood vessels (arrows). Original magnifications: ×100 (a); ×200 (b–d).](image1.png)

![Figure 2. Morphological appearance and growth rate of the two cell types isolated from the TSC human angiomyolipoma. A: H&E staining of flat-elongated smooth muscle-like cells at different magnifications (a, b) and at confluence (c), and rounder epithelial-like cells (d). B: Growth rate of smooth muscle-like and epithelial-like cells 4 and 8 days after plating. *P<0.05 and ***P<0.001 indicate significant differences versus smooth muscle-like cell proliferation. Scale bars: 40 μm (a, d), 20 μm (b, c).](image2.png)
Hamartin and tuberin regulate the RhoA promoter of stress fiber formation, and the absence of the TSC1/TSC2 complex leads to stress fiber disassembly and focal adhesion remodeling, thus deregulating cell dynamics.\textsuperscript{26,27} Because the epithelial-like cells were strongly stained by RhoA, they were christened R\textsuperscript{A} cells (Figure 3, h and h').

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**Mutation Analysis**

DNA sequencing has shown that blood, angiomyolipoma, and smooth muscle-like cells contained a germ-line TSC2 exon 18 mutation consisting of a base pair change in amino acid 698 from a lysine to a stop codon (K698X). This mutation inhibits tuberin tyrosine phosphorylation and the formation of the tuberin-hamartin complex.\textsuperscript{28} K698X-mutated tuberin should be unable to inhibit S6 kinase\textsuperscript{16} or interact with 14-3-3.\textsuperscript{29} This mutation was also heterozygously present in peripheral blood and the epithelial-like R\textsuperscript{A} cells. Sequencing of the A\textsuperscript{A} cells exclusively revealed mutant residue T at position 2110, thus indicating LOH of the TSC2 allele containing wild-type residue A (Figure 4A). The mutation detected in one copy in the A\textsuperscript{A} cells was consis-
tent with Knudson's two-hit tumor-suppressor gene model.30

LOH Analysis
We tested the blood and A<sup>+</sup> and R<sup>+</sup> cells of the TSC patient for LOH by means of PCR amplification using a panel of microsatellite markers near the TSC2 locus on chromosome 16p13.3. Five markers (D16S287, D16S291, D16S25, D16S665, and Kg8) were heterozygous in all of the samples (data not shown), but two (D16S3024 and D16S3394) showed LOH in the A<sup>+</sup> cells but not in the blood and R<sup>+</sup> cells (Figure 4, B and C).

Tuberin and Hamartin Expression
The 180-kd tuberin protein is expressed in many different cell and tissue types,31 and we evaluated the expression of the TSC complex in LOH A<sup>+</sup> cells, patient specimens, R<sup>+</sup> cells, and VSMCs. Western blots were made using two antibodies recognizing different regions of the COOH-terminal domain (tuberin and tuberin C20), an antibody reacting with the Thr1462-phosphorylated tuberin form, and another for hamartin. The blots are representative of three separate experiments.

Figure 4. A: Determination of bi-allelic inactivation by direct sequence analysis of blood, R<sup>+</sup> cells, and A<sup>+</sup> cells. The mutation site is indicated by a vertical arrow. A<sup>+</sup> cell sequencing revealed the mutation in TSC2 exon 18 at residue 2110. Representative PCR analysis of chromosome 16p13.3 microsatellite markers D16S3024 (B) and D16S3394 (C) in blood, R<sup>+</sup> cells, and A<sup>+</sup> cells.

Role of EGF and IGF-1 on A<sup>+</sup> Cell Growth and Survival

The A<sup>+</sup> cells were isolated and cultured in a medium containing EGF at a concentration of 10 ng/ml, as indicated by Arbiser and colleagues.21 EGF and IGF-1 receptors were detected in the A<sup>+</sup> cells, the liver, and AML of the TSC2 patient, and the VSMCs (Figure 6A). The role of EGF and IGF-1 in A<sup>+</sup> cell growth was evaluated by eliminating EGF from the culture medium or replacing EGF with IGF-1 at concentrations of 5 or 50 ng/ml for 21 days. Under all conditions, the A<sup>+</sup> cells survived but did not proliferate when EGF was removed from the medium or reduced to 1 ng/ml (these latter data are not shown), and so IGF-1 did not replace its proliferative action (Figure 6B). The opposite effect was observed in the VSMCs: their number markedly increased when IGF-1 was added to the culture medium, but their proliferation was quickly blocked by EGF supplementation (Figure 6C). The early slight effect of EGF on VSMC proliferation may be secondary to the reported brisk DNA synthesis with minimal cell division throughout 0 to 4 days that subsequently leads to cycle arrest.32 When rapamycin was added to the culture medium of A<sup>+</sup> cells at plating time, their growth rate was comparable with that of the VSMCs, and the action of rapamycin was not modified by the presence or absence of EGF (Table 1).

Depriving the medium of EGF for 21 days or replacing it with IGF-1 increased the phosphorylation of Akt in the A<sup>+</sup> cells, thus confirming the results of the LOH analysis (Figure 5). In response to PI3K activation, tuberin is directly phosphorylated by Akt at Thr1462 and Ser939, and a tuberin lacking PI3K-dependent phosphorylation sites can block the activation of S6K1.18 High levels of Thr1462-phosphorylated tuberin were detected in the liver and angiomyolipoma of the TSC2 patient, but they were low in the R<sup>+</sup> and VSMCs, and absent in the A<sup>+</sup> cells (Figure 5). Hamartin expression was comparable in all of the tested groups (Figure 5).

Figure 5. Expression of the TSC1/TSC2 complex in the liver and angiomyolipoma of the TSC2 patient and in A<sup>+</sup> and VSMCs. Western blots were made using two antibodies recognizing different regions of the TSC2 complex: tuberin and phospho-tuberin, and hamartin. The blots are representative of three separate experiments.
Angiomyolipoma of the TSC2 patient, and in A
Expression of EGF receptor (EGFR) and IGF-1 receptor (IGF-1R) in the liver
were counted in a Neubauer chamber after 4, 7, 10, 15, and 21 days of culture.
without EGF, and without EGF with the addition of IGF-1 50 or 5 ng/ml. The A
growth in complete medium (containing 10 ng/ml EGF), in the same medium
specific standard medium, in the presence of 10 ng/ml EGF, and in the presence
omission of EGF from the culture medium increased
not affected by IGF-1 (Figure 7A). The addition of PI3K
served in the cells grown in complete medium; this was
not change under any of the culture conditions. The
expression did not change under any of the experimental conditions.
EGF; IGF-1 failed to promote any increase. No reduction or mortality was
observed under any of the experimental conditions. Cell proliferation and survival.
EGF may act on both proliferation and survival.
These cells do not release EGF (data not shown).

Discussion
TSC is a tumor suppressor gene disorder associated with benign and malignant tumors. Lesions such as cortical tubers, subependymal giant cell astrocytomas, cardiac rhabdomyomas, and renal angiomyolipoma often show abnormal differentiation patterns, as well as deregulated cell growth and proliferation. Angiomyolipomas are uncommon renal tumors that have smooth muscle, fat, and vascular components, and belong to a group of neoplasms that co-express melanocytic and smooth muscle markers, including LAM of the lung.

Table 1. Cell Growth Rate

<table>
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<th>Days 3</th>
<th>Days 5</th>
<th>Days 10</th>
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<tr>
<td>VSMC</td>
<td>35%</td>
<td>60%</td>
<td>107%</td>
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<tr>
<td>A+ cells with RAPA and EGF</td>
<td>25%</td>
<td>35%</td>
<td>100%</td>
</tr>
<tr>
<td>A+ cells with RAPA, without EGF</td>
<td>24%</td>
<td>36%</td>
<td>100%</td>
</tr>
<tr>
<td>A+ cells in standard medium</td>
<td>36%</td>
<td>68%</td>
<td>240%</td>
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Percentage increase in cell number. Rapamycin (RAPA) at the concentration of 1 ng/ml was added at plating time. At time 0 cells (50 × 10⁶) were plated and the number of cells was evaluated at the indicated time.
human pathological TSC2 cells rather than knockout models are obvious and our results highlight some of them.

We here report the novel finding that the in vitro growth of smooth muscle-like cells derived from the renal angio-myolipoma of a TSC2 patient depends on the availability of EGF in the medium. Both the blood and angiomyolipoma cells showed a somatic TSC2 gene mutation in exon 18 consisting of a stop codon. The same gene modification was also present in isolated cells. To the best of our knowledge, only one previous study has shown that angiomyolipoma cells with TSC2 mutations can be grown in culture, although the number of such studies may have been limited by the loss of the entire primary culture.33

Our isolated angiomyolipoma cells can be grown in culture as a stabilized cell line, and so could be used as a continuous source and do not require any morphological, biochemical, and pharmacological modifications (see summary of A/H11001 and R/H11001 cell characterization in Table 2). They have been stored in liquid nitrogen and grown in culture for the past 2 years without any changes in their growth, pharmacological, or genetic characteristics. The subcloned smooth muscle cells (A/H11001) are LOH and do not express tuberin, whereas the 8/18 keratin-positive cells are non-LOH and contain tuberin and its phosphorylated form. Other angiomyolipoma cell cultures have been recently reported, but the cells did not carry TSC1 or TSC2 mutations, and immortalization required the introduction of simian virus 40 large T antigen and telomerase.21

EGF transiently activates Erk, a member of the MAPK family. EGF supplementation of the culture medium is necessary to promote the proliferation and maintenance of the A/H11001 cells, and its proliferative action cannot be replaced by the addition of IGF-1. Conversely, the proliferation rate of our control aorta smooth muscle cells (VSMCs) increased when IGF-1 was added to the growth medium and did not proliferate when exposed to EGF, thus demonstrating a clear difference between normal smooth muscle cells and our TSC2-deficient cells. The EGF-dependent growth is probably tuberin-dependent because blocking mTOR with rapamycin led to an A/H11001 cell growth rate that was comparable with that of the VSMCs. It therefore seems that the EGF-dependent growth is triggered by mTOR activation. The requirement of EGF supplementation for A/H11001 cell proliferation and the effect of anti-EGF-R antibodies on A/H11001 cell survival suggest a possible new therapeutic strategy for controlling smooth muscle cell growth in angiomyolipomas and LAM.

Akt is activated in response to insulin or IGF-1 receptor activation, and is thus capable of phosphorylating tu-
berin,16–18 This leads to the inhibition of tuberin GTPase activity against Rheb and an increase in Rheb-GTP that activates mTOR. Activated mTOR phosphorylates p70S6K and 4EBP1, and thus enables the translational machinery and promotes cell growth.19 There was no increase in the number of A+ cells cultured for 21 days in the absence of EGF or the presence of IGF-1, but Akt phosphorylation was greater than in the A+ cells grown in a medium containing EGF.

Hyperphosphorylation of p70S6K and its ribosomal protein S6 substrate has been observed in cells lacking tuberin from the Eker rat model of TSC2,19,34 in tumor cells containing TSC2 mutations,3,33 and in cells lacking hamartin from a murine model of TSC1,35 thus demonstrating that the hamartin-tuberin complex negatively regulates p70S6K. After 21 days of culture in a standard medium containing EGF, or in a medium deprived of EGF with or without the addition of IGF-1, phosphorylation of S6K and its substrate S6 in the A+ cells were unchanged which, as recently reported,19,34,36 indicates that the loss of TSC2 function in mammalian cells lead to constitutive S6K1 activation and phosphorylation of S6. Preliminary results suggest that S6K1 is constitutively activated also in R+ cells (unpublished data).

Hyperphosphorylation of 4EBP1 reduces its affinity for eukaryotic initiation factor (eIF)4E and its subsequent dissociation from eIF4E, and leads to the promotion of translation.37 The mechanisms of this event are regulated by the PI 3-kinase pathway and Akt phosphorylation when induced by insulin,38 and by means of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) when promoted by Erk stimulators.39 The regulation of 4E-BP1 and eIF4F via MEK/Erk signaling may be important for the control of translation by mitogenic signals that do not activate PI 3-kinase/Akt. The growth of A+ cells requires EGF supplementation, which also markedly increased 4EBP1 phosphorylation, which should lead to dissociation from eIF4E and consequent translation. The phosphorylation of Erk was not modified by any culture condition, but mTOR phosphorylation increased in the absence of EGF and IGF-1. It is possible that A+ cells grow in the presence of EGF because the EGF-activated pathway involving Erk modulates 4EBP1 and its translational function. It is known that the Erk pathway regulates the phosphorylation of multiple 4EBP1 sites to the point that 4EBP1 is released from eIF4E, and that this activation takes place through mechanisms requiring mTOR (Figure 9).40

It has been reported that exposure to anti-EGF receptor39,41 and anti-IGF-1 receptor42,43 inhibits the proliferation, survival, and differentiation of various cultured malignant human cell lines and tumors. Antibodies to EGF and IGF-1 receptors were added to the culture medium in the presence or absence of EGF, and both situations led to the progressive loss of A+ cells. The activity of A+ cell EGF receptors is therefore apparently involved in both proliferation and (perhaps) survival. The simple omission of EGF from the culture medium does not cause cell loss, possibly because of the presence of serum and the autocrine release of IGF-1 by the A+ cells themselves. This differentiates A+ smooth muscle-like cells from VSMCs: the latter do not release IGF-1 and their proliferation is stimulated when it is added to the culture medium, whereas A+ cells release a substantial amount of IGF-1 but, even when added at higher concentrations, does not promote A+ cell proliferation. However, blocking IGF-1R does cause cell loss, thus suggesting that IGF-1 may have switched from being a proliferative factor to a survival factor in A+ cells. This is confirmed by recent observations in our laboratory indicating the involvement of IGF-1 in the activation of an A+ cell anti-apoptotic pathway (unpublished data).

In conclusion, human LOH smooth muscle-like (A+) and non-LOH epithelial-like (R+) TSC2 cells from renal angiomyolipoma can be isolated, grown in culture, and indefinitely stored in liquid nitrogen. Unlike that of control aorta smooth muscle cells, the proliferation of A+ cells requires the addition of EGF to the culture medium, whereas IGF-1 is autocrinally secreted and may play a role in survival. Incubation with anti-EGFR and anti-IGF1R causes the loss of 50% of the A+ cells in 5 days and of 100% in 12 days. These effects of anti-EGFR and anti-

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**Table 2. Summary of the Characterization of A+ and R+ Cells**

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<thead>
<tr>
<th>Immunochemistry</th>
<th>Western Blotting</th>
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<tr>
<td>α-Actin</td>
<td>Tuberin</td>
</tr>
<tr>
<td>Keratin8/18</td>
<td>Phosphotuberin</td>
</tr>
<tr>
<td>HMB45</td>
<td>Hamartin</td>
</tr>
<tr>
<td>Hamartin</td>
<td>LOH</td>
</tr>
<tr>
<td>A+ cells</td>
<td>–</td>
</tr>
<tr>
<td>R+ cells</td>
<td>+</td>
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</tbody>
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Differences in protein expression between A+ and R+ cells. Immunocytochemical staining of A+ and R+ cells did not detect S100, vimentin, and CD68 proteins.
IGF-1R on A¹ cell survival may offer a new therapeutic perspective in TSC and LAM.

References


