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Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways

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Yu, Jane, Aristotelis Astrinidis, Sharon Howard, and Elizabeth Petri Henske. Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways. *Am J Physiol Lung Cell Mol Physiol* 286: L694–L700, 2004. First published August 15, 2003; 10.1152/ajplung.00204.2003.—Lymphangiomyomatosis (LAM) is a progressive lung disease affecting almost exclusively women. The reasons for this strong gender predisposition are poorly understood. Renal angiomyolipomas occur in 50–60% of sporadic LAM patients. The smooth muscle cells of pulmonary LAM and renal angiomyolipomas are nearly indistinguishable morphologically. Here, we report the first successful cell culture of a LAM-associated renal angiomyolipoma. The cells carried inactivating mutations in both alleles of the *TSC2* gene and expressed estrogen receptor α , estrogen receptor β , and androgen receptor. To elucidate the cellular pathways through which steroid hormones influence LAM pathogenesis, we treated the cells with both estradiol and tamoxifen. Cell growth was stimulated by estradiol, associated with phosphorylation of p44/42 MAPK at 5 min and an increase in c-myc expression at 4 h. Tamoxifen citrate also stimulated cell growth, associated with increased phosphorylation of p44/42 MAPK and expression of c-myc, indicating that tamoxifen has agonist effects on angiomyolipoma cells. This response to tamoxifen in human angiomyolipoma cells differs from prior studies of Eker rat leiomyoma cells, possibly reflecting cell type or species differences in cells lacking tuberin. Our data provide the first evidence that estradiol stimulates the growth of angiomyolipoma cells, that tamoxifen has agonist effects in angiomyolipoma cells, and that estradiol and tamoxifen impact both genomic and nongenomic signaling pathways in angiomyolipoma cells. The responsiveness of angiomyolipoma cells to estradiol may be related to the underlying reasons that LAM affects primarily women.

tuberous sclerosis complex; mitogen-activated protein kinase; tuberin; hamartin; estrogen receptor; lymphangiomyomatosis

PULMONARY LYMPHANGIOLEIOMYOMATOSIS (LAM) affects almost exclusively women, with an average age at diagnosis of 33 years (28, 54). LAM is characterized pathologically by a diffuse, bilateral proliferation of abnormal smooth muscle cells, accompanied by extensive reactive epithelial hyperplasia (40) and cystic degeneration of lung parenchyma. LAM can occur as an isolated disorder, referred to as sporadic LAM, or in patients with tuberous sclerosis complex (TSC). TSC is a tumor suppressor gene syndrome characterized by seizures, mental retardation, autism, and tumors in brain, retina, kidney, heart, and skin (21). Pulmo-

nary parenchymal changes consistent with LAM occur in 34–42% of women with TSC (13, 19, 43).

Renal angiomyolipomas occur in at least 70% of TSC patients and in ~50% of sporadic LAM patients (4). Angiomyolipomas are distinctive tumors with three components: dysplastic blood vessels, smooth muscle cells, and fat. The abnormal smooth muscle cells of pulmonary LAM and renal angiomyolipomas are nearly identical at the histological, immunohistochemical, and ultrastructural levels (10, 11). In prior work, we found that both angiomyolipoma cells and pulmonary LAM cells from some sporadic LAM patients contain somatic mutations in the *TSC2* gene (9). We and others (6, 29, 48, 59) have hypothesized that pulmonary LAM results from metastasis of angiomyolipoma cells.

The reasons that LAM occurs predominantly in women are not well understood. Estrogen receptor expression has been observed in pulmonary LAM cells (7, 33) and angiomyolipoma smooth muscle cells (39), and downregulation of the estrogen receptor has been observed after hormonal therapy for LAM (41). However, the effects of estrogen on LAM or angiomyolipoma cell growth have not, to our knowledge, been previously studied *in vitro*, in part because pure cultures of pulmonary LAM cells are difficult to establish. We report here the development of a primary cell culture from a LAM-associated renal angiomyolipoma. Genetic studies revealed mutations in both alleles of the *TSC2* gene. The growth of these cells was stimulated by both estradiol and tamoxifen, associated with phosphorylation of p44/42 MAPK and increased c-myc expression. These data demonstrate for the first time that steroid hormones stimulate the growth of angiomyolipoma cells and activate both cytoplasmic and genomic signaling pathways.

METHODS

Establishment of the angiomyolipoma culture. This study was approved by the Institutional Review Board of Fox Chase Cancer Center. Fresh angiomyolipoma tissue was minced and treated with 0.2% collagenase (Sigma, St. Louis, MO) in serum-free Ham's F-12 media at 37°C for 1 h. The cells were then washed and plated in 1.5 ml of IIA complete media (2) with 15% FBS for the first week and 7.5% FBS subsequently. Cells were used at passages 4–6 for all experiments.

Genetic analyses. DNA sequencing and single-strand conformation polymorphism (SSCP) analysis were performed as previously described (9).

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Immunoblotting and antibodies. Cells were lysed in RIPA buffer [$1\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM sodium orthovanadate supplemented with protease inhibitor cocktail I (Sigma)]. The cell lysates were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The following antibodies were used for Western blot analysis: anti-estrogen receptor α (ER α), anti-c-Myc, and anti-tuberin C-20 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ribosomal protein S6, anti-phospho-S6, anti-p44/42 MAPK, and anti-phospho-p44/42 MAPK (Cell Signaling Technology, Beverly, MA); anti-Kip1/p27 (BD Biosciences, Palo Alto, CA); anti-cyclin D1 (Neomarker, Fremont, CA); anti- β -actin (Sigma); and anti-hamartin (46).

RT-PCR. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). For the first-strand complementary DNA synthesis, 5 μ g of total RNA and oligo deoxythymidine primer were used. The RT reaction was performed using the SuperScript First-Strand Synthesis System (Invitrogen). ER α , estrogen receptor β (ER β), androgen receptor (AR), and 18S ribosomal RNA were amplified using previously reported primers and conditions (5, 51). PCR products were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide.

Hormonal treatments. Five hundred angiomyolipoma cells were seeded into each well of a 96-well plate. After 72 h, cells were fed with fresh media containing charcoal-stripped FBS (Atlanta Biologicals, Norcross, GA) supplemented with 0.1–100 nM 17- β -estradiol (Sigma), 0.2–20 μ M tamoxifen citrate (Sigma), or 0.1% ethanol (vehicle) in triplicate wells. Cells were fed with freshly prepared medium every other day. Cell growth was measured daily using Alamar blue (BioSource International, Camarillo, CA), an indicator dye that becomes fluorescent on mitochondrial reduction (1, 60).

Statistics. Results are presented as means \pm SD of experiments performed in triplicate. Statistical analysis was performed using a two-tailed paired Student's *t*-test.

RESULTS

Cultured angiomyolipoma cells have mutations inactivating both alleles of TSC2, express steroid hormone receptors, and have hyperphosphorylation of ribosomal protein S6. The cultured angiomyolipoma cells grew as a monolayer, with a homogeneous spindle-shaped morphology. In early passages, cells containing fat were also observed (Fig. 1A). The cells showed a variant band in *TSC2* exon 16 using SSCP analysis (data not shown), which was not present in normal kidney tissue from this patient, indicating that the mutation occurred somatically. Sequencing demonstrated that the angiomyolipoma cells contained a *TSC2* exon 16 missense mutation G1832A (Fig. 1B). This mutation, which results in an amino acid change from arginine to glutamine at position 611 of tuberlin, is one of the most frequently occurring mutations in patients with TSC (14). Tuberlin with the R611Q mutation is unable to inhibit S6 kinase (26) and does not interact with hamartin (45) or 14-3-3 (44).

Loss of heterozygosity of the wild-type allele containing the G at position 1832 was evident from the sequencing tracing, in which the wild-type G was absent. The G1832A mutation and the loss of the wild-type allele seen in the cell culture were identical to those in the paraffin-embedded angiomyolipoma tissue from this patient (9), proving that the cultured cells were derived from the angiomyolipoma.

Tuberlin, hamartin, and a low level of ER α were detected in the angiomyolipoma cells by Western immunoblot analysis (Fig. 2A). The amount of tuberlin relative to the amount of hamartin was decreased in 621 cells compared with other cell

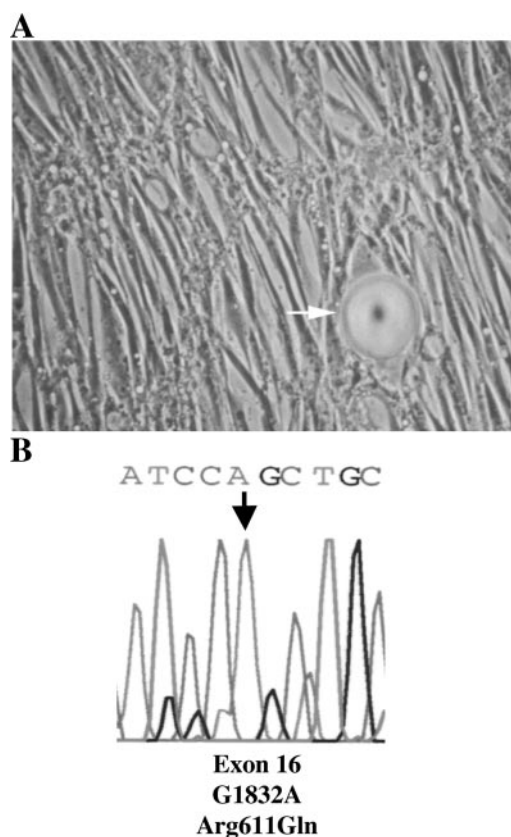


Fig. 1. Cultured angiomyolipoma cells contained *TSC2* gene mutations. **A:** photograph of the angiomyolipoma cells in culture. The majority of the cells were spindle shaped. In early passages, cells containing fat were occasionally seen (arrow). **B:** identification of the exon 16 mutation in the cultured angiomyolipoma cells by direct sequencing. The arrow indicates the position of the mutation (a nucleotide change G1832A) in *TSC2* exon 16, which results in an Arg611Gln missense mutation. Sequencing revealed exclusively the residue (A) at position 1832, indicating loss of heterozygosity of the *TSC2* allele containing the wild-type residue (G).

types, including MCF-7 cells (Fig. 2A) and HEK-293 cells (not shown), consistent with loss of one *TSC2* allele. Complete loss of tuberlin expression was not expected, because the G1832A missense mutation would not result in protein truncation. By RT-PCR, expression of ER α was confirmed (Fig. 2B). Expression of ER β and AR was also found by RT-PCR (Fig. 2B), suggesting that angiomyolipoma cells express multiple steroid hormone receptors. By RT-PCR, the level of ER α expression appeared to be lower in the angiomyolipoma cells than in MCF-7 cells, whereas the level of ER β expression was higher. The degree of phosphorylation of ribosomal protein S6 was higher in the angiomyolipoma cells than in MCF-7 cells (Fig. 2C), consistent with the known role of wild-type tuberlin in the inhibition of S6 kinase activity.

Estradiol and tamoxifen stimulate the growth of cultured angiomyolipoma cells. Because LAM occurs almost exclusively in women, and because LAM and angiomyolipoma cells are known to express ER α , we hypothesized that estradiol would stimulate the growth of the angiomyolipoma cells. Consistent with this hypothesis, we found that the growth of 621 angiomyolipoma cells was stimulated by estradiol at all four dose levels tested (Fig. 3A). The highest level of stimulation was at 0.1 nM, with an approximate threefold increase in

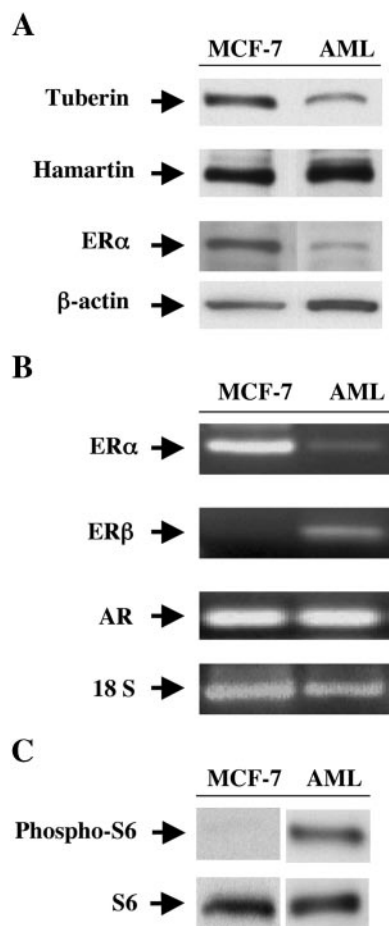


Fig. 2. Cultured angiomyolipoma cells expressed steroid hormone receptors and have hyperphosphorylation of ribosomal protein S6. *A*: expression of tuberin, hamartin, and estrogen receptor α ($ER\alpha$) was demonstrated by Western immunoblot in the angiomyolipoma (AML) cells. The amount of tuberin relative to the amount of hamartin was decreased in the AML cells compared with MCF-7 breast cancer cells, consistent with the loss of one *TSC2* allele as shown by sequencing. *B*: expression of $ER\alpha$, estrogen receptor β ($ER\beta$), and androgen receptor (AR) in the AML cells was demonstrated by RT-PCR. 18S ribosomal RNA was used as a control for the RT-PCR. MCF-7 cells were used as a control. *C*: by Western immunoblot, ribosomal protein p70S6 was hyperphosphorylated in the serum-starved AML cells compared with serum-starved MCF-7 cells.

growth at 6 days compared with vehicle control ($P < 0.05$). Although there appeared to be an inverse association between dose and proliferation, this was statistically significant only at the 6-day time point.

Unexpectedly, the growth of the angiomyolipoma cells was also stimulated by tamoxifen citrate (Fig. 3*B*). Tamoxifen citrate at 0.2 μM stimulated cultured cell growth by approximately threefold relative to the vehicle control level at 6 days ($P < 0.05$). These results indicate that tamoxifen acts as an estrogen agonist in these angiomyolipoma cells, in contrast to tamoxifen's estrogen antagonist action in Eker rat-derived ELT3 cells (25).

Estradiol and tamoxifen activate both cytoplasmic and genomic signaling pathways. To determine whether estradiol activates nongenomic (cytoplasmic) signaling pathways, we analyzed cell lysates within 60 min after 1 nM estradiol treatment. Estradiol increased p44/42 MAPK phosphorylation

at 5- and 60-min time points in the angiomyolipoma cells (Fig. 4*A*), linking estradiol to rapid, cytoplasmic signaling pathways in angiomyolipoma cells. Insufficient cells were available to test additional time points. MCF-7 cells treated with 1 nM estradiol also showed rapid activation of p44/42 MAPK (Fig. 4*A*). Tamoxifen citrate also increased p44/42 MAPK phosphorylation at 15-, 30-, 45-, and 60-min time points (Fig. 4*B*), suggesting that tamoxifen and estradiol are signaling through common cellular pathways. This is consistent with the hypothesis that tamoxifen acts as an estrogen agonist in angiomyolipoma cells.

To determine whether estradiol treatment of the angiomyolipoma cells was associated with transcriptional effects of the estrogen receptor, we studied cell extracts prepared after 4 and 8 h of estradiol treatment. Increased expression of *c-myc*, which is an estrogen-responsive gene (16), was observed at 4 h in the angiomyolipoma cells as well as in MCF-7 breast cancer cells treated with estrogen (Fig. 5*A*). Decreased p27 expression was seen in both angiomyolipoma cells and MCF-7 cells after estradiol treatment. Cyclin D1 expression was not changed.

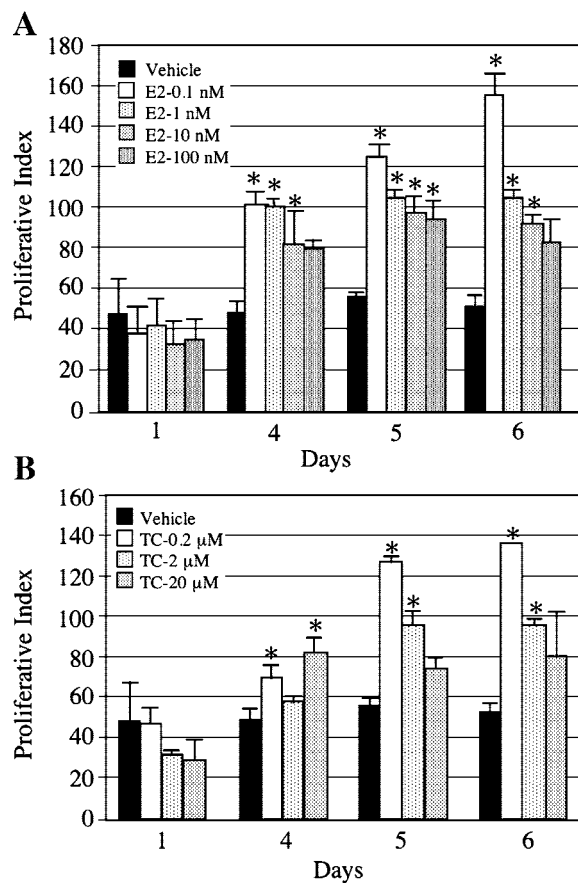


Fig. 3. AML cell growth was stimulated by estradiol and tamoxifen. *A*: cells were treated with estradiol (E2) at 0.1, 1, 10, or 100 nM or 0.1% ethanol (vehicle) in the presence of 10% charcoal-stripped serum. The proliferative index was determined by measuring the reduction of Alamar blue in triplicate wells. Estradiol (0.1 nM) resulted in an approximate threefold increase in growth at 6 days. Bars indicate the standard deviation. $*P < 0.05$ relative to vehicle control. Similar results were seen in 3 independent experiments. *B*: cells were treated with tamoxifen citrate (TC) at 0.2, 2, or 20 μM or 0.1% ethanol (vehicle) in 10% charcoal-stripped serum. The 2 lower doses, but not the highest dose, stimulated cell growth. $*P < 0.05$ relative to vehicle control. Similar results were seen in 2 independent experiments.

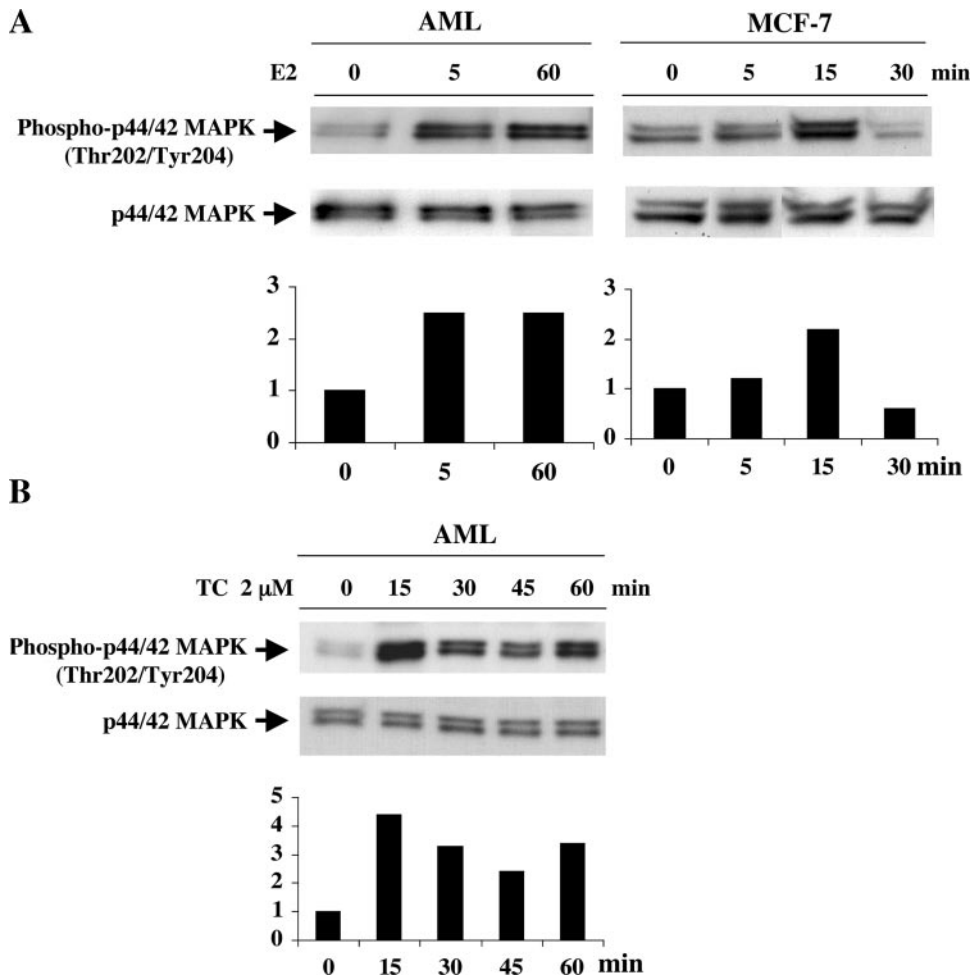


Fig. 4. Estradiol and tamoxifen citrate increased the phosphorylation of p44/42 MAPK. **A:** AML and MCF-7 cells were treated with 1 nM E2 in 10% charcoal-stripped serum. Increased phosphorylation of p44/42 MAPK was present at 5 and 60 min in the AML cells and at 15 min in MCF-7 cells. The total p44/42 MAPK is shown as a loading control. Bar graph indicates the fold change in phospho-MAPK, as determined by densitometry. Similar results were seen in a second independent experiment. **B:** AML cells were treated with 2 μ M TC in 10% charcoal-stripped serum. Increased phosphorylation of p44/42 MAPK was seen at 15, 30, 45, and 60 min.

After tamoxifen citrate treatment, increased expression of c-myc was seen at 8 h in the angiomyolipoma cells, again without a change in cyclin D1 (Fig. 5B).

DISCUSSION

LAM occurs almost exclusively in women, and LAM and angiomyolipoma cells are known to express ER α . Our data suggest that angiomyolipoma cells also express ER β , although this will require confirmation in additional specimens. In vivo (56) and in vitro data from the Eker rat model of *TSC2* indicate that tumorigenesis in TSC is hormonally driven, and the COOH terminus of tuberlin has been shown to interact in vitro with steroid hormone receptors as a transcriptional coactivator (23). Despite these compelling indications that steroid hormones contribute to angiomyolipoma pathogenesis, the impact of estrogen on angiomyolipoma cell growth and signaling has not been previously studied.

We report here that cells derived from a sporadic LAM-associated angiomyolipoma grew in response to both estradiol and tamoxifen citrate. This growth was associated with phosphorylation of p44/42 MAPK and increased expression of c-myc. An important feature of our work is that the angiomyolipoma had somatic *TSC2* gene mutations, allowing us to prove that the cultured cells were angiomyolipoma derived. To our knowledge, this is the first time that cells from an angiomyolipoma with *TSC2* gene mutations have been successfully

cultured. Our data indicate that estradiol and tamoxifen citrate stimulate both genomic, transcriptional responses (increased expression of c-myc) and nongenomic, cytoplasmic responses (rapid activation of p44/42 MAPK) in cultured angiomyolipoma cells.

The cellular pathways through which tuberlin could influence steroid hormone signaling are not clear. Hamartin and tuberlin function in multiple cellular pathways in mammalian cells, including vesicular trafficking (57), regulation of the G1 phase of the cell cycle (27, 42, 47, 50, 52), steroid hormone regulation (23), and Rho activation (3, 35). Tuberlin has a highly conserved domain with homology to rap1 GTPase activating protein (GAP), and tuberlin has been shown to possess GAP activity for rap1A (55) and rab5 (57). Recently, hyperphosphorylation of p70S6 kinase (p70S6K) and/or its substrate ribosomal protein S6 was observed in cells lacking hamartin from a murine model of *TSC1* (34), in cells lacking tuberlin from the Eker rat model of *TSC2* (22, 31) and in tumor cells containing *TSC2* mutations (30), demonstrating that the hamartin-tuberlin complex negatively regulates p70S6K. Tuberlin is also a substrate of the p38 and MK2 kinase cascade (37), mediating its interaction with 14-3-3 (38, 44, 49). Whether and how these pathways involving hamartin and tuberlin intersect with steroid hormone signaling will require additional studies.

Our data using a primary angiomyolipoma-derived culture provide a foundation for elucidating the role of steroid hor-

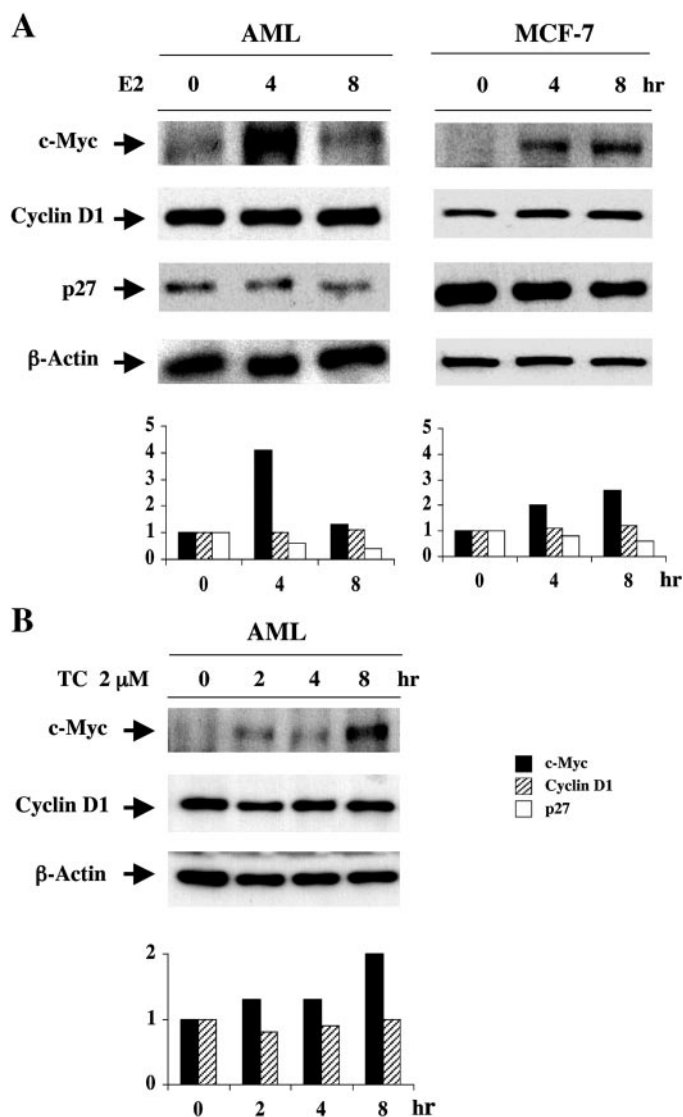


Fig. 5. Estradiol and tamoxifen citrate increase the expression of c-myc in AML and MCF-7 cells. *A*: AML cells were treated with 0.1 nM E2, and MCF-7 cells were treated with 1 nM E2, in 10% charcoal-stripped serum. Increased c-myc expression and decreased p27 expression were seen by immunoblot in both the AML and MCF-7 cells. There was no change in the expression of cyclin D1. β -actin was used as a loading control. Bar graph indicates the fold change in c-myc, cyclin D1, and p27, as determined by densitometry. Similar results were seen in 2 independent experiments. *B*: AML cells were treated with 2 μ M TC in 10% charcoal-stripped serum. Increased expression of c-myc was seen at 8 h. There was no change in the expression of cyclin D1.

mones in angiomyolipomas and LAM. The primary culture approach has inherent strengths and limitations. The limitations are clear: that the difficulties in obtaining fresh tissue for culture from patients with known mutations have limited our study to cells from a single patient and that the use of primary cultured cells limited the number of studies we could perform before losing the entire culture. The most important strengths of our approach are that transformation with an oncogene, which would itself affect cell growth pathways, was not needed and that our cells contained *TSC2* mutations, proving that they were angiomyolipoma derived. To our knowledge, only one previous culture of an angiomyolipoma has been reported (2). In that case, the cells did not contain mutations in *TSC1* or

TSC2, and immortalization required the introduction of both simian virus 40 large T antigen and telomerase.

Angiomyolipoma cultures derived from LAM patients could be an important additional model for LAM. Primary cultures of LAM cells have been established (22), but the close proximity of LAM cells to reactive cells is problematic and may result in mixing of cell types. ELT3 cells are a second cell culture model for LAM (24). ELT3 cells are derived from an Eker rat uterine leiomyoma, lack functional tuberlin, and express estrogen receptor. Estrogen treatment activates the phosphorylation of p44/42 MAPK in ELT3 cells (17), similar to our findings in angiomyolipoma cells. However, tamoxifen inhibits the growth of ELT3 cells (25), whereas tamoxifen stimulated the growth of angiomyolipoma cells, suggesting that there are species and/or cell type differences in the interactions between tuberlin and steroid-hormone signaling that affect selective estrogen receptor modulators such as tamoxifen.

Our results in cultured cells could have clinical implications. First, the medical literature contains at least 30 reports of rapid growth and/or spontaneous hemorrhage of angiomyolipomas during pregnancy (8, 18, 20, 32, 36, 53, 58), which could be related to the increase in angiomyolipoma cell growth we observed in response to estradiol in vitro. The levels of unconjugated estradiol rise markedly during pregnancy (15), from a mean of 0.5 ng/ml (1.8 nM) at week 6 to 17.3 ng/ml (63.5 nM) at week 40. Second, because the abnormal smooth muscle cells of pulmonary LAM and renal angiomyolipomas are nearly identical, studies of angiomyolipoma cells may contribute to the development of targeted therapies for pulmonary LAM and provide insight into the mechanisms underlying the predominance of LAM in women. Studies of cultured human angiomyolipoma cells could lead to the rational selection of hormonal therapies for patients with symptomatic or enlarging angiomyolipomas. Finally, there are also several reports of women with pulmonary LAM who died of progressive pulmonary disease within months after initiating tamoxifen therapy (12). Whether there is a link between the in vitro stimulation of angiomyolipoma cell growth by tamoxifen and these clinical outcomes is unknown. Tamoxifen had estrogen agonist effects in our angiomyolipoma cells but acted as an antagonist in Eker rat-derived ELT3 cells (25), suggesting species and/or cell type differences. The possibility that tamoxifen or other selective estrogen receptor modifiers have harmful agonist effects in LAM and TSC patients, therefore, needs to be further studied in human cells as well as in animal models.

In summary, we report here, for the first time, that the growth of cultured cells from a LAM-associated angiomyolipoma was stimulated by both estradiol and tamoxifen citrate. Estradiol or tamoxifen treatment was associated with phosphorylation of p44/42 MAPK and increased expression of c-myc, indicating that steroid hormone signaling in angiomyolipoma cells involves both cytoplasmic and genomic effects. LAM cells and angiomyolipoma cells are virtually indistinguishable, and it has been hypothesized that pulmonary LAM results from the metastatic spread of angiomyolipoma cells. Angiomyolipoma cell cultures, therefore, may be critical to the elucidation of LAM pathogenesis.

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