

The methylation of the *TSC2* promoter underlies the abnormal growth of *TSC2* angiomyolipoma-derived smooth muscle cells

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Running head: Promoter methylation in *TSC2*

Number of text pages:31

Number of tables:2

Number of figures:8

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ABSTRACT

Tuberous sclerosis complex (TSC) is an autosomal-dominant disease caused by mutations in *TSC1* or *TSC2* genes. Smooth muscle-like cells (ASM) were isolated from an angiomyolipoma of a TSC patient. These cells lacked tuberin and labeled by HMB45 and CD44v6 antibodies, and carried constitutive S6 phosphorylation. They bear a germline *TSC2* intron 8-exon 9 junction mutation, but DNA analysis and PCR amplification failed to demonstrate loss of heterozygosity. Testing for an epigenetic alteration as second hit, we detected the methylation of *TSC2* promoter. The biological relevance of this was confirmed by tuberin expression with reduction of HMB45 labeling and S6 constitutive phosphorylation following exposure to the chromatin remodelling agents, trichostatin A and 5-azacytidine. These cells were, named $TSC2^{-/meth}$ ASM cells. Their proliferation required epidermal growth factor (EGF) in the medium as we have previously described for $TSC2^{-/-}$ ASM cells. Blockade of EGF with monoclonal antibody caused the death of $TSC2^{-/meth}$ ASM cells. Also rapamycin effectively blocked the proliferation of these cells. Our data show for the first time that methylation of the *TSC2* promoter might cause complete loss of tuberin in *TSC2* cells, and that the AML pathogenesis might also be originated from epigenetic defects in smooth muscle cells. In addition the effect of chromatin remodeling agents in these cells suggests a further avenue for the treatment of TSC and lymphangioliomyomatosis.

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal-dominant disease characterized by hamartomas, in a wide array of tissues and organs, such as brain, kidney, skin, heart, and lungs.¹ Abdominal angiomyolipomas are often present in TSC patients; they may cause life-threatening hemorrhages and in such conditions their surgical resection is required.² The tumor suppressor genes, *TSC1* and *TSC2*, are associated with the development of TSC, and mutations in either genes are responsible for familial and sporadic forms of the disease.¹ The *TSC2* gene is located on chromosome 16p13 while *TSC1* on chromosome 9q34.^{3,4} Hamartin, the *TSC1* gene product, stabilizes tuberin, the *TSC2* gene product, through binding with it, thereby preventing tuberin from ubiquitination and degradation.⁵ Tuberin acts as a GTPase-activating protein to regulate Rheb function through the conversion of Rheb from the active GTP-bound form to the inactive GDP-bound form.^{6,7} Active Rheb activates mTOR, and the upregulation of the TSC/mTOR signaling pathway leads to increased protein synthesis, cell proliferation, and ultimately to tumorigenesis.⁸

TSC is due to a germline mutation in either *TSC1* or *TSC2*. In most hamartomas, TSC follows a second hit inactivating the wild type allele. The loss of heterozygosity (LOH) in *TSC1* or *TSC2* has been documented in angiomyolipomas (AMLs), cardiac rhabdomyomas, and lymphangiomyomatosis (LAM) cells, but it has only rarely been found in cerebral cortical tubers and skin lesions.^{9,10,11} So it is not clear whether inactivation of both alleles is the necessary step for hamartomas pathogenesis. Various explanations have been raised to define the inability to find a second somatic event in TSC lesions, and the failure to demonstrate such events has been attributed to either different genetic and epigenetic deficits in TSC genes or cell heterogeneity in TSC hamartomas.^{12,13} DNA methylation is an epigenetic change that induces chromatin modifications and repression of transcription via a methyl CpG binding protein

MeCP2, and recruitment of a Sin3A/HDAC corepressor complex.^{14,15} Twenty-four hamartomas from 10 patients were analyzed by Niida and colleagues¹¹ for second-hit mutations by promoter methylation of *TSC2*, but no evidences of such inactivation of the second allele were, then, detected.

Here we report the isolation and characterization of a homogenous population of smooth muscle-like cells from an AML cells (ASM cells) of a *TSC2* patient. The ASM cells were mostly positive for HMB45¹⁶ and CD44v6¹⁷, that are markers of TSC and LAM cells, and showed a germline *TSC2* intron 8-exon 9 junction mutation with no loss of heterozygosity (LOH). However, tuberin was undetectable by immunochemistry and western blotting. We found that these cells were methylated in the *TSC2* promoter, and the involvement of methylation in the inhibition of *TSC2* gene was confirmed by the cellular expression of tuberin after exposure to the chromatin remodeling agent, trichostatin A. Thus, ASM cells were named *TSC2*^{-meth} ASM cells. The proliferative, morphological and biochemical characteristics of *TSC2*^{-meth} ASM cells were very similar to *TSC2*^{-/-} smooth muscle cells with LOH that we previously isolated from an AML of a female *TSC2* patient (*TSC2*^{-/-} ASM cells).^{18,19} The growth of *TSC2*^{-meth} ASM cells requires the addition of epidermal growth factor (EGF) to the culture medium, while the exposure to specific monoclonal antibody raised against EGFR causes the blockade of proliferation and their death. Our data show for the first time that the methylation of the *TSC2* promoter might cause loss of tuberin in *TSC2* cells, and that such epigenetic alteration of smooth muscle cell function may underlie their abnormal growth and likely lead to AML development.

MATERIAL AND METHODS

Establishment of the Angiomyolipoma Culture

The renal angiomyolipoma sample was obtained during total nephrectomy from a 36-year-old man with a history of TSC who had given his informed consent according to the Declaration of Helsinki. The cells were obtained as shown in Lesma et al.¹⁸ Briefly, the tumor tissue was manually dissociated using collagenase type II (Sigma, Sigma, St. Louis, MO) by means of repetitive pipetting. The collagenase was neutralized with a serum-containing medium (50/50 mixture of Dulbecco's Eagle's medium/Ham F12; Euroclone, Paignton, UK) supplemented with hydrocortisone (2×10^{-7} M) (Sigma-Aldrich, St. Louis, USA), epidermal growth factor (EGF; 10 ng/ml) (Sigma-Aldrich), sodium selenite (5×10^{-8} M) (Sigma-Aldrich), insulin (25 μ g/ml) (Sigma-Aldrich), transferrin (10 μ g/ml) (Sigma-Aldrich), ferrous sulfate (1.6×10^{-6} M) (Sigma-Aldrich), and 15% fetal bovine serum (Euroclone) as indicated by Arbiser et al.²⁰

Vascular smooth muscle-like cells (VSMCs) and A549 cells were grown in DMEM supplemented with FBS 10%.

Cell Immunofluorescence Microscopy

The cells were cultured on glass slides, permeabilized with Cytoskelfix (Cytoskeleton, Denver, CO, USA) and dried in air. The primary antibody against α -actin (1:100; Sigma-Aldrich), vimentin (1:70; Santa Cruz, Santa Cruz, USA), S100 (1:8000; DAKO, Carpinteria, CA, USA) keratin 8/18 (1:100; New Marker, Fremont, CA, USA), HMB45 (1:100, DAKO), CD44v6 (1:100; Invitrogen, Carlsbad, CA USA), hamartin (1:100, Santa Cruz), tuberin (C-20) (1:100; Santa Cruz) were applied overnight at 4°C. The samples were incubated for 3 hours at room temperature with fluorescein isothiocyanate-conjugated anti-mouse antibody (Alexa, Eugene, OR, USA) for α -actin, HMB45, and keratin 8/18, with fluorescein isothiocyanate-conjugated anti-goat antibody (Alexa) for vimentin and hamartin, with rhodamin-conjugate anti-rabbit antibody (Alexa) for tuberin.

HMB45 positivity was evaluated by immunofluorescence. Cells were incubated for 72 hours with trichostatin (3.3 mM). HMB45 labeling was defined as strong (+), and negative (-). Quantitation of HMB45 intensity was achieved with a confocal microscopy (Leica TCS-SP2) and analyzed by Leica Confocal software using the profile through stack/series methods as follows: 30 equal polygonal areas covering the cells were randomly selected on each image. The fluorescence intensity values of the selected cells were quantified and the mean and the standard deviation of the data were calculated by the GraphPad software. The level of statistical significance was determined by Student's *t*-test.

Western Blotting

The cells were lysed in lysis buffer (5 mmol/L EDTA, 100 mmol/L deoxycholic acid, 3% sodium dodecyl sulfate), boiled, electrophoretically run on a 10% sodium dodecyl sulfate-polyacrylaide 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, phospho-tuberin (Thr1462) (1:1000; Cell Signaling), hamartin (a gift from Dr. Nellist and dr. Halley, Erasmus University, Rotterdam, The Netherlands), phospho-Akt (Ser473) (1:1000; Cell Signaling), phospho-S6 ribosomal protein (Ser235/236) (1:1000; Cell Signaling, Beverly, USA), S6 ribosomal protein (1:1000; Cell Signaling), phospho-extracellular signal-regulated kinase (Erk) (Thr202/Tyr204) (1:1000; Cell Signaling), Erk (1:1000; Cell Signaling), EGF receptor (1:200; Santa Cruz), β -actin (1:10000 ; Sigma-Aldrich), p53 (1:100; Santa Cruz), p21 (1:1000; Cell Signaling). The membrane were washed and incubated for 1 hour with anti-rabbit antibody (1:10,000; Chemicon, Temecula, CA, USA). The reaction was revealed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA). Densitometric analysis was performed by Kodak MJ project program. Data were expressed as optical density.

Mutation study

The DNAs were extracted from peripheral blood, angiomyolipoma and isolated cultured cells using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). All of the exons and intron-exons junctions of *TSC1* and *TSC2* from the genomic DNAs were amplified by means of standard polymerase chain reaction (PCR) and described primers.²¹ The sequencing reactions were performed as previously reported by Lesma et al.¹⁸ and repeated at least twice.

Loss of heterozygosity analysis

The panel of microsatellite markers near the *TSC2* locus on chromosome 16p13.3 consisted of D16S525, D16S3024, D16S3394, D16S291, D16S664 and D16S663. The 5' sense primers were labelled with 6-FAM fluorescent dyes (M-Medical, Cornaredo, Italy). The primer sequences were obtained from the Genome Database (<http://www.ncbi.nlm.nih.gov/>). Microsatellite orientation and position were obtained from UCSC Genome Browser (<http://genome.ucsc.edu/>). Loss of heterozygosity was analyzed as previously described in Lesma et al.¹⁸

Methylation Assays

The methylation status of *TSC2* and *ZFX* promoter regions, was analyzed by PCR using as template the genomic DNA previously digested with the methylation sensitive enzyme HpaII and with its isoschizomer MspI, insensitive to methylation (New England Biolabs, Beverly, MA). The primers used for amplifications spanned several methylation-sensitive restriction sites (7 for *TSC2* gene) in the 5' of the tested genes. The UCSC Browser (<http://genome.ucsc.edu/>) and the CpG island searcher (<http://cpgislands.usc.edu/>) were consulted in order to identify *TSC2* promoter region and CpG island.

The primers used to *TSC2* promoter analysis were sense 5'-atgccgctaccggaagtgc-3' (chr16:2,037,961-2,037,979, UCSC Genome Browser) and antisense 5'- tggctagagagctctctact-3' (chr16:2,038,391-2,038,371, UCSC Genome Browser). After denaturation at 94°C for 5 minutes,

35 cycles of PCR were done; each cycle consisted of 45 seconds at 95°C, 45 seconds at 54°C, and 45 seconds at 72°C. ZFX primers and methods are fully described in Gilbert and Sharp.²²

To quantify the methylation levels of *TSC2* promoter, pyrosequencing technology was used to analyze the same region studied by methylation-sensitive digestion assay. The bisulphite conversion of genomic DNA (1µg) was obtained using EZ DNA methylation kit (Zymo Research, Orange, CA, USA). After bisulphite treatment, PCR was carried out in a final volume of 50 µl with 2.5 unit of Promega Go-Taq Hot Start Polymerase (Promega, Madison, WI, USA). The primer sequences for modified sequences are: forward primer 5'-ttygttagaggggyggtatagaat-3' (chr16: 2,037,899-2,037,921, UCSC Genome Browser) and biotinylated reverse primer 5'-acactacraaatccrcctctc-3' (chr16: 2,038,141-2,038,121, UCSC Genome Browser). The PCR conditions were: Step 1: 95°C, 2 minutes; Step 2: 95°C, 30 seconds; Step 3: 61.2 °C decrease 0.5°C per cycle, 30 seconds; Step 4: 72°C, 30 seconds; Step 5: repeat steps 2-4 14 more times; Step 6: 95°C, 30 seconds; Step 7: 54°C, 30 seconds; Step 8: 72°C, 30 seconds; Step 9: repeat steps 6-8 31 more times; Step 10: 72°C, 5 minutes. 40 µl of PCR product were used for pyrosequencing assay using the sequencing primers 5'-atyggaagtgygggt-3' (chr16: 2,037,969-2,037,983, UCSC Genome Browser).

Pyrosequencing reactions were performed in the PSQ HS 96 System (Biotage, Uppsala, Sweden) by using Pyro Gold Reagent kits (Biotage). Methylation was quantified using Pyro Q-CpG Software (Biotage) which calculates the ratio of converted C's (T's) to unconverted C's at each CpG and expresses this as a percentage of methylation. To assess the methylation pattern in normal condition we analyzed different normal DNA from peripheral blood lymphocytes.

Evaluation of Cell Growth and Proliferation

The proliferation growth factor dependence of the smooth muscle-like cells and VSMCs were assayed in the presence or absence of EGF (10ng/ml) or by replacing EGF with insulin-like

growth factor 1 (IGF-1) (50 ng/ml), by counting the cells after 4, 7, 17, 21, and 25 days of culture in a Neubauer chamber. Anti-EGFR antibody (clone 225; Calbiochem, Darmstadt, Germany), and IGF-1R (clone α IR3; Calbiochem) were added at a concentration of 5 μ g/ml to the complete medium, and cell proliferations were evaluated after 4,7, 17, 21, and 25 days of culture. Each data point of the proliferation experiments is the mean of four independent experiments.

Cell treatments

Trichostatin A (Sigma-Aldrich) was incubated for 72 hours at the concentration of 3.3 mM. 5-azacytidine (Sigma-Aldrich) was incubated for 96 hours at the concentration of 1 or 10 μ M.

In the western blotting experiments to study S6 and Erk phosphorylation, cells were incubated with rapamycin (1ng/ml) or anti-EGFR antibody (5 μ g/ml) for 24 or 48 hours.

Cells were incubated for 2 hours with IGF-1 (50 ng/ml) with or without LY294002 (20 μ M) (Sigma).

The action of rapamycin (Rapamune-Sirulimus, Wyeth Europa, UK) was evaluated by adding 1 ng/ml and 5ng/ml to the TSC2^{-meth} cells at plating time and 3 hours after plating, and measuring cell proliferation after 5, 8 and 10 or evaluating the apoptotic effect by TUNEL analysis after 10 day incubation. For TUNEL analysis VSMCs were incubated with staurosporin (100mM) for 1h 30'.

TUNEL analysis

Apoptotic cells were examined by TUNEL analysis using an *in situ* cell death DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) following the manufacturer's instruction. Briefly, cells were fixed for 25 minutes in 4% paraformaldehyde, washed twice in PBS for 5 minutes, permeabilized in 0.2% Triton X-100 for 5 minutes and washed twice in PBS

for 5 minutes. Slides were incubated with rTdT reaction Mix for 1 hour at 37°C, then endogenous peroxidases were blocked with 0.3% hydrogen peroxide for 5 minutes at room temperature. Following incubation with streptavidin HRP, apoptotic cells were localized using 3',3'-diaminobenzidine tetrahydrochloride. The number of TUNEL-positive cells was counted and the data presented are mean of the percentage of TUNEL –positive cells.

Statistical Analysis

The data are expressed as mean values \pm SEM, and were statistically analyzed using Student's *t*-test; significance is indicated for *P* values of * <0.05 , ** <0.01 and *** <0.001 .

RESULTS

Cellular characterization

From the angiomyolipoma of a male TSC patient, a homogenous cellular population was isolated and cultured in monolayers. The characteristic elongated shape strongly resembled TSC2 smooth muscle (TSC2^{-/-} ASM) cells isolated from an angiomyolipoma of a female TSC2 patient and previously described by our group.¹⁸ The majority of these isolated cells were strongly labeled by α -actin antibody, the marker for smooth muscle cells (Figure 1), while the lack of detection of vimentin, keratin 8-18 and S100 suggested that fibroblasts, epithelial-like cells or adipocytes were absent from this cellular population (Figure 1). The angiomyolipoma-derived smooth muscle (ASM) cells were also labeled by the typical markers of TSC and LAM cells, HMB45 antibody¹⁶ and CD44v6 antibody¹⁷ (Figure 1).

Mutations analysis

A germline TSC2 intron 8-exon 9 junction heterozygous mutation (c.867-2A>G) was detected by DNA sequencing in patient blood and angiomyolipoma (data not shown) and in

angiomyolipoma-derived ASM cells (Figure 2A). The LOH of these ASM cells was tested by means of PCR amplification, using a panel of microsatellite markers near the *TSC2* locus on chromosome 16p13.3, but we failed to detect LOH (data not shown). On the other hand, these newly isolated ASM cells lacked tuberin as immunofluorescence and western blotting failed to reveal any positive reactivity to specific antibodies. This negative result can be compared to what observed in *TSC2*^{-/-} ASM cells, while VSMCs and A549 cells, used as control, expressed tuberin, as expected (Figure 2B). Differently, hamartin was present at comparable levels in all tested cells and β -actin was evaluated as protein control (Figure 2B).

Methylation of the TSC2 promoter region

The absence of LOH and the lack of tuberin expression in ASM cells led us into assessing whether the Knudson second hits could be epigenetic. We analyzed the DNA methylation of the CpG island within the *TSC2* promoter region, using methylation-sensitive digestion with HpaII restriction enzyme. Methylation (presence of PCR amplification after HpaII digestion) of the promoter in ASM cells was found while normal VSMCs cells were unmethylated (absence of PCR amplification after HpaII digestion) (Figure 3A).

To control the enzyme cleavage efficiency we chose two different approaches. In the first case, it was used the digestion with MspI, an isoschizomer of HpaII that, unlike HpaII, can cleave the sequence when the internal C of the restriction site CCGG is methylated. In the second case the cleavage efficiency was tested with the amplification of *ZFX* promoter, which is always unmethylated. As expected, we found a complete DNA digestion in both controls, and this resulted in no PCR amplification in all samples (Figure 3A). In addition we observed the *TSC2* promoter methylation in the original AML tissue, although there was a reduced extent probably due to the heterogeneity of AML (Figure 3A). To confirm the above reported qualitative methylation data we performed the pyrosequencing quantitative assay on the same promoter

regions analyzed with methylation-sensitive digestion. This method allows to reveal quantitatively the methylation of all CpGs position. In normal samples *TSC2* resulted demethylated in all CpGs analyzed (Figure 3B). In the *TSC2*^{-/meth} ASM cells, consistently with our previous results, we found a promoter methylation pattern indicating an epigenetic silencing. The low methylation levels (29%, 6%, 6%) obtained with the pyrosequencing analysis were indicative of methylation of only one allele.

In the isolated ASM cells the epigenetic silencing of *TSC2* was confirmed by transcriptional reactivation with consequent tuberin expression following treatment with the histone deacetylase inhibitor trichostatin A for 72 hours or with the DNA methylase inhibitor 5-azacytidine for 96 hours (Figure 3C). Trichostatin A and 5-azacytidine are chromatin remodelling agents able to reactivate genes epigenetically silenced^{23,24,25} Following the demonstration of the DNA methylation of the CpG island, the angiomyolipoma-derived ASM cells were named *TSC2*^{-/meth} ASM cells, and thereafter we shall use this name. Following 5-azacytidine exposure we monitored for a week the proliferation of *TSC2*^{-/meth} cells; the rate of proliferation was comparable to control. With trichostatin A the overproliferation was eliminated (data not shown). To evaluate the effects of trichostatin A-induced tuberin expression on the phenotype of *TSC2*^{-/meth} ASM cells, gp100 expression was quantified by exposure to HMB45 antibody, a marker of TSC and LAM cells.^{16,18} After 72 hours of trichostatin A exposure, the labeling was greatly reduced. HMB45 reactivity was quantified as positive (+) or negative (-). Normally, about 70% of untreated *TSC2*^{-/meth} ASM cells were strongly labeled by HMB45 (Figure 3C), while after incubation with trichostatin A, HMB45-labeled cells were reduced to 45%, and the negative ones increased from 30% to 55% (Figure 3D).

Effect of trichostatin A on *TSC2*^{-/meth} ASM cell phenotype

To further investigate the effects of trichostatin A in TSC2^{-meth} ASM cells, we evaluated S6 and Erk phosphorylation. After 72 hour incubation with trichostatin A there was a reduction of S6 and Erk phosphorylation in TSC2^{-meth} ASM cells (Figure 4). This was not observed in TSC2^{-/-} ASM cells (Figure 4). The expression of S6 and Erk were not changed by trichostatin A in both cell lines (Figure 4). Thus the induced expression of tuberin by trichostatin A exposure correlated with downregulation of the constitutive phosphorylation of S6. As expected, trichostatin A led to a slowing proliferation rate even within these 72 hours (data not shown).

Role of PI3K pathway in TSC2^{-meth} ASM cells

We had previously shown that the specific inhibitor LY294002 was unable to inhibit PI3K in TSC2^{-/-} ASM cells.²⁶ Here we report that a similar phenomenon was also present in TSC2^{-meth} ASM cells (Figure 5). Addition of IGF-1 increased Akt and PTEN phosphorylation without affecting constitutive S6 phosphorylation and the co-incubation with LY 294002 failed to modify the extent of both basal and IGF-1-mediated phosphorylations (Figure 5). Following incubation with trichostatin A for 72 hours, the LY294002 addition was able of reducing the extent of both basal and IGF-1-stimulated Akt phosphorylation in TSC2^{-meth} ASM cells (Figure 5). In this latter condition, also the IGF-1-promoted increase of S6 phosphorylation was inhibited by LY294002 (Figure 5).

Regulation of growth of TSC2^{-meth} ASM cells

We have detected EGFR by western blotting analysis of TSC2^{-meth} ASM cells, and the level of expression is comparable to that of TSC2^{-/-} ASM cells, vascular smooth muscle cells (VSMCs) and A549 cells (insert in Figure 6A). Also, the proliferation of TSC2^{-meth} ASM cells required EGF addition to the culture medium and the substitution with another growth factor, such as IGF-1, could not substitute for EGF-proliferative action (Figure 6A). Moreover, the exposure to anti-EGFR and anti-IGF-1R antibodies reduced the proliferation and caused the progressive loss

of TSC2^{-/meth} ASM cells. The rate of proliferation of the TSC2^{-/meth} ASM cells in presence or absence of EGF and the ability of anti-EGFR or anti-IGF-1R antibodies to kill them are comparable to those observed for TSC2^{-/-} ASM cells (Figure 6B).

Rapamycin was applied at 1 and 5 ng/ml at time of plating or 3 hours later. Rapamycin significantly reduced the rate of cell proliferation either when applied at plating time or 3 hours after plating causing a significant loss of cells at the concentration of 5 ng/ml (Table 1). The percentage of attached cells was 80±1,6% and 96±0.7% at 3 and 16 hours after plating respectively. The effect of the delayed addition is different to what observed in TSC2^{-/-} ASM cells, which were affected by rapamycin only when the drug was applied at plating time.²⁶

Effect of anti-EGFR antibody and rapamycin on S6 and Erk phosphorylation in TSC2^{-/meth} ASM cells

The cellular effects of anti-EGFR antibody on TSC2^{-/meth} ASM cell proliferation were also evaluated biochemically. It is well known that TSC protein abnormalities may lead to dysregulation of mTOR activity with increased p70 S6 kinase activity, and, as expected, TSC2^{-/meth} ASM cells presented constitutive phosphorylation of S6. Following 24 or 48 hour incubation with rapamycin or anti-EGFR antibody the extent of such phosphorylation was reduced without changes in S6 expression (Figure 7). As the Erk signaling pathway is activated by EGF and is a major contributor to cellular growth, we have studied the phosphorylation of this kinase after exposure to anti-EGFR antibody or rapamycin. The extent of Erk phosphorylation was reduced by anti-EGFR, mainly after 48 hour incubation while the Erk levels were unchanged (Figure 7). The effect of rapamycin on Erk phosphorylation was, however, rather minor (Figure 7). The rapamycin-mediated reduced ability of TSC2^{-/meth} cells to proliferate was not accompanied by enhancement of apoptosis. Rapamycin was added to the medium at 1 or 5 ng/ml at plating time or 3 hours after plating for 10 days; this did not change the expression of p21 and p53 (Figure 8);

moreover, the number of TUNEL-positive cells remained comparable to control untreated cells (Table 2). Differentially, 57.3% of VSMCs, used as control, underwent apoptosis after exposure to 100mM staurosporin for 1h30' (Table 2).

DISCUSSION

TSC is a tumor suppressor gene disorder associated with benign and malignant tumors, and angiomyolipomas are the most common renal tumors. They are composed of smooth muscle, fat, and vascular cells.²⁷ Tuberin is the *TSC2* gene product; it functions as a renal tumor suppressor gene and regulates cell growth and cell cycle progression.²⁸ The lack of tuberin may lead to abnormal cell proliferation.²⁹ LOH in TSC cells occurs in 60% of the angiomyolipomas of women with sporadic LAM.³⁰ We have previously reported the isolation and the characterization of *TSC2*^{-/-} ASM cells from an angiomyolipoma with a mutation in exon 18 consisting of a stop codon associated to LOH of *TSC2* locus.¹⁸ The lack of tuberin appears to be strictly related to their EGF requirement for growth.¹⁸ In the current study we report that the lack of tuberin in *TSC2* cells may also be caused by an epigenetic silencing such as the methylation of the *TSC2* gene promoter.

Blood and ASM cells of a *TSC2* patient bear the somatic mutation of *TSC2* gene in intron8-exon9 junction (867-2AG), on the other hand the newly purified angiomyolipoma cells did not express tuberin and LOH was not detected. This inability to find a deletion of *TSC2* locus in the newly isolated angiomyolipoma cells could have been explained by a number of different possibilities such as a second mutations, as shown for the *PDK1* and *PDK2* genes in autosomal dominant polycystic kidney disease,³¹ or the methylations of the wild-type allele frequently observed in human cancers.^{32,33} In these *TSC2* ASM cells we detected a methylation of the CpG

island leading to the inhibition of tuberin expression. Thus the methylated purified ASM cells were named $TSC2^{-/meth}$ ASM cells.

DNA methylation at CpG dinucleotides is a common and important epigenetic mechanism of inactivation of tumor-suppressor in cancers, and such a genetic modification is carried out by DNA methyltransferase.³⁴ Several human genes contain a CpG island within their promoter region, and unmethylated CpG islands are generally protected from methylation in normal cells.³⁵ However, the *de novo* methylation of CpG islands which overlaps with promoter regions is a common feature in human cancer, and results in the loss of gene function, similar to other mechanisms such as deletion or point mutation.^{33,36} The methylation of the *TSC2* promoter is biologically very relevant in the case of $TSC2^{-/meth}$ ASM cells since the functional effect on tuberin transcription is a complete blockade due to the present germline mutation of *TSC2* intron 8-exon 9 junction on the other allele.

In a previous report no evidence of methylation within *TSC2* promoter region of the wild-type allele was found in hamartomas obtained from TSC patients.¹¹ Failure to demonstrate promoter methylation may be attributed to heterogeneity of tumor tissues while in the case of $TSC2^{-/meth}$ ASM cells we could analyzed a pure population. On the other hand, there are several examples in cancer of functional modification of *TSC* gene by methylation. One is the clinical outcome of patients with breast cancer, that turns unfavorable, where the *TSC* promoter is methylated.³⁷ Also the downregulation of *TSC* gene function in oral squamous cell carcinoma can be ascribed to an epigenetic alteration by methylation of the *TSC* promoter.³⁸

The role of promoter methylation of the wild-type allele, in the abnormal development of $TSC2^{-/meth}$ cells is confirmed by the normalizing effects of chromatin remodeling agents, trichostatin A and 5-azacytidine. These agents promoted the expression of tuberin and downregulated the constitutive phosphorylation of S6 and the labeling by HMB45 antibody.

Histone deacetylase (HDAC) inhibitors, such as trichostatin A, are a new class of targeted anticancer agents, which are potent inducers of cellular differentiation, growth arrest and cell death, and are effective *in vitro* and *in vivo*.³⁹ By reversible acetylation, HDAC inhibitors modify the structure and function of histones and proteins in transcription factor complexes, which are involved in the regulation of gene expression. The inhibition of the HDAC could increase the remission rate of many kinds of solid tumors, obviously by cooperation with other anticancer drugs.⁴⁰ It is thus, conceivable that, in cases such as this, the use of HDAC inhibitors may result in an important additional therapeutic approach to the treatments for TSC now in development.^{18,41}

As described for TSC2^{-/-} ASM cells¹⁸, TSC2^{-/meth} ASM cell growth required the addition of EGF in the growth medium, and the exposure to anti-EGFR antibody caused the blockade of cell proliferation and, then, death. The growth rate of the two types of TSC2 cells is similar, thus the lack of tuberlin, resulted from two different genetic lesions, leads to EGF dependence in both cases. The EGFR signaling pathways regulate cell differentiation, proliferation, and migration and, in the case of TSC2 cells, might also be involved in the constitutive S6 phosphorylation. Anti-EGFR antibody has been shown to be efficacious in several types of cancer, such as colorectal and head and neck cancers⁴² and we suggest that might be also of therapeutic value in TSC.¹⁸

Rapamycin, a mTOR inhibitor, has been identified as a potential therapeutic agent for TSC and LAM^{43,44} and a recently reported clinical trial shows its potential efficacy in promoting the regression of TSC angiomyolipomas.⁴¹ The exposure to rapamycin downregulated the constitutive S6 phosphorylation in TSC2^{-/meth} ASM cells and it was capable of inhibiting cellular proliferation either when added at plating time or in a delayed application. In TSC2^{-/-} ASM cells, rapamycin regulated proliferation only when added at plating time, and this is a clear

pharmacological difference with $TSC2^{-/meth}$ ASM cells.¹⁸ The inhibitory effects of rapamycin upon $TSC2^{-/meth}$ ASM cell proliferation did not require any activation of apoptosis.

In addition to the proliferative effects caused by the lack of tuberin, we observed that LY294002 failed to inhibit IGF-1-mediated Akt phosphorylation in $TSC2^{-/meth}$ ASM cells. When trichostatin A was added to the growth medium, the ability of LY294002 to inhibit IGF-1-mediated and basal Akt phosphorylation was apparent. A similar phenomenon was observed in $TSC2^{-/-}$ ASM cells and the introduction of the *TSC2* gene restored the normal pharmacological activity of this drug.²⁶ Thus, in both *TSC2* cell types the lack of tuberin affects also the sensitivity to drugs.

In conclusion, this study provides the first evidence that the methylation of the *TSC2* promoter as a second hit may cause the blockade of tuberin expression and full deployment of *TSC2* cellular phenotype. The de-methylation ability of an HDAC inhibitor, trichostatin A, and a DNA methylase inhibitor, 5-azacytidine, allowed the expression of tuberin and reverted the biochemical, pharmacological and phenotypical characteristics. The growth of these cells requires EGF in the medium, and the blockade of EGFR leads to their death. These methylated cells, differently from $TSC2^{-/-}$ ASM cells¹⁸ are very sensitive to the cytostatic action of rapamycin.

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Figure legends

Figure 1. Immunocytochemical characterization of TSC angiomyolipoma-derived cells. Characterization was performed using a panel of antibodies related to the AML cellular constituents. TSC cells were positively labeled by α -actin antibody showing their nature of smooth muscle cells. Cells were negative for S100, a marker of lipid-containing cells, vimentin, a marker of fibroblasts, and keratin 8-18, a marker for epithelial-like cells. Antibodies to HMB45 and CD44v6, markers of TSC and LAM cells, positively immunolabeled these TSC cells. All the bars = 50 μ m.

Figure 2. Genetic and biochemical analysis of TSC angiomyolipoma-derived cells **A.** The shown mutation was determined by direct sequence analysis of blood from a control and the TSC patient, and the isolated cells. The mutation site is indicated by a vertical arrow. Cell sequencing revealed the mutation in TSC2 intron 8-exon 9 junction mutation (c.867-2A>G). **B.** The expression of tuberin and hamartin in the ASM cells was determined by western blotting and immunofluorescence. Tuberin was undetectable in the purified ASM cells and TSC2^{-/-} ASM cells while it was expressed in VSMCs, and A549 cells. Hamartin was expressed in all cell types. The blots are representative of four separate experiments. The immunocytochemical staining is referred only to the purified ASM cells (bars= 50 μ m)

Figure 3. DNA methylation in *TSC2* promoter and effect of chromatin remodelling agents, trichostatin A (TSA) and 5-azacytidine (5-AZA). **A.** The lack of tuberin in TSC2 ASM cells was evaluated by DNA methylation of the CpG island within the *TSC2* and *ZFX* promoter regions, using methylation-sensitive digestion with HpaII restriction enzyme and its isoschizomer MspI. The determination was performed three times. Methylation occurs at the promoter site in the TSC2 ASM cells (left panel) and AML tissue (right panel). **B.** Pyrograms indicating the percentage of methylation (light blue boxes) of 3 explicative CpGs within TSC2 promoter region

of the normal control (left panel) and $TSC2^{-/meth}$ ASM cells (right panel). These CpGs are a part of the methylation sites analyzed by methylation-sensitive digestion assay. **C.** Following incubation with TSA (3.3 μ M) for 72 hours (left panel) or 5-azacytidine (5-AZA) (1 and 10 μ M) for 96 hours (right panel), tuberin and phospho-tuberin (Thr1462) expression was promoted in $TSC2^{-/meth}$ ASM cells as evaluated by western blotting. The phosphorylated form was detectable only after 5-AZA incubation. As expected TSA did not have any effect on tuberin expression in $TSC2^{-/-}$ ASM cells. Experiments were done four times. The expression of tuberin induced by treatments with TSA or 5-AZA was also confirmed by immunocytochemical staining (bars = 50 μ m) **D.** Effect of trichostatin A on HMB45 labeling of $TSC2^{-/meth}$ ASM cells. The positive cells are indicated with +, and the negative with -. The exposure to trichostatin A reduced HMB45 labeling very significantly. Mean values \pm SEM. Significant differences (*** $P < 0.001$) versus control were evaluated by student's *t*-test.

Figure 4. Effect of trichostatin A (TSA) (3.3 μ M) incubation (72 hours) on S6 and Erk phosphorylation in $TSC2^{-/-}$ and $TSC2^{-/meth}$ ASM cells. In $TSC2^{-/meth}$ ASM cells, trichostatin A markedly reduced the constitutive phosphorylation of S6 (Ser235/236) and also Erk (Thr202/Tyr204) phosphorylation. No effect by trichostatin A was observed in $TSC2^{-/-}$ ASM cells. The blots are representative of three independent experiments. These data were confirmed by the densitometric analysis. Mean values \pm SEM. Significant differences (*** $P < 0.001$) versus control were evaluated by student's *t*-test

Figure 5. Effect of trichostatin A (TSA) on PI3K pathway. $TSC2^{-/meth}$ ASM cells were incubated with or without trichostatin A (3.3 μ M) for 72 hours and then with IGF-1 (50 ng/ml) and LY294002 (LY) (20 μ M) for 2 hours. IGF-1 exposure of $TSC2^{-/meth}$ ASM cells induced Akt (Ser473) and PTEN (Ser380/Thr382/Thr383) phosphorylation without affecting S6 (Ser235/236)

phosphorylation. The co-incubation with IGF-1 and PI3K inhibitor, LY294002, did not modify the extent of phosphorylation of Akt, PTEN and S6. The expression of Akt, and S6 was unaffected by any treatment. After trichostatin A exposure, LY290042 was capable of antagonizing IGF-1-mediated and basal phosphorylation of both Akt and S6 while the phosphorylation of PTEN was unaffected as reported for *TSC2*^{-/-} ASM cells after retroviral transfection of the *TSC2* gene.²⁶ The expression of Akt, and S6 proteins was unaltered. The blots are representative of four independent experiments. These data were confirmed by the densitometric analysis (graphs at the right). Mean values \pm SEM. Significant differences (**P*<0.05; ***P*<0.01; ****P*<0.001) versus control were evaluated by student's *t*-test

Figure 6. Evaluation of EGF and IGF-1 role in *TSC2*^{-meth} ASM proliferation. Cells were counted after 4, 7, 17, 21 and 25 days of culture. **A:** The growth and proliferation of *TSC2*^{-meth} ASM cells required the addition of EGF (10 ng/ml) in the culture medium; IGF-1 (50 ng/ml) failed to promote any increase in cell number. The treatment with either anti-EGFR or anti-IGF-1R antibodies limited the growth rate in the early days of drug exposure and then started to dye. Experiments were done four times. **Insert:** expression of EGF receptor (EGFR). **B:** Comparison of the proliferation of the *TSC2*^{-/-} (full line) and *TSC2*^{-meth} (dashed line) ASM cells in presence or absence of EGF, and with anti-EGFR or anti-IGF-1R antibodies. The beginning of the treatment was considered 100% of the percentage of the cells. The growth rate of the two cell line was similar. Experiments were done three times each. Mean values \pm SEM. Significant differences (****P*<0.001) vs control were evaluated by Student's *t* test.

Figure 7. Effect of rapamycin (1 ng/ml) and anti-EGFR antibody (5 μ g/ml) on phosphorylation of S6 (Ser235/236) and ERK in *TSC2*^{-meth} ASM cells. The blots are representative of three experiments. Incubation with rapamycin for inhibited constitutive S6 phosphorylation within 24

hours, while inhibition of Erk (Thr202/Tyr204) phosphorylation required 48 hours. The effect of anti-EGFR on Erk phosphorylation was superior to that of rapamycin. These data were confirmed by the densitometric analysis. Mean values \pm SEM. Significant differences (***) $P < 0.001$) versus control were evaluated by student's *t*-test

Figure 8. Exposure to rapamycin (1 and 5 ng/ml) added at plating time or 3 hours after plating failed to modify the expression of p21 and p53. The levels of β -actin were evaluated as protein control.

Table1. Cell proliferation rate and rapamycin effect

Days	5	8	10
TSC2 ^{-meth} ASM cells	180%	230%	286%
TSC2 ^{-meth} ASM cells with rapa 1ng/ml at plating time	143%	143%	125%
TSC2 ^{-meth} ASM cells with rapa 1 ng/ml 3 hours after plating	152%	160%	168%
TSC2 ^{-meth} ASM cells with rapa 5ng/ml at plating time	89%	89%	45%
TSC2 ^{-meth} ASM cells with rapa 5 ng/ml 3 hours after plating	116%	125%	45%

Percentage increase in cell number. We plated 2.8×10^4 cells and the number of cells was evaluated at the indicated time.

Table 2. Effect of rapamycin on apoptosis

	% of TUNEL positive cells
TSC2 ^{-/meth} ASM cells	4.5
TSC2 ^{-/meth} ASM cells + 1 ng/ml rapamycin at plating time	6.1
TSC2 ^{-/meth} ASM cells + 5 ng/ml rapamycin at plating time	6.9
TSC2 ^{-/meth} ASM cells + 1 ng/ml rapamycin 3 hours after plating	4.4
TSC2 ^{-/meth} ASM cells + 5 ng/ml rapamycin 3 hours after plating	5.2
VSMC	5.1
VSMC + staurosporin	57.3

Percentage of TUNEL positive cells. Rapamycin was incubated for 10 days at the indicated conditions and TUNEL-positive and negative cells were counted. VSMCs were incubated with 100mM staurosporin for 1h 30'.