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Molecular Pathogenesis of Genetic and Inherited Diseases

Novel Proteins Regulated by mTOR in Subependymal Giant Cell Astrocytomas of Patients with Tuberous Sclerosis Complex and New Therapeutic Implications

Magdalena Ewa Tyburczy,* Katarzyna Kotulska,† Piotr Pokarowski,‡ Jakub Mieczkowski,* Joanna Kucharska,* Wieslawa Grajkowska,† Maciej Roszkowski,§ Sergiusz Jozwiak,† and Bozena Kaminska*

From the Laboratory of Transcription Regulation, the Nencki Institute of Experimental Biology, Warsaw; the Departments of Neurology and Epileptology,† and Neurosurgery,§ the Children's Memorial Health Institute, Warsaw; and the Faculty of Mathematics, Informatics, and Mechanics,‡ University of Warsaw, Poland*

Subependymal giant cell astrocytomas (SEGAs) are rare brain tumors associated with tuberous sclerosis complex (TSC), a disease caused by mutations in *TSC1* or *TSC2*, resulting in enhancement of mammalian target of rapamycin (mTOR) activity, dysregulation of cell growth, and tumorigenesis. Signaling via mTOR plays a role in multifaceted genomic responses, but its effectors in the brain are largely unknown. Therefore, gene expression profiling on four SEGAs was performed with Affymetrix Human Genome arrays. Of the genes differentially expressed in TSC, 11 were validated by real-time PCR on independent tumor samples and 3 SEGA-derived cultures. Expression of several proteins was confirmed by immunohistochemistry. The differentially-regulated proteins were mainly involved in tumorigenesis and nervous system development. *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, *SFRP4*, and *NPTX1* genes were likely to be mTOR effector genes in SEGA, as their expression was modulated by an mTOR inhibitor, rapamycin, in SEGA-derived cells. Inhibition of mTOR signaling affected size of cultured SEGA cells but had no influence on their proliferation, morphology, or migration, whereas inhibition of both mTOR and extracellular signal-regulated kinase signaling pathways led to significant alterations of these processes. For the first time, we identified genes related to the occurrence of SEGA and regulated by mTOR and demonstrated an effective modulation of SEGA growth by pharmacological inhibition of both mTOR and extracellular signal-regulated kinase signaling pathways, which could represent a novel therapeutic approach. ***(Am J Pathol 2010, 176:1878–1890; DOI: 10.2353/ajpath.2010.090950)***

Subependymal giant cell astrocytomas (SEGAs) are rare, low-grade brain tumors (World Health Organization Grade I) of a mixed glioneuronal lineage.[1,2] They are observed in 10% to 20% of patients with tuberous sclerosis complex (TSC) and are the major cause of morbidity in children and young adults with TSC.[3] The disease affects about one in 6000 people, is characterized by the formation of benign tumors in multiple organs (mainly brain, heart, kidneys, skin, or lungs), and is often associated with epilepsy, mental retardation, and autism.[4,5] Tuberous sclerosis complex is caused by mutation in one of two tumor suppressor genes, *TSC1* and *TSC2*, which encode Hamartin and Tuberin, respectively.[6,7] Both proteins form the TSC complex that inhibits the mammalian target of rapamycin complex 1 (mTORC1). Within the TSC complex, TSC1 stabilizes TSC2, whereas TSC2 acts as a GTPase-activating protein for the small GTPase RHEB (Ras homolog enriched in brain).[8–10] Mutation in one of the genes leads to elevated RHEB-GTP levels and activation of mTORC1, which further triggers a downstream kinase signaling cascade, including phosphorylation of eukaryotic translation initiation factor 4E-binding proteins and p70 S6 kinases, proteins involved in translation initiation and ribosome biogenesis.[11] Increased

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Address reprint requests to Prof. Bozena Kaminska, Laboratory of Transcription Regulation, The Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland. E-mail: bozenakk@nencki.gov.pl.

activation of mTOR kinase results in disorganized cellular overgrowth, abnormal differentiation, and formation of neoplasms.[12,13]

Clinical trials with rapamycin (sirolimus), a specific inhibitor of mTORC1, have been initiated and showed a regression of SEGAs after a systemic administration of the drug.[14,15] However, molecular mechanisms underlying a regression of SEGAs in TSC patients are still poorly understood. By combining the use of rapamycin, transcriptional profiling, and RNA interference, more than 400 genes in yeast and 90 genes in *D. melanogaster* were identified to be up- or down-regulated by mTOR inhibition.[16–19] Moreover, the gene expression analysis in Tsc2 null murine neuroepithelial progenitor cells revealed altered expression of many genes encoding proteins involved in cell growth, adhesion, and neuronal transmission.[20] However, understanding of mTOR signaling and its downstream targets in the human brain remains far from complete.

In the current study, gene expression profiling on SEGA samples was performed and we identified specific genes involved in tumorigenesis (up-regulated) and the nervous system development (down-regulated) in SEGAs or SEGA-derived cell cultures when compared with the normal brain or cultured human astrocytes. Immunohistochemistry on paraffin-embedded sections confirmed up-regulated levels of several identified proteins in SEGAs. Rapamycin affected the expression of selected genes in SEGA-derived cell cultures showing their dependence on mTOR signaling. Moreover, pharmacological inhibition of mTOR and extracellular signal-regulated kinase (ERK) signaling pathways in cultured SEGA cells affected their proliferation, size, morphology, and migration. Specific expression of the identified genes in the pathological brain and the influence of mTOR and ERK signaling on biology of SEGA cells may provide explanation of how these pathways contribute to the pathogenesis of SEGA and neurological alterations associated with tuberous sclerosis complex.

Materials and Methods

Patient Samples

Ten SEGA samples and three control brain tissues were accessed from the Department of Pathology and Department of Pediatric Neurology, The Children's Memorial Health Institute, Warsaw, Poland. SEGA specimens were originally obtained from tumors immediately after resection from TSC patients diagnosed clinically according to the criteria of Roach. A genetic analysis proved that four of five analyzed patients had mutations in *TSC2*. Control tissues consisted of periventricular regions of non-TSC patients. Two additional controls were: FirstChoice® Human Brain Reference RNA pooled from 23 donors (Applied Biosystems, Darmstadt, Germany) and Human Brain Total RNA pooled from 2 donors (Clontech, Saint-Germain-en-Laye, France).

RNA Isolation, Affymetrix GeneChip Microarray, and Data Analysis

Total RNA was prepared by Tri-Reagent (Sigma-Aldrich, Munich, Germany) extraction from snap-frozen tissues. RNA was cleaned up using RNeasy Mini Kit (Qiagen, Hilden, Germany) which was also used to isolate total RNA from harvested cells. The quality and quantity of total RNA were verified using the Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (5 μg) was converted to double-stranded cDNA. Biotin-labeled cRNA was generated after an *in vitro* transcription reaction. The cRNA was fragmented and then hybridized to a control microarray (Test3) and then, after sample quality evaluation, to the arrays HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA). Immediately after hybridization, the arrays underwent automated washing and staining steps. Finally, they were scanned and the software computed intensities for each cell. Samples hybridization was done in the Department of Nuclear Medicine and Endocrine Oncology, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland, using a standard protocol provided by Affymetrix. Microarray data were analyzed using five popular preprocessing methods: RMA,[21] MAS5.0 (Affymetrix Inc. 2002,), GC-RMA,[22] MBEI pmonly,[23] and PDNN.[24] This was done to identify changes in gene expression robust to a particular choice of a preprocessing method. Probe set measurements were transformed into measurements for genes using annotation provided in the Ensembl database. SEGA gene expression profiling data were deposited at ArrayExpress, accession: E-MEXP-2351. Additionally, to remove a possible cross-hybridization effect, all probe sets with annotation to more than one gene were excluded from further analysis. Furthermore, expression measurements computed for probe sets annotated explicitly to the same gene were averaged using robust Tukey biweight function. Changes in gene expression were examined separately for each preprocessing algorithm using Welsh *t* test. Next, to obtain a robust estimator of *P* values, five values of *t* test computed for each gene were averaged with Tukey biweight function, and the mean values were used to obtain *P* values. Finally, we computed q values for all analyzed genes. That allowed us to select a set of differentially expressed genes in which false discovery rate was at 5% level. Most of preprocessing and all statistical computations were done with the R programming environment and Bioconductor packages.[25] Only the PDNN expression measure was computed with the original PerfectMatch software.[24]

Reverse Transcription and Real-Time PCR Analysis

Total RNA (1 μg) was used to synthesize cDNA by extension of oligo(dT)$_{15}$ primers (2.5 mmol/L) with 200 units of M-MLV reverse transcriptase (Sigma-Aldrich, Munich, Germany). Real-time PCR amplifications were performed in duplicate on cDNA equivalent to 25 ng RNA in 20-μl reaction volume containing 1xSYBR GREEN PCR Master Mix

(Part. No. 4309155, Applied Biosystems, Darmstadt, Germany) and the primer sets QuantiTect Primer Assays (200; Qiagen, Hilden, Germany): Hs_ANXA1_1_SG, Hs_CNDP1_1_SG, Hs_GPNMB_1_SG, Hs_KIAA1189_1_SG, Hs_LTF_2_SG, Hs_MBP_1_SG, Hs_NEUROD1_1_SG, Hs_NPTX1_1_SG, Hs_RND3_1_SG, Hs_S100A11_1_SG, and Hs_SFRP4_2_SG. 18SrRNA was used as an internal standard reference. 18SrRNA primers were designed with the Primer Express Software (Applied Biosystems, Foster City, CA): forward 5′-CGGACATCTAAGGGCATCAC-3′ and reverse 5′-AACGAACGAGACTCTGGCAT-3′. The thermal cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C for denaturation, and 1 minute at 60°C for annealing and extension. The relative quantification of gene expression was determined with ABI PRISM 7700 Sequence Detection System using the comparative CT method.

Immunohistochemistry

Immunostaining was performed on 5-μm sections of paraffin-embedded brain tissues of five SEGAs and five control brains obtained from the Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland. The sections were deparaffinized in xylene, hydrated with a descending ethanol series, and rinsed with deionized water. For each washing 0.05 mol/L Tris-buffered saline, pH 7.4 was used. To retrieve the antigen, sections were boiled for 10 minutes in citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked for 30 minutes with 0.3% hydrogen peroxide. Sections were then incubated overnight at 4°C with the following antibodies: anti-human-Annexin A1 (Abcam, Cambridge, UK, diluted 1:300), anti-human-GPNMB (Abcam, Cambridge, UK, diluted 1:300), and anti-human-S100A11 (ProteinTech Group, Manchester, UK, diluted 1:50) in 1% Swine Serum (Dako, Hamburg, Germany) in Tris buffered saline. Sections were incubated sequentially with biotinylated secondary anti-rabbit or anti-mouse antibodies and extravidin- or streptavidin-peroxidase conjugate (Sigma-Aldrich, Munich, Germany). Peroxidase activity was revealed by 3,3′-diaminobenzidine (DAB, 10 minutes), and then counterstaining was performed with hematoxylin (Sigma-Aldrich, Munich, Germany). Finally, sections were dehydrated through ethanols, cleared in xylene, and mounted. Sections without primary antibodies were used as negative controls.

Cell Culture and Treatment

Freshly resected SEGA samples from three patients were collected, cut into small fragments, and trypsinized for 20 minutes in 37°C. Afterward, cell suspension was centrifuged and suspended in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen, Karlsruhe, Germany) and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin; Sigma-Aldrich, Munich, Germany). Cells were maintained for about 2 weeks before they were used in experiments. Normal Human Astrocytes (Clonetics® NHA) were cultured in Astrocyte Medium (Lonza, Walkersville, MD). SEGA and NHA were treated with rapamycin (LC Laboratories, Woburn, MA), U0126 (Cell Signaling Technology, Danvers, MA), or dimethyl sulfoxide (DMSO; Sigma-Aldrich, Munich, Germany), a solvent for both drugs. During the treatment cells were grown in DMEM with 2% FBS.

Western Blot Analysis

Whole-cell protein extracts from SEGA and NHA cells were prepared as described,[26] loaded onto 10% polyacrylamide gel, electrophorezed, and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were incubated overnight at 4°C with primary antibodies: anti–phospho-p70 S6 Kinase (T389), anti-p70 S6 Kinase (clone 49D7), anti–phospho-ERK1/2 (T202/Y204), and anti-ERK1/2 (1:1000, Cell Signaling Technology, Danvers, MA). After blocking in 5% low-fat milk in TBS-T (0.1% Tween 20/Tris-buffered saline, pH 7.6), the membranes were incubated overnight with primary antibodies diluted in a blocking buffer and then, for one hour, with a secondary anti-rabbit antibody linked to horseradish peroxidase (1:2000, Cell Signaling Technology, Danvers, MA). Membranes were reprobed with a monoclonal anti–β-Actin–Peroxidase conjugated antibody (1:50000, Sigma-Aldrich, Munich, Germany) that served as a loading control. Immunocomplexes were visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

MTT Metabolism Test

Cells were cultured in 24-well plates with the addition of rapamycin, U0126, or DMSO as a control. After treatment, MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, Munich, Germany) was added to each well to a final concentration of 0.5 mg/ml. After 3 hours, formazan crystals that formed from MTT in actively metabolizing cells were dissolved in lysis buffer containing 20% SDS and 50% DMF. Optical densities were measured at 570 nm using a scanning multiwell spectrophotometer (Thermo labsystem Multiscan EX).

BrdU Proliferation Assay

Cells were cultured in 24-well plates with the addition of rapamycin, U0126, or DMSO as a control. Cell Proliferation ELISA BrdU assay (Roche Diagnostics GmbH, Mannheim, Germany) was used to estimate cell proliferation rate. Briefly, BrdU labeling solution was added to each well 6 hours before fixing and incubating with a mouse monoclonal anti-BrdU antibody conjugated with peroxidase. Next, 1 mol/L H_2SO_4 was added and optical densities were measured at 450 nm using a scanning multiwell spectrophotometer (Thermo labsystem Multiscan EX).

Measurement of Cell Size

Cells were cultured on glass slides with the addition of rapamycin, U0126, or DMSO as a control. Medium with

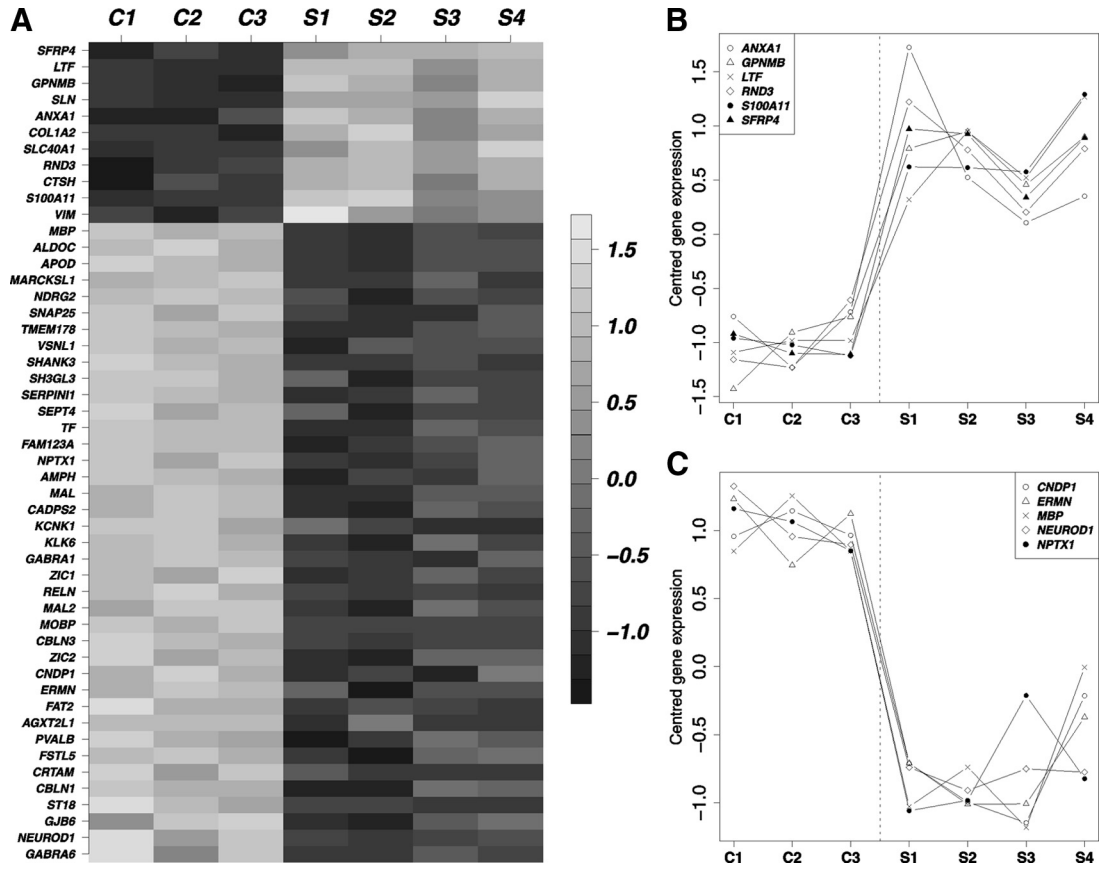

Figure 1. Microarray analysis of gene expression in SEGA and control brain. C1-3, control brain samples: C1 - control brain tissue from one donor, C2 - commercial control brain RNA pooled from 2 donors (Clontech), and C3 - commercial control brain RNA pooled from 23 donors (Ambion); S1-4, SEGA samples. **A:** Heat map representation of GC-RMA normalized microarray expression data for genes significantly altered in SEGA samples compared with control brain samples ($q < 0.05$). For a given gene an average value was computed and subtracted from each observation. Next, every value was divided by SD of all observations. **B** and **C:** Scatter plots of genes up- and down-regulated in SEGA compared with control brain, respectively, chosen for further studies according to literature data.

the inhibitors was changed every second day. After 6 days cells were fixed with 4% paraformaldehyde, stained with Phalloidin-TRITC (0.1 μg/ml, Sigma-Aldrich, Munich, Germany) for 1 hour, and mounted. Cells were viewed with a Leica SP5 laser confocal microscope (Leica, Mannheim, Germany) using the 561 nm line of the DPSS laser and a 20×, 0.7 an PlanApo oil-immersion objective. Optical sections were collected at 0.16-μm increments, and 2D projections were made with the LAS AF software. Image stacks were processed using the 3D Constructor plug-in to Image-Pro Plus 6.3 software. Individual cells were analyzed for cell volume and surface area.

Scratch Assay

Cells were cultured in 6-well plates and pre-incubated with rapamycin, U0126, or DMSO as a control for 3 hours before wounding the cells. Next, a scratch was created in the center of the cell monolayer using a pipette tip. Immediately thereafter, cells were washed with PBS to remove cellular debris and incubated with the inhibitors for additional 27 hours. The ability of cells to migrate into the scratch area was measured by counting of DAPI-stained nuclei along the scratch at 0 time point and after 30 hours of cell exposure to the inhibitors.

Results

Identification of Genes Differentially Expressed in SEGA versus Control Brain with Microarray Analysis

Gene expression profiling was performed on four SEGA samples from patients with TSC (S1-4), one control brain tissue (C1), and two commercial control brain RNA (C2 [from two donors] and C3 [from 23 donors]) using Affymetrix HG-U133 Plus 2.0 array sets. Figure 1 shows the results of microarray analysis obtained for probe sets called Present after the filtration as described in *Materials and Methods*. We first identified genes significantly altered in SEGAs versus control brain using false discovery rate of <5%, then we selected 50 differentially expressed genes with the biggest difference in expression level between SEGAs and control brain samples, and the highest similarity within these groups using the MultiExperiment Viewer 4.0 software (Figure 1A). A list of genes differentially expressed in SEGAs is shown in Table 1.

The 50 selected genes (11 up-regulated and 39 down-regulated in SEGAs) were categorized into functional groups using the Ingenuity Pathway Analysis software. Based on their known biological functions, the genes were mainly involved in tumorigenesis (14 genes) and the ner-

Table 1. Summary of Genes with Highest Up- or Down-Regulation Scores in SEGAs Compared with Control Brains

Unigene	Gene symbol	Gene name	Fold change over controls
Tumorigenesis			
Hs.658169	***SFRP4***	**secreted frizzled-related protein 4**	**2.514**
Hs.610567	***LTF***	**lactotransferrin**	**2.197**
Hs.190495	***GPNMB***	**glycoprotein (transmembrane) nmb**	**2.139**
Hs.494173	***ANXA1***	**annexin A1**	**1.791**
Hs.489142	*COL1A2*	collagen, type I, alpha 2	1.757
Hs.6838	***RND3***	**Rho family GTPase 3**	**1.690**
Hs.148641	*CTSH*	cathepsin H	1.451
Hs.593414	***S100A11***	**s100 calcium binding protein a11**	**1.390**
Hs.702229	*APOD*	apolipoprotein D	0.686
Hs.525205	*NDRG2*	NDRG family member 2	0.668
Hs.444212	*VSNL1*	visinin-like 1	0.632
Hs.287518	*SEPT4*	septin 4	0.580
Hs.591255	*FAT2*	FAT tumor suppressor homolog 2 (Drosophila)	0.423
Hs.655499	*ST18*	suppression of tumorigenicity 18	0.386
Nervous system development			
Hs.551713	***MBP***	**myelin basic protein**	**0.757**
Hs.75061	*MARCKSL1*	MARCKS-like 1	0.682
Hs.167317	*SNAP25*	synaptosomal-associated protein, 25kDa	0.660
Hs.149035	*SHANK3*	SH3 and multiple ankyrin repeat domains 3	0.632
Hs.270055	*SH3GL3*	SH3-domain GRB2-like 3	0.608
Hs.478153	*SERPINI1*	neuroserpin	0.607
Hs.518267	*TF*	transferrin	0.573
Hs.702002	***NPTX1***	**neuronal pentraxin I**	**0.540**
Hs.80395	*MAL*	mal, T-cell differentiation protein	0.525
Hs.708214	*CADPS2*	Ca2+-dependent activator protein for secretion 2	0.521
Hs.79361	*KLK6*	kallikrein-6	0.514
Hs.175934	*GABRA1*	gamma-aminobutyric acid (GABA) A receptor, alpha 1	0.506
Hs.647962	*ZIC1*	Zic family member 1 (odd-paired homolog, Drosophila)	0.506
Hs.655654	*RELN*	reelin	0.504
Hs.121333	*MOBP*	myelin-associated oligodendrocyte basic protein	0.463
Hs.653700	*ZIC2*	Zic family member 2 (odd-paired homolog, Drosophila)	0.451
Hs.400613	***CNDP1***	**carnosine dipeptidase 1 (metallopeptidase M20 family)**	**0.444**
Hs.443894	***ERMN***	**ermin, ERM-like protein**	**0.431**
Hs.591255	*FAT2*	FAT tumor suppressor homolog 2 (Drosophila)	0.423
Hs.295449	*PVALB*	parvalbumin	0.412
Hs.458423	*CBLN1*	cerebellin 1 precursor	0.388
Hs.709709	***NEUROD1***	**neurogenic differentiation 1**	**0.342**
Hs.90791	*GABRA6*	gamma-aminobutyric acid (GABA) A receptor, alpha 6	0.311
Others and unknown			
Hs.334629	*SLN*	sarcolipin	2.021
Hs.643005	*SLC40A1*	solute carrier family 40, member 1	1.719
Hs.628678	*VIM*	vimentin	1.314
Hs.155247	*ALDOC*	aldolase C, fructose-bisphosphate	0.708
Hs.40808	*TMEM178*	transmembrane protein 178	0.634
Hs.528335	*FAM123A*	family with sequence similarity 123A	0.555
Hs.592182	*AMPH*	amphiphysin	0.538
Hs.208544	*KCNK1*	potassium channel, subfamily K, member 1	0.519
Hs.201083	*MAL2*	mal, T-cell differentiation protein 2	0.473
Hs.207603	*CBLN3*	cerebellin 3 precursor	0.463
Hs.106576	*AGXT2L1*	alanine-glyoxylate aminotransferase 2-like 1	0.422
Hs.591707	*FSTL5*	fstl5	0.408
Hs.159523	*CRTAM*	cytotoxic and regulatory T cell molecule	0.407
Hs.511757	*GJB6*	gap junction beta-6 protein	0.381

Genes used for further studies are printed in bold type.

vous system development (23 genes). Interestingly, all genes related to the nervous system development were down-regulated in SEGAs when compared with control brain samples (Table 1). Of the 50 genes, we chose 11 differentially expressed genes for further studies by a systematic PubMed search for genes with potential relevance to TSC biology and pathology. The expression pattern of these genes: six up- and five down-regulated in SEGAs showed evident differences between SEGAs and control brain samples (Figure 1, B and C). The selected genes up-regulated in SEGA: *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, and *SFRP4* are involved in tumorigenesis, and genes down-regulated in SEGA: *CNDP1*, *ERMIN*, *MBP*, *NEUROD1*, and *NPTX1* are potentially related to the nervous system development. Among them *ANXA1*, *GPNMB*, and *NPTX1* were previously shown to be differentially expressed in Tsc2 null murine neuroepithelial progenitor cells compared with wild-type neuroepithelial progenitor cells.[20]

Real-Time PCR Verification of Differentially Regulated Genes and Immunohistochemical Detection of ANXA1, GPNMB, and S100A11 in SEGA Tissue Sections

To verify the results of microarray analysis, we performed real-time PCR and immunohistochemical validation, wherever antibody was available, mostly on an independent set of tumor samples. We selected 11 differentially expressed genes for a quantitative validation using real-time PCR. Analysis was performed on 10 SEGAs and 5 control brain samples and included tumor samples used for microarray analysis and additional samples from different individuals: six SEGAs and two control brain samples. As shown in Figure 2, real-time PCR confirmed the findings of the microarray experiment, with ANXA1, GPNMB, LTF, RND3, S100A11, and SFRP4 mRNAs up-regulated in SEGAs and CNDP1, ERMIN, MBP, NEUROD1, and NPTX1 mRNAs down-regulated in SEGAs. Differences in the levels of expression between SEGAs and control brains were significant for all tested genes ($P < 0.05$, Figure 2, A and B) and paralleled those observed in the microarray study.

We evaluated ANXA1, GPNMB, and S100A11 expression immunohistochemically on a panel of SEGA tissue sections with commercially available antibodies. A staining was performed on paraffin-embedded SEGAs from five TSC patients (distinct from those used in the microarray and real-time PCR experiments) and five non-tumoral brain tissue samples. Representative micrographs in Figure 3 show that all tested SEGA tissues were immunoreactive for ANXA1, GPNMB, and S100A11, and the levels of studied proteins were higher in SEGA relative to the non-tumoral brain. Cell membrane and cytoplasmic ANXA1 and GPNMB staining was detected in all SEGA samples, mostly in giant cells (Figure 3, A and B, left panels, arrows). On the other hand, S100A11 expression was apparently both cytoplasmic and nuclear (Figure 3C, left panel). Only a faint staining was observed in non-tumoral brain tissue samples (Figure 3, A, B, and C, right panels). Negative controls without primary antibodies showed no positive staining (Figure 3D). The results demonstrate an increased expression of ANXA1, GPNMB, and S100A11 proteins in SEGAs compared with control brain consistently with up-regulation of these genes expression.

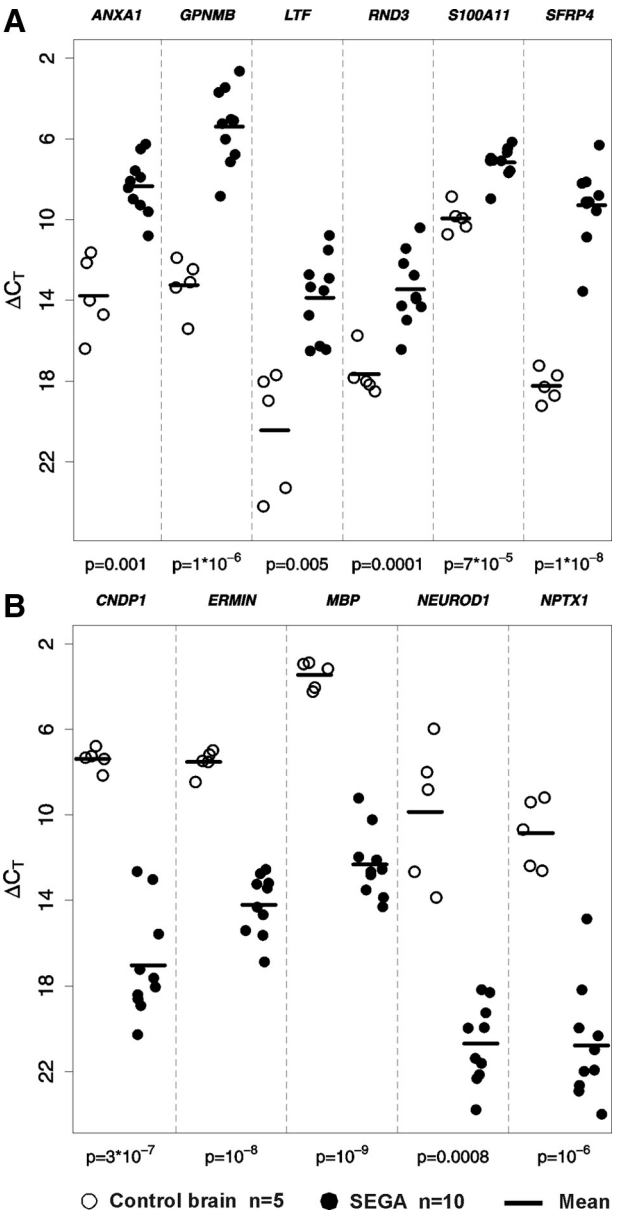

Figure 2. Real-time PCR validation of candidate genes in SEGAs and control brain samples. **A** and **B:** The difference in expression of up- and down-regulated genes in SEGAs compared with control brain samples was confirmed on samples used for microarray analysis, six additional SEGAs, and two additional nontumoral control brain samples from non-TSC patients. Lower ΔC_T are consistent with higher gene expression. Statistical analysis was done by Student *t* test, $P < 0.05$ are considered to be significant.

Genes Differentially Expressed in SEGA Samples Have Similar Expression Profile in SEGA-Derived Cell Cultures

To examine a role of mTOR signaling in the expression of identified genes, we established primary cell cultures derived from SEGAs from three TSC patients. First, we investigated whether the expression of the 11 selected genes in cultured SEGA cells was comparable with the pattern observed in SEGA samples. We performed real-time PCR analysis using RNA isolated from SEGA cells and five control brain samples used previously in the microarray and real-time PCR experiments. We found that the levels of ANXA1, GPNMB, LTF, RND3, S100A11, and SFRP4 mRNAs were higher (Figure 4A) and those of CNDP1, ERMIN, MBP, NEUROD1, and NPTX1 were lower in SEGA cells (Figure 4B). Differences in the levels of expression between SEGA-derived cell cultures and control brains were statistically significant for all tested genes. It confirmed that expression profiles of these genes were similar in the homogenous population of cultured SEGA cells, as well as in the whole tumor samples.

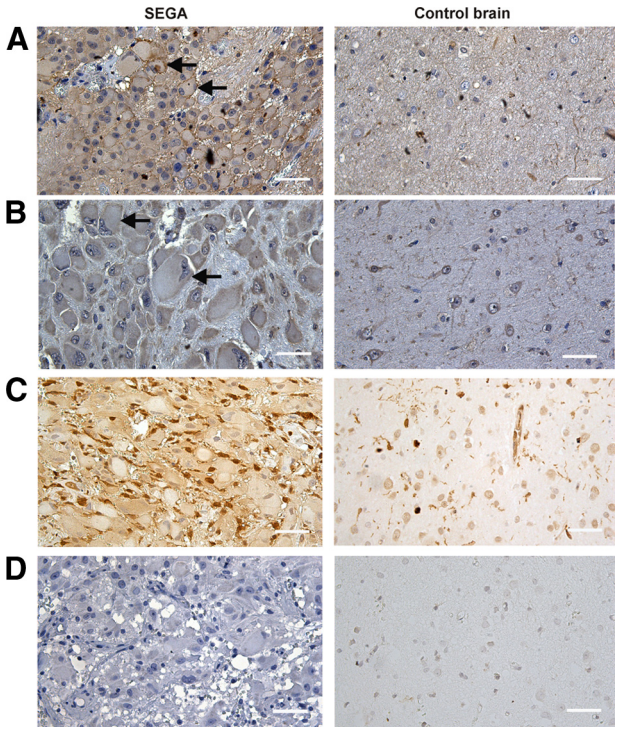

Figure 3. Immunohistochemical validation of selected markers expression in SEGA cells and control brain tissues. Representative images show a positive 3,3′-diaminobenzidine (DAB) staining (brown) for ANXA1 (**A**), GPNMB (**B**), and S100A11 (**C**) in SEGA cells (**left panels**). **D:** negative controls without primary antibodies. Nuclei were counterstained with hematoxylin (blue). **Arrows** indicate giant cells. The expression of studied proteins was confined to tumor cells. Scale bar = 50 μm.

Expression of Tumorigenesis-Related Genes and MBP *Is Increased in SEGA Cells in Comparison with Normal Human Astrocytes*

The cell origin of SEGA is unknown and controversial. Although the tumors are classified as astrocytomas, they seem to have a mixed glioneuronal lineage and express both neuronal and glial markers.[1] We compared expression of the newly identified genes in three SEGA-derived cell cultures to their expression patterns in NHA. Cultured NHA resemble SEGA cells morphologically, however SEGA giant cells are bigger than normal astrocytes (Figure 5A). Moreover, SEGA cells were immunoreactive for glial fibrillary acidic protein used as a marker of astrocytes (data not shown). We found that the majority of tested genes (6 of 11)—*ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, and *SFRP4*—showed the same expression patterns in SEGA cells versus NHA as versus control brain. Furthermore, the expression of *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, *SFRP4*, and *MBP* was greatly elevated in SEGA cell cultures when compared with normal human astrocytes. Briefly, *SFRP4* expression was ≈300 times (P = 0.0480), *LTF* >200 times (P = 0.0267), *GPNMB* ≈100 times (P = 0.0052), *MBP* ≈50 times (P = 0.0428), *RND3* ≈30 times (P = 0.0022), and *ANXA1* (P = 0.0103) and *S100A11* ≈10 times (P = 0.0001) increased in SEGA-derived cells compared with NHA (Figure 5B). Our results indicate that normal human astrocytes are an appropriate control for cultured SEGA cells.

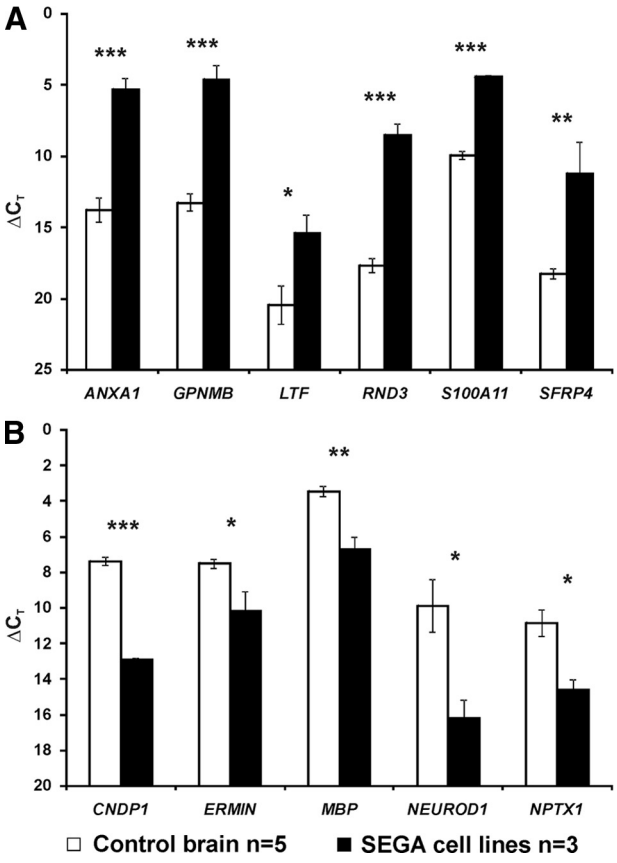

Figure 4. Real-time PCR validation of candidate genes in primary SEGA cell cultures and control brain. **A** and **B:** The difference in expression of genes up- and down-regulated in SEGA cells compared with control brain samples, respectively. Bars represent means ± SEM of five control brain samples and three SEGA cell lines. Lower ΔC_T values are consistent with higher gene expression. Statistical analysis was done by Student t test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Inhibition of mTOR Activity Alters Expression Levels of Selected Genes in SEGA Cells But Not in Normal Human Astrocytes

To investigate the influence of mTOR on the regulation of selected genes expression, the mTOR kinase activity was inhibited in SEGA cells and normal human astrocytes using rapamycin. We observed that the levels of phospho-S6 kinase and its substrate phospho-S6 were significantly higher in SEGA cells than in NHA cultures. Moreover, rapamycin at concentration 10 nmol/L inhibited the kinase activity of mTOR and reduced the S6 kinase and S6 phosphorylation in SEGA cells, whereas the treatment had no influence on the levels of phosphorylation of these proteins in NHA (Figure 5C). Therefore, we sought to examine whether inhibition of mTOR activity would have a greater effect on the expression of genes regulated by mTOR in SEGA cells than in normal human astrocytes. The expression of 11 genes in SEGA and NHA was determined 24 hours after exposure to 10 nmol/L rapamycin. The reduction of mRNA levels of six genes highly up-regulated in SEGAs was observed along with the increase of *NPTX1* expression in the drug-treated SEGA cells. Briefly, we observed ≈3-fold decrease in *LTF* (P =

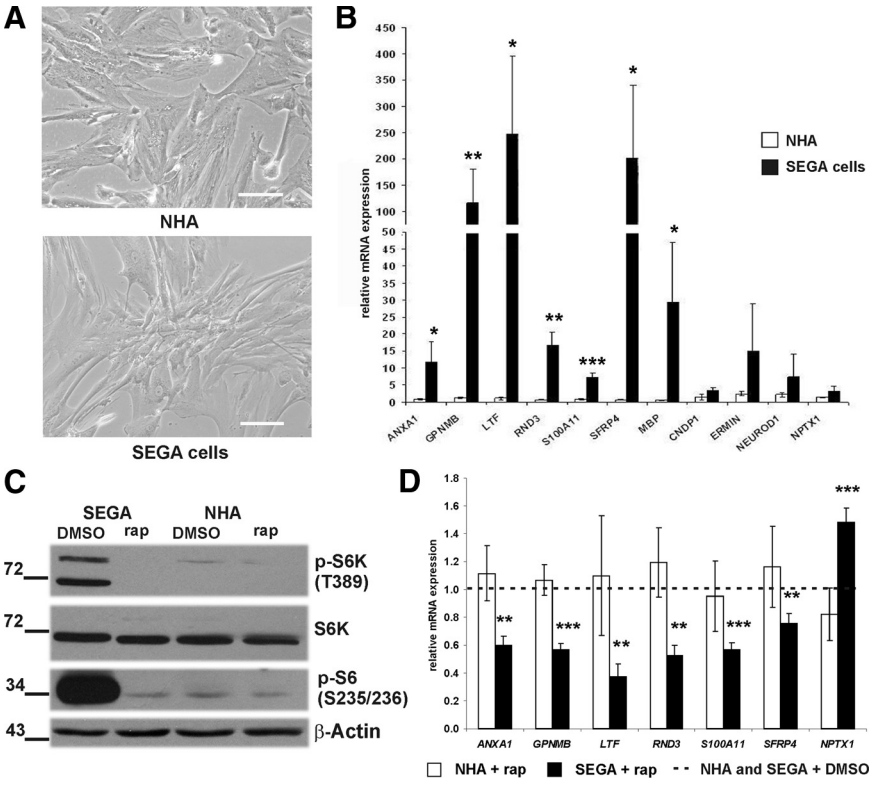

Figure 5. Rapamycin alters the expression of *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, *SFRP4*, and *NPTX1* in SEGA cells but not in normal human astrocytes. **A:** Phase-contrast images of normal human astrocytes (NHA) and cultured SEGA-derived cells. Scale bar = 100 μm. **B:** Real-time PCR analysis of candidate genes expression in NHA and SEGA-derived cells. Bars represent means ± SEM of the mRNA levels of each gene in SEGA cells (means of three independently derived SEGA cell lines) relative to those expressed in NHA (means of three cell cultures). Statistical analysis was done by Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **C:** Representative Western blot analysis of phospho-S6K (T389) and phospho-S6 (S235/236) levels in SEGA-derived cell cultures and NHA after treatment with 10 nmol/L rapamycin for 24 hours. S6K and β-Actin served as protein loading controls. **D:** Real-time PCR analysis of expression levels of *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, *SFRP4*, and *NPTX1* in three SEGA-derived cell cultures and NHA cells after rapamycin treatment. The mRNA levels of investigated genes in cells treated with rapamycin were related to their expression in control (DMSO-treated) cells. Bars represent means ± SEM of three independent experiments. Statistical analysis was done by Student *t* test, $^{**}P < 0.01$, $^{***}P < 0.001$.

0.0018), ≈2-fold decrease in *ANXA1* ($P = 0.0024$), *GPNMB* ($P = 0.0002$), *RND3* ($P = 0.0013$), and *S100A11* ($P = 0.0003$), and ≈1.5-fold decrease in *SFRP4* ($P = 0.0092$) expression levels in SEGA cells exposed to rapamycin. Furthermore, the treatment increased the expression of *NPTX1* by ≈1.5 fold ($P = 0.0007$) in these cells. On the other hand, according to our assumption, the expression levels of the investigated genes were not significantly changed in NHA after rapamycin treatment (Figure 5D). These results demonstrate that the expression of *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, *SFRP4*, and *NPTX1* is regulated by mTOR signaling pathway in SEGA cells.

mTOR and ERK Signaling Pathways Regulate Proliferation, Size, Morphology, and Migration of SEGA Cells

Recent studies demonstrate that, in addition to mTOR, ERK signaling pathway is activated in the TSC brain lesions.[27–29] To investigate a role of mTOR and ERK signaling in pathology of SEGA, primary SEGA cell cultures were treated for 3 hours with 10 nmol/L rapamycin or 40 μmol/L U0126 (an inhibitor of MEK1/2 kinase activity) alone or in combination. The corresponding dose of DMSO was added to control cells. Rapamycin or U0126 completely inhibited mTOR and MEK1/2 kinase activities, which was estimated by the lack of phosphorylated substrates of the kinases: phospho-S6K and phospho-ERK1/2, respectively. Neither of the inhibitors affected the other signaling pathway (Figure 6A). Because both pathways transmit the prosurvival signals, we investigated viability and proliferation of SEGA cells after treatment with the inhibitors. The ≈30% decrease of the number of living cells was observed after 72 hours exposure of SEGA cells to rapamycin and U0126 added in combination to culture media ($P = 0.002$), as was determined using MTT metabolism test. Rapamycin or U0126 alone had no significant effects on viability of SEGA cells (Figure 6B). Furthermore, U0126 alone and together with rapamycin inhibited the proliferation of SEGA cells by ≈30% ($P = 0.046$) and ≈40% ($P = 0.005$), respectively. Rapamycin alone did not affect cell proliferation (Figure 6C).

Because mTOR hyperactivity is responsible for cell overgrowth and ERK signaling has been shown to control remodeling of actin cytoskeleton and cell motility,[30,31] we studied the influence of rapamycin and U0126 on morphology and migration of SEGA cells. Figure 6D shows staining of actin cytoskeleton in SEGA cell cultures 6 days after exposure to rapamycin or/and U0126. Profound changes in cellular morphology were observed after U0126 treatment (cells became more round than elongated) with a distinctive arrangement of actin filaments, which was even more noticeable on concomitant treatment with rapamycin and U0126. Rapamycin alone had no apparent influence on the shape of SEGA cells. However, the measurement of SEGA cell size with confocal microscopy and the Image-Pro Plus 6.3 software demonstrated a decrease of cell volume by ≈50% ($P = 0.0002$) and cell area by 40% ($P = 0.0004$) after 6 days of exposure to rapamycin. Similar results were observed after concomitant treatment with rapamycin and U0126: volume and area of SEGA cells were reduced by ≈40%

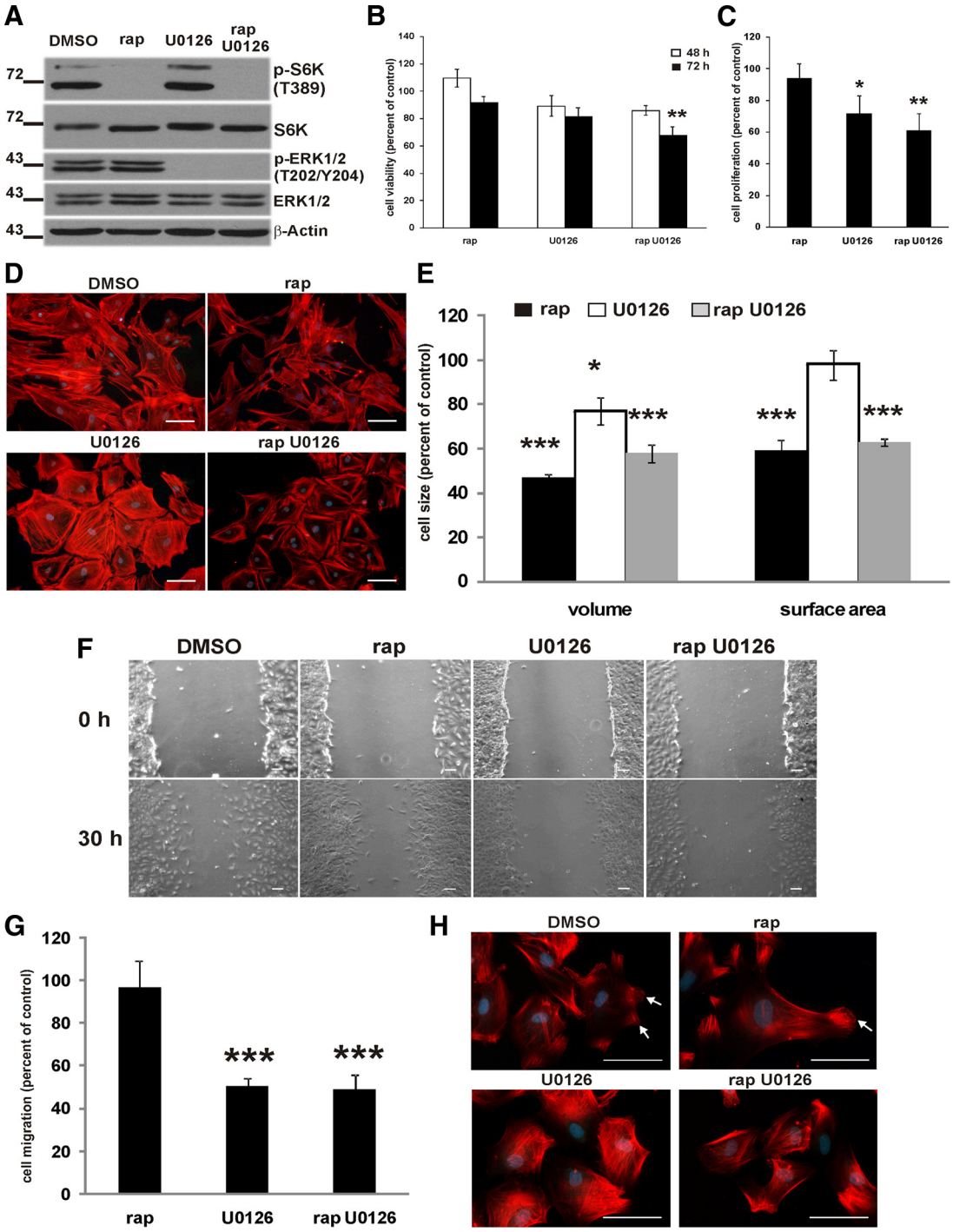

Figure 6. Combined inhibition of mTOR and ERK signaling pathways affects proliferation, shape, size, and migration of SEGA-derived cells. **A:** Western blot analysis of the levels of phospho-S6K (T389), S6K, phospho-ERK1/2 (T202/Y204), ERK1/2, and β-Actin in SEGA-derived cells 3 hours after treatment with 10 nmol/L rapamycin or/and 40 μmol/L U0126. DMSO was used as a control. **B:** Cell viability was determined 48 and 72 hours after exposure to rapamycin or/and U0126 using MTT metabolism test. Bars represent viability of cells treated with the inhibitors related to control cells (means ± SEM of four independent cell cultures, each in triplicate). **C:** Cell proliferation was determined 48 hours after exposure to rapamycin or/and U0126 using BrdU test. Bars represent the percentage of proliferating cells treated with the inhibitors related to control cells (means ± SEM of four independent cell cultures, each in triplicate). **D:** Representative images show fluorescent phalloidin staining of the actin cytoskeleton in SEGA cells 6 days after exposure to rapamycin or/and U0126. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar = 100 μm. **E:** Cell size was analyzed by confocal microscopy in phalloidin- and DAPI-stained SEGA cell cultures 6 days after exposure to rapamycin or/and U0126 and quantified with Image-Pro Plus 6.3 software. Bars represent the percentage of volume and surface area of cells treated with the inhibitors related to control cells (means ± SEM of two independent cell cultures, ≈150 randomly selected cells per condition). **F:** Scratch assay - representative phase-contrast images along the scratch area at 0 time point and after 30 hours of exposure of SEGA cells to rapamycin or/and U0126. Scale bar = 100 μm. **G:** Cell migration into the wound area was estimated at 27 hours after scratching by counting of DAPI-stained nuclei along the scratch. Bars represent the percentage of nuclei of cells treated with the inhibitors related to control cells (means ± SEM of three independent cell cultures, each in duplicate, ten nonoverlapping microscopic fields at each well were analyzed). **H:** Representative images show fluorescent phalloidin staining of the actin cytoskeleton of SEGA cells along the scratch 30 hours after exposure of cells to rapamycin or/and U0126. DMSO was used as a control. **Arrows** indicate lamellipodia at the leading edges of migrating cells. Nuclei were stained with DAPI. Scale bar = 100 μm. Statistical analysis was done by one-way analysis of variance followed by Newman-Keuls test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

($P = 0.0003$). U0126 alone did not affect size of SEGA cells area and had a slight effect on their volume (≈20% decrease, $P = 0.01$; Figure 6E). To assess an influence of the inhibitors on migration of SEGA cells into the wound area, we performed scratch assay. As shown in Figure 6, F and G, U0126 decreased a number of migrating cells by 51% after 30 hours of treatment ($P = 0.0003$). Exposure to both drugs in combination produced very similar results (inhibition of cell migration by 50%, $P = 0002$), indicating that mTOR signaling does not participate in this process in SEGA cells. This was also confirmed by the lack of any effect on migration after an exposure to rapamycin alone. These results were in agreement with the observed formation of lamellipodia consisting of branched actin filaments at the leading edges of migrating cells mainly in control and rapamycin-treated cells (Figure 6H). The data show that inhibiting of both mTOR and ERK signaling pathways results in the most significant changes in SEGA cell growth.

Discussion

The present study shows the results of the first genome-wide expression profiling on SEGAs—brain tumors occurring in TSC, a disease characterized by the aberrant activity of mTOR kinase. We performed microarray analysis, real-time PCR, and immunohistochemistry to identify genes that are differentially expressed in SEGA compared with nontumoral brain. We used a relatively small number of SEGA samples for global gene profiling, therefore the microarray data analysis was very restrictive. Human control brain RNAs are difficult to obtain, thus we used the commercial human brain reference RNA pooled from 23 or 2 donors to increase the total number of control samples in the microarray experiment. The whole brain controls were considered a better choice than those collected from specific brain regions, because SEGAs have a mixed glioneuronal lineage. Moreover, as we aimed at the identification of genes which expression was modulated by the aberrantly active TSC1/2–mTOR signaling pathway, it was essential to obtain controls from non-TSC donors. Of the fifty genes identified as differentially expressed in SEGA, the expression of eleven genes selected for further studies was altered in six additional SEGAs from independent TSC patients compared with control brain samples, as was validated by real-time PCR. Seven genes: *ANXA1*, *GPNMB*, *LTF*, *RND3*, *S100A11*, *SFRP4*, and *NPTX1* were shown to be mTOR effector genes in SEGA-derived cell cultures, because treatment with rapamycin (an inhibitor of mTOR) modulated their expression. Microscopic analysis of stained sections confirmed overexpression of Annexin A1, GPNMB, and S100A11 in tumor samples from five different TSC patients.

Identified genes encode proteins that fall into two major categories: proteins involved in tumorigenesis, up-regulated in SEGA and proteins implicated in the nervous system development/differentiation, down-regulated in SEGA. Genes up-regulated in SEGAs and dependent on the mTOR signaling have been shown to be involved in progression or suppression of various tumors. Annexin A1 (ANXA1), a calcium- and phospholipid-binding protein with anti-inflammatory properties, plays roles in diverse cellular functions, such as membrane aggregation, inflammation, phagocytosis, proliferation, and apoptosis. *ANXA1* was up-regulated in astrocytomas or pancreatic cancers.[32–33] On the other hand, down-regulation of ANXA1 was reported in many types of cancers, including head and neck and breast cancers.[34–35] Annexin A1 interacts with S100A11 (also called calgizzarin or S100C), an EF hand–type Ca^{2+} binding protein of the S100 family of proteins. S100A11 plays distinct roles depending on the type of tumor.[36] In bladder and renal cancers, the expression of S100A11 was related to tumor suppression,[37,38] but in prostate and breast cancers to tumor promotion.[39–40] Intranuclear localization of S100C/S100A11 was implicated in the contact inhibition of fibroblasts and HeLa cell growth.[41] Next, glycoprotein nonmetastatic melanoma protein B (GPNMB) was shown to be highly expressed in malignant gliomas and in metastatic breast cancer.[42–43] GPNMB is suggested to be an adhesion molecule mediating intercellular interactions and contributing to the acquisition of the invasive nature of malignant tumor cells. However, it was also reported to be up-regulated in low metastatic melanoma cell lines.[44]

Furthermore, *LTF*, *RND3*, and *SFRP4* are other genes identified in the present study and having potential tumor suppressor activity. Lactotransferrin (LTF), a transcription factor which modulates cell growth and a variety of cellular and immune responses,[45] is down-regulated in malignant gliomas and nasopharyngeal carcinoma.[46,47] Similarly, Rho family GTPase 3 (RND3), a protein involved in organization of actin cytoskeleton and cell cycle regulation,[48] was observed to inhibit cell cycle progression and induce apoptosis in glioblastoma and prostate cancer cells,[49–50] which may imply its role as a tumor suppressor protein. Secreted frizzled-related protein 4 (SFRP4), an antagonist of Wnt pathway controlling cell proliferation, differentiation, and migration, was down-regulated in endometrial cancers and chronic lymphocytic leukemia.[51,52] We postulate here that Annexin A1, GPNMB, S100A11, LTF, RND3, and SFRP4 are candidate markers of subependymal giant cell astrocytomas. GPNMB, LTF, RND3, and SFRP4 may be responsible for benign phenotype and relatively low invasiveness of these tumors.

A second category consists of genes which are down-regulated in SEGA and encode proteins implicated in the nervous system development and differentiation. Our study revealed reduced expression of many genes encoding oligodendrocyte-specific proteins such as myelin basic protein (MBP), myelin-associated oligodendrocyte basic protein, transferrin, or ermin, a marker of myelinating oligodendrocytes that binds to filamentous actin and regulates cell morphology.[53] Next, expression level of neuronal pentraxin 1 (NPTX1), an early neuronal lineage marker shown to take part in the synapse formation and remodeling,[54,55] was also down-regulated in SEGAs. Furthermore, neurogenic differentiation 1 (NeuroD1/BETA2), a member of the bHLH transcription factor family, emerges to be a gene of particular

importance. It is known to influence the fate of specific neuronal, endocrine, and retinal cells[56,57] and plays a pivotal role in terminal differentiation of neural progenitors.[58] Overexpression of human NeuroD1 in fetal glial cell line resulted in appearance of the early neuronal development markers.[59] It is possible that reduced expression of these genes might be related to the postulated defects in differentiation and mixed glioneuronal origin of SEGA. Moreover, NeuroD1-null mice were shown to develop malformations in the dentate gyrus and exhibit spontaneous limbic seizures.[60] Carnosine dipeptidase 1 (CNDP1) was another protein with reduced expression in SEGA, which has been associated with neuropathological processes. CNDP1 deficiency was reported in syndromes characterized by mental retardation, developmental delay, seizures, neurosensory hearing loss, or progressive childhood dementia.[61,62] Down-regulation of these genes might be linked with the occurrence of neurological dysfunctions, such as seizures or mental retardation, among TSC patients.

A role of mTOR signaling in the regulation of expression of identified genes was studied in primary SEGA cell cultures derived from three TSC patients. We observed that the genes had the same expression patterns in SEGA cells as in tumor samples in comparison with the control brain and normal human astrocytes. We observed that elevated expression of genes related to tumorigenesis was reduced by inhibition of mTOR signaling with rapamycin in SEGA cells. The expression level of neuronal pentraxin 1 was elevated after exposure to the drug.

The previous study performed on a single SEGA cell line indicated that 20 nmol/L rapamycin inhibited cell viability after 24 hours of treatment.[27] However, our results, reproduced on three independent SEGA cell lines, showed that inhibition of mTOR activity did not affect SEGA cell viability and proliferation even after six days of exposure to rapamycin. Furthermore, rapamycin did not influence either SEGA cell migration or actin cytoskeleton arrangement, but it decreased SEGA cell size. This observation is in agreement with a hypothesis that mTOR is responsible for SEGA giant cell overgrowth. On the other hand, we also showed that the inhibition of MEK1/2 activity with U0126 diminished proliferation of SEGA cells without affecting their survival. Moreover, it significantly decreased SEGA cell migration. Reduction of ERK1/2 phosphorylation did not influence SEGA cell size but it changed their shape by modulation of actin cytoskeleton. Combined suppression of mTOR and ERK signaling pathways resulted in the most remarkable decrease of SEGA cell viability, proliferation, and actin cytoskeleton rearrangement. These results suggest that mTOR and ERK signaling pathways may be responsible for pathogenesis and progression of SEGA. Combined inhibition of both pathways should be considered as a promising strategy for treatment of tumors harboring deregulation of these pathways.

In conclusion, this is the first report to show genes differentially expressed in subependymal giant cell astrocytomas when compared with the control brain. Moreover, we identified seven novel genes in the human brain dependent on mTOR kinase at the transcriptional level. These genes should be further investigated as potential diagnostic biomarkers or target genes for the development of effective therapeutics. Furthermore, we suggest that targeting at both mTOR and ERK signaling pathways may give better results in the treatment of SEGAs than using mTOR inhibitors alone.

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