Cortical Tubers: Windows into Dysregulation of Epilepsy Risk and Synaptic Signaling Genes by MicroRNAs

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Abstract

Tuberous sclerosis complex (TSC) is a multisystem genetic disorder caused by mutations in the TSC1 and TSC2 genes. Over 80% of TSC patients are affected by epilepsy, but the molecular events contributing to seizures in TSC are not well understood. Recent reports have demonstrated that the brain is enriched with microRNA activity, and they are critical in neural development and function. However, little is known about the role of microRNAs in TSC. Here, we report the characterization of aberrant microRNA activity in cortical tubers resected from 5 TSC patients surgically treated for medically intractable epilepsy. By comparing epileptogenic tubers with adjacent nontuber tissue, we identified a set of 4 coordinately overexpressed microRNAs (miRs 23a, 34a, 34b*, 532-5p). We used quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomic profiling to investigate the combined effect of the 4 microRNAs on target proteins. The proportion of repressed proteins among the predicted targets was significantly greater than in the overall proteome and was highly enriched for proteins involved in synaptic signal transmission. Among the combinatorial targets were TSC1, coding for the protein hamartin, and several epilepsy risk genes. We found decreased levels of hamartin in epileptogenic tubers and confirmed targeting of the TSC1 3’UTR by miRs-23a and 34a.

Key words: expression profiling, hamartin, proteomics, synapse, tuberous sclerosis complex

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder resulting from mutations in the TSC genes, leading to activation of mammalian/mechanistic target of rapamycin (mTOR), and is characterized by hamartomatous lesions in many organs, including brain lesions termed tubers (Roach and Sparagana 2004). Tubers are characterized by abnormal cortical lamination and the presence of dysmorphic neurons and balloon cells. Epilepsy afflicts over 80% of TSC patients, and many suffer seizures that do not respond to pharmacological therapy and require surgical resection of epileptogenic tubers (Thiele 2004; Weiner et al. 2004). The molecular events that contribute to seizures in TSC are not well characterized.

MicroRNAs are endogenous noncoding RNAs that provide post-transcriptional regulation of many genes and most cell processes. Approximately one-third of all protein-coding genes are believed to be regulated by microRNAs, and aberrant microRNA expression has been implicated in a wide range of diseases (Filipowicz et al. 2008). Mature microRNAs are ~22 nucleotides in length and anneal to complementary sites in the 3’ untranslated region (UTR) of target transcripts as part of an RNA-induced silencing complex, resulting in either transcript degradation or inhibition of protein translation. There are currently over 2000 known mature microRNAs in the human genome, and each microRNA may regulate dozens to hundreds of target transcripts.
The 3′ UTR in a single-messenger RNA may contain binding sites for numerous microRNAs, and a transcript can be concurrently repressed by multiple microRNA species. Compared with other organs, the brain is highly enriched for microRNA activity, likely due to the remarkable level of structural and functional complexity in the tissue (Fineberg et al. 2009). Numerous microRNAs are differentially expressed during corticogenesis and neuronal differentiation (Krichevsky et al. 2003; Nielsen et al. 2009). Recent reports have also implicated microRNAs in epileptogenesis (Jimenez-Mateos et al. 2012; You et al. 2012). However, little is known about the involvement of microRNAs in TSC, and there are currently no published reports on their role in cortical tubers. A potential role for microRNAs in neurological manifestations of TSC is suggested by the prominent role of microRNAs in neurodevelopment and evidence indicating microRNA regulation downstream of mTOR. MicroRNAs have been reported to be regulated by p53 in different cell types, and p53 levels and activity are regulated by mTOR (Lee et al. 2007; Shin et al. 2012). In addition, inflammation, which is present in epilepsy and cortical tubers (Maldonado et al. 2003; Aronica and Crino 2011), has been shown to induce microRNA expression through inflammatory cytokines (Funken et al. 2009). In this work, we characterized microRNA expression patterns in cortical tubers resected to treat intractable epilepsy in TSC patients and compared expression levels with patient-matched adjacent nontuber tissue that was concurrently resected due to epileptiform electroencephalogram (EEG) activity. Furthermore, we used quantitative proteomics to assess the effects of altered microRNA levels on protein expression, as well as investigate the potential roles for p53 and inflammation as mechanisms involved in regulating microRNA levels in TSC.

Materials and Methods

TSC Brain Tissue

Microarray analysis was performed on brain tissue samples obtained from 5 patients who underwent epilepsy surgery for medically refractory epilepsy at Children’s Hospital of Michigan, Detroit. Table 1 shows patient demographics and mutation types. Inclusion criteria were: 1) diagnosis of TSC defined by clinical criteria developed in a consensus conference (Roach et al. 1999); and 2) resective surgery for treatment of medically refractory epilepsy; 3) both epileptogenic tuber and adjacent nontuber tissue available; and 4) written informed consent of parent or guardian. Exclusion criteria were: history of previous brain surgery that may have affected the original epileptic focus (including epilepsy surgery, surgery for subependymal giant cell astrocytoma, shunt placement for hydrocephaalus). Preoperative assessment included clinical evaluation, neuroimaging using magnetic resonance imaging (MRI) and positron emission tomography (PET) with alpha-[C-11]methyl-L-tryptophan (AMT) (Chugani et al. 2013), and ictal/interictal EEG. Two tissue types were sampled from the surgical resection determined by clinical parameters for each patient: 1) tuber characterized by independent epileptiform activity (seizure onset); and 2) nontuber tissue adjacent to the epileptogenic tuber (nononset). Portions of each sample from frozen blocks were immediately placed in extraction buffer for RNA and protein isolation. All protocols were approved by the Human Investigation Committee at Wayne State University.

RNA Isolation and Quality

Total RNA, including small RNAs, was isolated from brain tuber and nontuber samples using 25 mg of each tissue sample and the “All-in-One Purification” kit (Norgen Biotek, Thorold, ON, Canada). All steps were performed according to the vendor’s protocol. RNA-free DNase (Qiagen, Valencia, CA, USA) was used to remove potential gDNA contamination from the samples. RNA integrity was tested using Agilent 2100 Bioanalyzer with the RNA 6000 Nano Assay kit or RNA 6000 Pico Assay kit (Agilent Technologies, Palo Alto, CA, USA). The Bioanalyzer was used to determine if the 18S and 28S ribosomal bands were defined and to ensure minimal degradation of RNA. Optimal concentration of total RNA with the Nano assay was 100–200 ng/μL, and 0.5–1 ng/μL for testing with Pico assay. All steps were done according to the vendor’s protocol.

Sample Labeling and microRNA Microarray Hybridization

For each sample, 100 ng of total RNA along with Agilent microRNA spike-in controls were treated with calf intestine alkaline phosphatase (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and incubated at 37 °C for 30 min. Then the samples were denatured and labeled with Cyanine 3 per the vendor’s protocol (Agilent Technologies). The labeled microRNAs were cleaned using micro Bio-Spin 6 columns (Bio-Rad Laboratories, Hercules, CA, USA), and dried in a speed-vac. Dried samples were resuspended in 17 μL of nuclease-free water. Also, 1 μL of Hyb Spike-in, 4.5 μL of 10× GE Blocking, and 22.5 μL of 2× Hi-RPM hybridization buffer (Agilent) were added, and the hybridization mixture was incubated at 100 °C for 5 min and then put on ice for 5 min. Samples were immediately added to the microarrays in an Agilent SureHyb hybridization chamber and rotated at 20 rpm in a hybridization oven for 20 h at 55 °C. Agilent Human miRNA V3 microarrays were used. Microarrays were processed using the miRNA Microarray System protocol v2.2 (Agilent Technologies). Slides were scanned using an Agilent dual laser scanner. Tiff images were analyzed using Agilent’s feature extraction software (version 10.7.1.1).

Microarray Data Analysis

Microarray data were imported into GeneSpring version 12 for normalization and analysis (Agilent Technologies). The data on

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gender</th>
<th>Age</th>
<th>Gene</th>
<th>Location of resected tissue</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1409</td>
<td>F</td>
<td>2 years 6 months</td>
<td>TSC1</td>
<td>Right temporal</td>
<td>Frameshift, 8-bp insertion, nt 1609</td>
</tr>
<tr>
<td>E3007</td>
<td>F</td>
<td>10 years</td>
<td>TSC1</td>
<td>Left frontal</td>
<td>Frameshift, 5-bp deletion, nt 1020–1024</td>
</tr>
<tr>
<td>H2407</td>
<td>M</td>
<td>8 years 9 months</td>
<td>TSC2</td>
<td>Left temporal</td>
<td>Frameshift, 1-bp deletion nt 5444</td>
</tr>
<tr>
<td>81603</td>
<td>M</td>
<td>8 years 11 months</td>
<td>TSC2</td>
<td>Right frontal</td>
<td>Transition nt 2410 t-&gt;c, codon 804 Cys-&gt;Arg</td>
</tr>
<tr>
<td>90602</td>
<td>M</td>
<td>7 years 10 months</td>
<td>TSC2</td>
<td>Left occipital/parietal</td>
<td>Transition nt 5227 c-&gt;t, codon 1743 Arg -&gt; Trp</td>
</tr>
</tbody>
</table>
each array were quantile normalized, an interarray normalization procedure that ensures equivalent signal distributions for all arrays. Quantile normalization has been shown to be robust and preferable for analysis of microRNA expression in tissue samples (Rao et al. 2008). We then performed a series of stringent filtering and statistical analyses, as outlined below. Filtering microarray data using detection calls and variance metrics are useful methods to improve statistical power while controlling the false discovery rate (Hackerst and Hess 2009). Following this approach, we selected probes flagged as detected and also having an expression level above the 25th percentile in at least 75% of the samples for at least one condition being compared (tuber or nontuber). Additionally, we filtered out all probes having a coefficient of variation ≤5.0%, calculated from all samples, as these microRNAs are most likely to not be differentially expressed. Statistical analysis was performed using a moderated t-test with the Westfall and Young family-wise error rate (FWER) correction. The FWER indicates the probability that one or more false positives exist among the identified set.

**Quantitative RT-PCR of microRNA Expression**

cDNAs of all microRNAs were prepared using a single reverse transcription reaction for each sample. About 100 ng total RNA and Universal cDNA Synthesis Kit (Exiqon, Inc., Woburn, MA, USA) were used in a total volume of 20 μL. Incubation at 42 °C for 60 min was followed by heat inactivation at 95 °C for 5 min and immediate cooling to 4 °C. Undiluted cDNA was stored at −20 °C. Immediately before use, cDNA of each sample was ×40 diluted and 8 μL used for the real-time PCR reaction with Exiqon SYBR Green master mix. ROX passive reference dye (Affymetrix, Inc., Santa Clara, CA, USA) was diluted 1:10 in nuclease-free water and 0.4 μL of the ROX dilution was added per 20 μL qPCR reaction. LNA primer sets for miRs-23a, 34a, and 532-5p were obtained from Exiqon. Standard cycling condition for SybrGreen with LNA primer sets for miRs-23a, 34a, and 532-5p were obtained from Exiqon. The full-length TSC1 3’ UTR-luciferase reporter construct (SC222100), a hsa-miR-34a precursor expression plasmid (SC400356), and control expression vector (pCMV-Mir) were obtained from Origene (Rockville, MD, USA). The mir-23a precursor expression plasmid (HmiR0298-MR04) and scrambled expression control vector (CmiR0001-MR04) were purchased from GeneCopoeia (Rockville, MD, USA). A pGL4.73 (SV40) Renilla-luc expression vector was used to control for transfection efficiency (#E6911, Promega, Madison, WI, USA). Cells were plated in DMEM (high glucose, Gibco, Life Technologies, Grand Island, NY, USA) with 10% FBS and 1% nonessential amino acids. The cells were plated in a 96-well flat bottom white plate on the day of transfection. After 3 h cells were transfected with Renilla-luc reporter, 3’ UTR-luc reporter, and microRNA expression vector or microRNA expression control vector (empty or scrambled) in the concentration ratio of 0.3:2.5:4:0 (Origene vector) or 0.25:2.5:4:25 (GeneCopoeia vector) respectively, with Fugene 6 (Promega) as per manufacturer’s instructions. Transfections were performed using at least 3 replicates for each condition. After 48 h of transfection, the Renilla and Firefly luciferase activities were quantified by the Dual Glo luciferase assay system (Promega) measured on a FlexStation 3 universal plate reader (Molecular Devices, Madison, WI, USA). Data analysis was performed using Softmax Pro. The activities of 3’ UTR reporter gene were normalized by those of control Renilla-luc and expressed as a fold change by comparing cells transfected with the mir-34a or 23a expression vector to those transfected with empty or scrambled expression vector. A two-tailed t-test with equal variance was used to determine statistical significance.

**Protein Extraction**

Fresh 1× cell lysis buffer was prepared from 10× cell lysis buffer (p/n 9803, Cell Signaling, Beverly, MA, USA) and protease inhibitor cocktail (DMSO solution) (Sigma p/n P8340) was added in 1:100 dilution. Frozen brain tissue (25–50 mg) was homogenized with a mortar and a pestle for each sample. Liquid nitrogen was added into the mortar during homogenization to keep the tissue frozen. Cold 1× cell lysis buffer (500–800 μL) with protease inhibitor cocktail was added to the frozen tissue powder, mixed and scooped into a 2-mL eppendorf tube. Then the protein extract was centrifuged for 10 min at 14 000 × g in a cold centrifuge. Supernatant was transferred into a fresh 1.5-mL eppendorf tube and smaller aliquots of each protein extract were stored at −80 °C.

**Quantitative LC-MS/MS Proteomics Analysis**

Each protein sample (95 μg) was reduced with dithiothreitol and alkylated and iodoacetamide (Sigma, St Louis, MO, USA). Samples were then diluted so that the Triton-X concentration was 0.05% and digested with trypsin. Five microgram was analyzed for complete digestion by SDS-PAGE, and the remaining material was desalted using an Oasis 30-mg HLB cartridge (Waters, Milford, MA, USA). A micro BCA assay (ThermoFisher Scientific, Waltham, MA, USA).
USA) was used to confirm protein concentrations and then 43 µg of each sample was taken for TMT-10plex labeling (ThermoFisher Scientific). In addition, an equal aliquot of each sample was pooled for a reference sample that was also 43 µg. Eight samples and the reference pool were labeled according to the manufacturer’s protocol; one channel was left empty. The labeled samples were combined and 200 µg was taken for analysis. Detergent was removed by serial solid phase extraction using reversed phase, strong cation exchange, and reversed phase materials. An Oasis HLB cartridge (Waters), 4 strong cation exchange spin columns (Nest Group, Southborough, MA) and a Pierce 100 µL C18 tip (ThermoFisher Scientific) were used, respectively. The sample was then dried and resuspended and fractionated at pH 10 using an Agilent PLRP-S 0.5 x 150 mm column (Agilent, Santa Clara, CA, USA) with elution by a gradient of acetonitrile into 33 fractions. The fractions were then dried and resuspended and submitted for LC-MS² analysis on an Orbitrap Fusion mass spectrometer equipped with an EASY-nLC UHPLC (ThermoFisher Scientific). Peptides from each fraction were eluted into the mass spectrometer over a period of 110 min. Peptide fragmentation and reporter ion detection were performed using simultaneous precursor selection, where the top 10 most abundant MS² fragments were selected for higher energy collisional dissociation fragmentation (McAlister et al. 2012). MS² fragmentation was at 30% collision energy and MS3 fragmentation was at 65% collision energy.

Proteome Discoverer 1.4.1.14 was used to extract and search mass spectra against the Uniprot human canonical database (February 2014; 20 264 entries) using Sequest HT with the percolator algorithm to assign confidence to identifications. Because of the large number of spectra, each fraction was searched and quantified separately, and the search results were then combined in Proteome Discoverer. Peptides that were identified with high confidence (1% false discovery rate) were used for subsequent analysis. Proteins that were identified on the basis of those peptides were grouped to find the smallest list of proteins that explains all identified peptides. Reporter ions were quantified relative to the all-sample-pool reporter intensity and the ratios for each fraction were normalized to a median of 1. The resultant proteomics data were imported into GeneSpring v12.6.1 for quantitative analysis. Ratios reflecting relative protein abundance to the all-sample pool were quantile normalized for each of the samples. Statistical significance of alterations in protein level between tuber and nontuber samples was determined using a two-sided student’s t-test and the Storey bootstrap false discovery rate method (GeneSpring). Fisher’s exact test was used to calculate significance of enrichment for repressed target proteins (JMP 11).

For analysis of p53 pathway activation, we used a published list of genes with multiple levels of evidence demonstrating direct induction by p53 transcriptional regulation (Riley et al. 2008). Of the 136 genes annotated with p53 activator sites in Supplementary Table 2 of Riley et al., we found 28 with proteomics spectra for all samples in our dataset. These were subsequently used to assess p53 activity. Likewise, we used a set of direct target genes to measure NF-κB activity. We used the curated list of NF-κB target genes from http://bioinfo.lifl.fr/NF-KB/ and downloaded all genes identified as “human gene with checked binding sites.” Of the 104 genes meeting this criterion, 24 genes had proteomics spectra for all of our TSC samples. Significance of p53 and NF-κB pathway activation was determined by analysis of \( \log_{10}(N/J) \) values for target proteins, where \( N \) represents protein expression in tuber and \( J \) is expression in nontuber. The one-sample t-test against zero was used to determine statistical significance (one-sided, JMP 11). Empirical cumulative distributions of the \( \log_{10}(N/J) \) values from the proteome (5748 proteins), p53-activated proteins (28), and NF-κB target proteins (24) were derived with JMP 11. Statistical significance of the difference between the proteome distribution and each of the p53 and NF-κB distributions was determined using the two-sample Kolmogorov–Smirnov test (two-sided, JMP 11).

### Results

#### Identification of Aberrant microRNA Expression in Cortical Tubers

We performed microRNA expression analysis on matched pairs of epileptogenic tubers (seizure onset) and adjacent nontuber tissue (nononset) for 5 subjects: 2 with TSC1 mutations and 3 with TSC2 mutations (Table 1). Agilent microRNA microarrays having probes for more than 900 human microRNAs were used to quantify microRNA expression for each of the 10 tissue samples. Stringent filtering and statistical analyses (Materials and Methods) were used to arrive at a high-confidence list of differentially expressed microRNAs. A FWER correction was used to ensure that the probability of one or more false positives being among the selected microRNAs was only 5%. This approach identified 5 statistically significant microRNAs having a minimum 2-fold difference between tuber and nontuber tissues (Table 2). Four microRNAs exhibited higher expression in tuber compared with nontuber tissue (miRs-34a, 23a, 34b*, 532-5p), while one microRNA had lower expression (miR-381).

#### Quantitative RT-PCR Confirms microRNA Overexpression in Cortical Tubers

Computational analysis (below) suggested that miR-34b* targets few transcripts, while miRs 23a, 34a, and 532-5p were predicted to have a combinatorial effect on many genes involved in neurological processes. Therefore, we performed qRT-PCR analysis on the latter group of microRNAs to confirm the microarray results. Expression levels for each microRNA were compared between patient-matched tuber and nontuber tissue and the mean fold change calculated from the 5 patients (Table 2). All 3 microRNAs were confirmed to have higher expression in tubers than the adjacent nontuber tissue, consistent with the microarray results.

### Table 2 Statistically significant microRNAs differentially expressed in TSC tubers

<table>
<thead>
<tr>
<th>microRNA</th>
<th>P</th>
<th>P (corrected)</th>
<th>Microarray FC</th>
<th>qRT-PCR FC</th>
<th>Chr</th>
<th>miRBase accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-34a</td>
<td>0.0012</td>
<td>0.0000</td>
<td>3.04</td>
<td>6.79</td>
<td>1</td>
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</tr>
<tr>
<td>hsa-miR-23a</td>
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<td>0.0000</td>
<td>2.65</td>
<td>2.80</td>
<td>19</td>
<td>MIMAT0000778</td>
</tr>
<tr>
<td>hsa-miR-532-5p</td>
<td>0.0101</td>
<td>0.0202</td>
<td>2.89</td>
<td>1.86</td>
<td>X</td>
<td>MIMAT0002888</td>
</tr>
<tr>
<td>hsa-miR-34b*</td>
<td>0.0007</td>
<td>0.0000</td>
<td>3.58</td>
<td>11</td>
<td>11</td>
<td>MIMAT0006854</td>
</tr>
<tr>
<td>hsa-miR-381</td>
<td>0.0098</td>
<td>0.0101</td>
<td>–2.18</td>
<td>–2.18</td>
<td>14</td>
<td>MIMAT0007364</td>
</tr>
</tbody>
</table>
Comparison of microRNA Expression in Tubers and Nontuber Tissue to Normal Cortex

Tissue was designated as tuber or nontuber by a neuropathologist, although the nontuber tissue may not be entirely normal. The tissue was adjacent to the seizure-onset zone (tuber) and also exhibited interictal spiking activity on the intracranial EEG. Additionally, both tuber and nontuberal tissues are haploinsufficient for TSC1 or TSC2. We found differential expression of several microRNAs in the matched tissue sample; however, this approach did not indicate how the expression levels relate to normal cortical tissue from non-TSC subjects. To address this question, we utilized microRNA data from a postmortem (PM) study of primate brain tissue that included samples from human, chimpanzee, and macaque (Somel et al. 2011). The study investigated microRNA expression in prefrontal cortex and cerebellar cortex from subjects representing a wide range of ages. We downloaded the dataset from the NCBI Gene Expression Omnibus repository (GEO ID GSE29356) and utilized the data for prefrontal cortex specimens from 8 human subjects ranging in age from 2 days to 88 years. These data were generated using the same microRNA microarray platform and version that we used in our TSC study, thus facilitating direct comparison to our results. To further ensure compatibility between the 2 datasets, we co-normalized the primate PM data with our TSC data using quantile normalization. This procedure results in matched quantiles of expression level across all microarrays. To determine if the normalization approach was effective, we compared expression levels between the 2 datasets for 51 microRNAs that were invariant in our TSC tuber versus nontuber analysis. These microRNAs had expression coefficients of variations <5%, indicating that they are not differentially expressed in tubers compared with nontuber tissue, and we would therefore expect similar expression levels in the PM controls. Using normalized expression values for the 51 microRNAs, a scatter plot comparison of the PM values versus the TSC data provided an excellent linear correlation, with a Pearson’s correlation coefficient = 0.93 and a linear fit of $y = 0.997x$ (data not shown). These metrics indicate that the normalized expression values from the 2 datasets are similar, thus enabling the below comparison of microRNA expression in TSC tissues to their levels in normal PM cortex.

Analysis of the expression level of miR-34a in the PM control samples revealed a pronounced postnatal increase in expression that plateaus after the teenage years. Age-dependent elevation of miR-34a expression in brain and cardiac tissue has previously been reported (Somel et al. 2010; Li et al. 2011; Boon et al. 2013). Since our pediatric subjects are within the timeframe of rapid increase in miR-34a expression, it is imperative that we consider subject age when investigating microRNA expression events in this population. We did not observe age-dependent expression of miRs-23a, 34b*, 532-5p, and 531 in the PM dataset. Figure 1A presents miR-34a expression as a function of age for each of the PM subjects and the TSC samples in our study. Expression levels for PM controls are represented by gray points and are fit with a logarithmic curve (blue). Expression in nontuber tissue from our TSC subjects is indicated by yellow inverted triangles, and these points are generally situated on or near the curve fit to the PM controls, indicating that miR-34a expression in the TSC nontuber tissue is near normal. However, the expression of miR-34a in epileptogenic tubers is clearly elevated above the expected age-associated level for each subject, as indicated by the red diamonds. Expression of miRs-23a, 34b*, and 532-5p in nontuber tissue was similar to levels in the PM controls, and the corresponding expression level in epileptogenic tubers was clearly elevated for each microRNA (Fig. 1B). A measurable difference between PM controls and nontuber tissue was observed for miR-381, with expression of tuber < nontuber < PM (data not shown).

Quantitative LC-MS/MS Proteomic Profiling Demonstrates Significant Repression Among Combinatorial Targets and Enrichment for Synaptic Signal Transmission Proteins

We used liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics with tandem mass tags (TMT) to quantify protein levels in tuber and nontuber tissues. This approach allowed us to: 1) perform quantitative analysis that is superior to traditional Western blots (Aebersold et al. 2013); 2) perform global analysis for a large portion of the proteome; and 3) perform concurrent analysis of all samples (TMT) thus providing an important internal control for the comparison of tuber and nontuber protein levels. One limitation of LC-MS/MS is the requirement for an ample amount of protein. We had sufficient protein from tuber and nontuber from 3 of the 5 patients (E3007, 81603, H2407). Patient 90602 had sufficient protein from nontuber tissue. We were also able to extract protein from an additional tuber from subject 81603. Thus, the proteomics analysis was performed on 8 samples in total: 4 tuber and 4 nontuber tissues. Overall 614 115 MS2 spectra were submitted and 136 208 were identified (22%). About 42 060 peptides and 6309 proteins were identified on the basis of these spectra. We obtained quantifiable mass spectra for 5749 proteins in all 8 samples. The set of 5749 proteins quantified represents somewhat less than half of all the proteins expected to be present in these tissues (Michalski et al. 2011; Nagaraj et al. 2011). Very low abundance proteins and proteins that do not generate tryptic peptides that produce good spectra are unlikely to be identified. We first identified proteins with a significant change in abundance between tuber and nontuber samples. Statistical selection using a 10% false discovery rate and minimum 1.5-fold change resulted in 1745 significant proteins. Of these, 842 proteins were elevated and 903 had lower levels in tubers.

To investigate protein expression altered by the overexpressed microRNAs, we first identified high-confidence target transcripts using a prediction consensus approach. It is widely recognized that computational methods for microRNA target prediction have a high false-positive rate (Yue et al. 2009; Reyes-Herrera and Ficarra 2012). The number of false positives can be reduced by applying a consensus of multiple prediction methods (Kuhn et al. 2008). We used a consensus tool (miRWalk) to identify putative targets for each of the 4 microRNAs overexpressed in tubers (Dweep et al. 2011). A consensus score was defined for each microRNA/transcript interaction as the number of algorithms (of 10) that predicted that the transcript is targeted by the microRNA. We then focused on the set of transcripts that are targets of more than one of the 4 microRNAs. Since a single microRNA may target many transcripts, and a given transcript 3’ UTR may have target sites for multiple microRNA species, it is important to consider potential combinatorial targeting among a set of differentially expressed microRNAs (Dombkowski et al. 2011). To account for such combined effects a combinatorial consensus score (CCS) was calculated for each transcript by summing the individual consensus score from all 4 of the microRNAs. The target transcripts were ranked using the CCS.

Our proteome profiling allowed us to empirically determine the minimal CCS associated with significant target repression. Since the 4 microRNAs were overexpressed in tubers their activity...
would be evident by a reduction in target protein levels. Using the set of 1745 differentially expressed proteins, we binned the proteins by CCS and then determined the fraction of proteins in each bin (score) that were repressed in tubers. Considerable enrichment for repressed proteins was observed with targets having a CCS of 13 or greater. We found that 68% (85/125) of proteins having a CCS $\geq 13$ were repressed as compared with 50.5% (818/1620) of proteins with a CCS $\leq 12$ ($P = 0.00018$) (Fig. 2).

The results demonstrate significant repression among proteins having a high-confidence combinatorial target score, evidence of targeting by the 4 overexpressed microRNAs.

We performed a functional analysis of the 85 repressed combinatorial target proteins (CCS $\geq 13$) using DAVID (Database for Annotation, Visualization, and Integrated Discovery) to identify gene ontologies and functional categories associated with the repressed targets (Huang da et al. 2009). Using the gene ontology (GO) biological process, the most significant ontology was "transmission of nerve impulse." By definition of the GO Consortium, this ontology is assigned to genes/proteins involved in synaptic signaling and consequent electrochemical polarization/depolarization in neurons. The fraction of repressed target proteins with this ontology (14/85, Table 3) is highly significant ($P = 1.6e^{-8}$) and represents a 7.8-fold enrichment. The enrichment value indicates that the number of repressed target proteins involved in synaptic signaling is nearly 8 times what is expected by chance, given the number of genes with this ontology found throughout the entire genome. The proportion of proteins in this functional category (16.5%) is markedly higher in the repressed combinatorial targets than in the group of repressed nontarget proteins (5.9%). The results demonstrate that the set of transcripts targeted by the collective group of 4 microRNAs is highly enriched for genes/proteins involved in synaptic signaling. Additionally, 9 genes known to confer risk of epilepsy are among the 85 repressed combinatorial target genes (Table 3), including the TSC1 protein hamartin, synapsin II (SYN2), $\gamma$-aminobutyrate (GABA) A receptor beta3 (GABRB3), doublecortin (DCX), and neurofibromin (NF1). The consensus score is shown for each predicted microRNA/target interaction. The scores for miR-34b* are consistently low, suggesting that this microRNA is not significantly involved in the regulation of these transcripts. Conversely, the scores for miR-34a are...
consistently high indicating that this microRNA has a prominent role in the post-transcriptional regulation of these genes. Other high-ranking and significantly enriched ontologies among the repressed target proteins included “neurological system process” and “neuron development.”

Hamartin is Repressed in Tubers

A notable repressed protein among the combinatorial targets is hamartin, the protein product of the TSC1 gene. The quantitative proteomics data show that, on average, hamartin levels were 2.2-fold lower in epileptogenic tubers compared with nontuber tissue (Fig. 3). Interestingly, hamartin levels were decreased in tubers of the TSC1 patient and the TSC2 patients. TSC1 is predicted to be a target of miRs-23a, 34a, and 532-5p based on a consensus of 5, 4, and 5 prediction algorithms, respectively (Table 3).

Luciferase Reporter Assays Confirm Predicted microRNA/Target Interactions of TSC1

We utilized a luciferase reporter assay to validate targeting of the 3′ UTR of TSC1 by miRs-23a and 34a. A vector construct created with the firefly luciferase reporter gene and the 3′ UTR of TSC1 was transfected into COS-7 cells. This cell line has been widely used for microRNA target validation, including studies of neural development (Peng et al. 2012; Zhao et al. 2012). Importantly, we used a construct that included the entire TSC1 3′ UTR since truncated UTRs may alter microRNA/UTR interactions (Kuhn et al. 2008). Co-transfection with a vector expressing pre-miR-34a resulted in a 71% decrease in expression of the luciferase reporter compared with control expression vector (P = 0.0005) (Fig. 4). A 40% decrease in reporter expression was observed when co-transfecting with pre-miR-23a compared with control expression vector transfection.

Table 3 Combinatorial target proteins significantly repressed in tubers and associated with synaptic signaling or epilepsy risk

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>UniProt Accession</th>
<th>Protein (T/N)</th>
<th>Trans. nerve impulse</th>
<th>Epilepsy risk</th>
<th>Prediction score</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABRA3</td>
<td>Gamma-aminobutyric acid receptor subunit alpha-3</td>
<td>P34903</td>
<td>–6.7</td>
<td>♦</td>
<td></td>
<td>5 3 3 2</td>
</tr>
<tr>
<td>CACNA1E</td>
<td>Voltage-dependent R-type calcium channel subunit alpha-1E</td>
<td>Q15878</td>
<td>–5.0</td>
<td>♦</td>
<td></td>
<td>5 5 2 2</td>
</tr>
<tr>
<td>SCN3B</td>
<td>Sodium channel subunit beta-3</td>
<td>Q9NY72</td>
<td>–4.4</td>
<td>♦</td>
<td></td>
<td>5 5 4 2</td>
</tr>
<tr>
<td>GABBR2</td>
<td>Gamma-aminobutyric acid type B receptor subunit 2</td>
<td>Q75899</td>
<td>–3.0</td>
<td>♦</td>
<td></td>
<td>6 0 5 3</td>
</tr>
<tr>
<td>GABBR3</td>
<td>Gamma-aminobutyric acid receptor subunit beta-3</td>
<td>P28472</td>
<td>–2.9</td>
<td>♦</td>
<td></td>
<td>5 6 2 2</td>
</tr>
<tr>
<td>SLC6A1</td>
<td>Sodium- and chloride-dependent GABA transporter 1</td>
<td>P30531</td>
<td>–2.7</td>
<td>♦</td>
<td></td>
<td>6 5 5 0</td>
</tr>
<tr>
<td>SYN2</td>
<td>Synapsin-2</td>
<td>Q92777</td>
<td>–2.7</td>
<td>♦</td>
<td></td>
<td>4 3 5 3</td>
</tr>
<tr>
<td>DCX</td>
<td>Neuronal migration protein doublecortin</td>
<td>O43602</td>
<td>–2.6</td>
<td>♦</td>
<td></td>
<td>7 2 6 2</td>
</tr>
<tr>
<td>SLC30A3</td>
<td>Zinc transporter 3</td>
<td>Q99726</td>
<td>–2.4</td>
<td>♦</td>
<td></td>
<td>7 4 0 2</td>
</tr>
<tr>
<td>APBA1</td>
<td>Amyloid beta A4 precursor protein-binding family A member 1</td>
<td>Q02410</td>
<td>–2.3</td>
<td>♦</td>
<td>♦</td>
<td>7 4 1 2</td>
</tr>
<tr>
<td>NPTX1</td>
<td>Neuronal pentraxin-1</td>
<td>Q15818</td>
<td>–2.2</td>
<td>♦</td>
<td>♦</td>
<td>6 1 4 2</td>
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<tr>
<td>TSC1</td>
<td>Hamartin</td>
<td>Q92574</td>
<td>–2.2</td>
<td>♦</td>
<td>♦</td>
<td>4 5 5 2</td>
</tr>
<tr>
<td>KCNMA1</td>
<td>Calcium-activated potassium channel subunit alpha-1</td>
<td>Q12791</td>
<td>–1.9</td>
<td>♦</td>
<td>♦</td>
<td>5 5 5 3</td>
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<tr>
<td>PVRL1</td>
<td>Poliovirus receptor-related protein 1</td>
<td>Q15223</td>
<td>–1.9</td>
<td>♦</td>
<td>♦</td>
<td>6 3 4 2</td>
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<tr>
<td>SNPH</td>
<td>Syntaphilin</td>
<td>O15079</td>
<td>–1.7</td>
<td>♦</td>
<td>♦</td>
<td>5 2 5 2</td>
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<tr>
<td>NF1</td>
<td>Neurofibromin</td>
<td>P21359</td>
<td>–1.6</td>
<td>♦</td>
<td>♦</td>
<td>3 5 5 1</td>
</tr>
<tr>
<td>KIF1B</td>
<td>Kinesin-like protein KIF1B</td>
<td>O60333</td>
<td>–1.6</td>
<td>♦</td>
<td>♦</td>
<td>5 5 5 3</td>
</tr>
</tbody>
</table>

Note: Shading corresponds to the consensus prediction score.
To assess p53 pathway activation, we used 28 proteins known to be directly induced by p53 through transcriptional regulation (Materials and Methods). For each of these proteins, we calculated \( \log_2(T/N) \) using the average expression value for tuber (T) and nontuber (N). This parameter is zero when no change in expression is measured, negative when repressed in tubers, and positive when overexpressed in tubers compared with nontuber. This metric was also calculated for all 5749 proteins having proteomics spectra for all samples. We refer to this set as the “proteome.” Expression levels for the group of 28 p53-activated proteins were significantly elevated \((P = 0.015)\), with mean \( \log_2(T/N) = 0.35 \) (1.27-fold). Mean \( \log_2(T/N) \) for the proteome was 0.0029, very close to the expected value of zero.

We performed the same analysis for a group of 24 proteins established as direct targets of NF-κB, a key mediator of inflammation (Materials and Methods). We found that expression levels of the overall set of NF-κB target proteins to be significantly elevated in tubers \((P < 0.0001)\), with mean \( \log_2(T/N) = 0.96 \) (1.94-fold). The distributions of \( \log_2(T/N) \) values for the p53- and NF-κB-regulated proteins were significantly different than the overall proteome and reflect overall induction of the proteins. These results are evident in the empirical cumulative distributions shown in Figure 5. Cumulative distributions provide the probability \((y\text{-axis})\) of observing a specified value or less \((x\text{-axis})\). The cumulative distribution of \( \log_2(T/N) \) values for the proteome is shown by the red, solid curve. Distributions for p53- and NF-κB-regulated proteins are clearly shifted to the right, reflecting increased expression in tubers for proteins regulated by the 2 transcription factors. Comparison of each distribution to the overall proteome reveals statistical significance, \( P = 0.0112 \) and 0.0005, respectively, for p53 and NF-κB target sets. Tables with proteomics data for p53 and NF-κB target proteins are found in the supplementary files.
miR-34a during this critical period, as well as in utero, would be expected to disrupt normal neurodevelopment. Several groups have reported increased levels of miR-34a in animal models of epilepsy (Hu et al. 2011, 2012; Sano et al. 2012; Risbud and Porter 2013). MiRs 23a and 381 have also been reported as differentially expressed in epilepsy, and we found these microRNAs differentially expressed in epileptogenic tubers as well (Song et al. 2011; Hu et al. 2012; Risbud and Porter 2013). Dysregulation of miR-34a has been associated with a range of neurological disorders. An analysis of genomic copy-number variants (CNVs) associated with autism identified miR-34a as one of 10 “hub” microRNAs (microRNAs targeting multiple autism risk genes) resident in the autism CNV regions (Vaishnavi et al. 2013). MiR-34a has also been identified as a potential blood-based biomarker of schizophrenia (Lai et al. 2011). In a case/control comparison, miR-34a was found to be 2.5-fold higher in mononuclear leukocytes of schizophrenia patients. This observation is consistent with a PM study that found miR-34a overexpression in the prefrontal cortex of patients with schizophrenia (Kim et al. 2010). Overexpression of miR-34a was also reported in the cerebral cortex of a mouse model of Alzheimer’s disease (Wang et al. 2009).

The end point of microRNA activity is best observed at the protein level since many microRNA/transcript interactions impede translation and may not alter transcript levels (Selbach et al. 2008; Li et al. 2012; Liu et al. 2013). We used quantitative LC-MS/MS proteomic profiling to investigate alterations in protein abundance for targets of the 4 overexpressed microRNAs. We demonstrated significant repression in tubers for proteins of transcripts with a high-confidence combinatorial target score. The set of repressed target transcripts was highly enriched for genes involved in synaptic signaling. Additionally, the proteins for a number of genes known to confer risk of epilepsy were among the combinatorial targets repressed in tubers. Notably, among the repressed target proteins associated with epilepsy risk and synaptic signal transmission was hamartin, the product of the TSC1 gene. We used a luciferase reporter assay to demonstrate targeting of the TSC1 3′ UTR by miRs-34a and 23a. This finding is consistent with a recent publication, and our own computational analysis, that predict targeting of TSC1 by miR-23a (Romaker et al. 2014).

We also found decreased hamartin in tubers from the TSC1 patient, as well as those with TSC2 mutations.

Investigations from a number of laboratories suggest the TSC genes may be post-transcriptionally regulated. A number of studies have reported repressed hamartin and/or tuberin levels in cortical tubers, even where loss of heterozygosity (LOH) or other second-hit mutations of the genes could not be identified (Kerfoot et al. 1996; Mizuguchi et al. 1996, 1997, 2000; Mizuguchi and Takashima 2001; Vinai et al. 2004; Boer et al. 2006). While reduced expression of one of the proteins could be expected in the presence of inactivating mutations in the corresponding gene (e.g., truncations), many of the reports noted simultaneous repression of hamartin and tuberin with no clear causative explanation. It has been speculated that mutation of one of the TSC genes may reduce expression of the other gene product, but no mechanism has been uncovered (Jozwiak et al. 2004; Nida et al. 2001) noted that one potential explanation is that “expression of the wild-type protein being ‘turned off’ or reduced as a result of epigenetic events,” yet they found no evidence of methylation-based gene silencing. Collectively, these observations describe hallmarks of microRNA activity. We have found aberrant microRNA expression in cortical tubers and their targeting of TSC1. Subsequent mechanistic studies are warranted to determine if the aberrant microRNAs represent a type of “second hit,” contributing to tuber formation in the absence of LOH (Crino et al. 2010) or if their dysregulation is an outcome of tuber pathology.

In addition to TSC1, our integrated microRNA/proteomic profiling revealed a number of target transcripts which are known to cause epilepsy when deficient or deleted (Table 3). Knockout of SYN2 was shown to cause seizures in a mouse model (Rosalie et al. 1995). Mice with inactivated GABRB3 have a seizure phenotype (Romanics et al. 1997). Mutations in DCX result in type I lissencephaly, with clinical features of disorganized cortical layering, epilepsy, and mental retardation (Gleeson et al. 1998). Mutations in NF1 lead to neurofibromatosis type I, a neurocutaneous syndrome characterized by benign brain lesions, cognitive disorders, and seizures in ~10% of patients (Ostendorf et al. 2013). Each of these genes is a high-confidence combinatorial target of the 4 overexpressed microRNAs, and their protein levels were significantly decreased in tubers.

A model for the mechanism of microRNA dysregulation and their role in TSC tuber pathology emerges from our findings and the current literature (Fig. 6). Considerable evidence in the literature has shown that: 1) loss of TSC1 or TSC2 results in activation of p53; and 2) activated p53 induces miRs-34a and 23a. Using cell line knockouts of TSC1 and TSC2, a study confirmed that “loss of TSC1 or TSC2 results in a dramatic elevation of p53 protein” due to increased translation stimulated by mTOR activation (Lee et al. 2007). The same study reported elevated p53 levels in human angiomylipomas due to TSC mutations. The authors presented an intriguing hypothesis that the proapoptotic effect of p53 might be the reason why TSC lesions are generally benign. A recent study found that, in addition to increased synthesis of p53, mTOR activates p53 through phosphorylation of Ser46 (Krzezniak et al. 2014). Evidence of elevated p53 activity in TSC tubers is evident in our proteomics dataset. We analyzed expression levels for the proteins produced from a set of known p53-activated genes. The overall set of proteins was significantly increased in tubers compared with nontuber tissue, consistent with p53 activation. A number of reports have demonstrated the direct
transactivation of miR-34a by p53, and this regulatory axis has gained substantial interest (Chang et al. 2007; Raver-Shapira et al. 2007; Rokavec et al. 2014). Induction of both miR-34a and p53 due to loss of TSC1 was reported in a study detailing the role of TSC1 in the survival of mast cells (Shin et al. 2012). MiR-34b has also been shown to be activated by p53 (He et al. 2007), and a comparison of p53 null and wild-type colon cancer cell lines identified miR-23a as a probable target of p53 (Chang et al. 2007). Recently, 2 studies have confirmed that miR-23a is induced by p53 in hepatocellular carcinoma (Wang et al. 2013, 2014). Collectively, these reports and our results indicate that p53 activation is a likely mechanism involved in aberrant expression of microRNAs with loss of TSC1 or TSC2 in cortical tubers, and downstream microRNA targeting may contribute to tuber pathogenesis and epileptogenesis. Previous reports have shown that mammalian/mechanistic target of rapamycin activation due to TSC mutations results in induction of miRs-23a and 34a mediated through p53. We have shown that these microRNAs are overexpressed in epileptogenic tubers, along with miR-532-5p, and they target transcripts involved in synaptic signaling, neuron development, and a number of genes known to confer risk of epilepsy. The repression of target proteins may contribute to tuber pathogenesis as denoted by MRI showing the locations of multiple tubers. Events leading to inflammation in a subset of tubers may further exacerbate overexpression of the microRNAs mediated by proinflammatory cytokines. The combined effects of these mechanisms may lead to epileptogenesis in a subset of tubers, as highlighted by the MRI and AMT-PET imaging shown in the figure. The red arrow indicates an epileptogenic tuber with high AMT uptake, a marker of inflammation. The remaining arrows note the locations of nonepileptogenic tubers.

**Figure 6.** Mechanistic model depicting roles of microRNAs and signaling pathways in TSC tuber pathogenesis and epileptogenesis. Previous reports have shown that mammalian/mechanistic target of rapamycin activation due to TSC mutations results in induction of miRs-23a and 34a mediated through p53. We have shown that these microRNAs are overexpressed in epileptogenic tubers, along with miR-532-5p, and they target transcripts involved in synaptic signaling, neuron development, and a number of genes known to confer risk of epilepsy. The repression of target proteins may contribute to tuber pathogenesis as denoted by MRI showing the locations of multiple tubers. Events leading to inflammation in a subset of tubers may further exacerbate overexpression of the microRNAs mediated by proinflammatory cytokines. The combined effects of these mechanisms may lead to epileptogenesis in a subset of tubers, as highlighted by the MRI and AMT-PET imaging shown in the figure. The red arrow indicates an epileptogenic tuber with high AMT uptake, a marker of inflammation. The remaining arrows note the locations of nonepileptogenic tubers.
pathogenesis through repression of genes involved in neuronal development and synaptic signaling, as well as epilepsy risk genes.


Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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References


