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Construction and analysis of a dysregulated lncRNA-associated ceRNA network in a rat model of temporal lobe epilepsy

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ABSTRACT

Purpose: The aim of this work was to investigate expression and cross-talk between long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) in a rat model of temporal lobe epilepsy (TLE).

Methods: Noncoding RNA chips were used to explore the expression and relationship between lncRNAs and miRNAs in a rat model of TLE. The expression of different lncRNAs and mRNAs was analysed by Pearson's correlation coefficient, and the function of each lncRNA was annotated by co-expressed genes based on gene ontology classification using DAVID. MiRNA-lncRNA interactions were predicted by using StarBase v2.0, and the competing endogenous RNA (ceRNA) relationship between lncRNAs and miRNAs was built by using Cytoscape software. Real-time PCR was used to verify chip results.

Results: According to the expression profile analysis, 54 lncRNAs, 36 miRNAs and 122 mRNAs were dysregulated in TLE rat model compared to normal controls. The functions of lncRNAs in epilepsy were annotated by their co-expressed genes based on the "guilt by association" strategy. DAVID analysis revealed that differentially expressed lncRNA functions were involved in "potassium channel activity", "metal ion transmembrane transporter activity", and "voltage-gated potassium channel activity". Based on the ceRNA theory, 13 mRNAs, 10 miRNAs and 11 lncRNAs comprise the lncRNA-miRNA-mRNA ceRNA relationship in epilepsy.

Conclusions: The molecular functions of the differentially expressed genes play an important role in the pathogenesis of voltage-gated potassium channel activity. Further ceRNA analyses suggest that modulation of lncRNAs could emerge as a promising therapeutic target for TLE.

1. Introduction

The role of the non-coding genome in epilepsy has primarily been focused on the widespread disruption of microRNA (miRNA) expression and function [1]. However, the involvement of long non-coding RNAs (lncRNAs) in epilepsy remains unknown. LncRNAs are a heterogeneous group of non-coding transcripts (longer than 200 nt) that play roles in many biological processes [2] and comprise the largest portion of the mammalian non-coding transcriptome. LncRNAs are involved in many transcriptional regulatory processes [3]; for example, lncRNAs mediate the efficiency of upstream promoter region transcription of protein-coding genes and disrupt the downstream expression of genes, such as the SER3 gene in yeast [4]. Furthermore, lncRNAs also mediate the inhibition of RNA polymerase II, chromatin remodelling and histone modification, thus modulating downstream gene expression, such as in p15AS [5]. LncRNAs have been reported to interact with protein-coding gene transcription and form the complementary double-strand, which then interferes with precursor RNA splicing [6], such as that of siRNA or miRNA transcriptional precursor molecules [7].

Typically, the "guilt by association" strategy has been used to characterize lncRNA function [8,9]. In principle, any RNA molecule that possesses at least one miRNA response element (MRE) that is accessible to microRNA binding could act as a competing endogenous RNA (ceRNA) [10]. Based on a simple set of interactions between one miRNA and two target mRNAs (or lncRNAs), an increasing number of investigators have proposed that a near equimolar equilibrium of all these elements is required for optimal ceRNA-mediated cross-regulation [11]. Transcription and degradation rates for both miRNA and lncRNA, as well as the association, dissociation, and degradation rates of miRNA/lncRNA complexes, represent key parameters of the competing model [12]. For example, the muscle-specific lncRNA linc-MD1 regulates the timing of muscle differentiation by sequestering miR-133 to

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modulate expression of MAML1 and MEF2C [13]. A recent study identified a lncRNA-associated ceRNA network in glioblastoma multiforme, identifying prognostic lncRNAs using network analysis [14]. In our previous work, we analysed DNA methylation profiles in temporal lobe epilepsy (TLE) patients for ncRNAs, primarily focusing on lncRNAs and miRNAs by reannotating data from a DNA methylation BeadChip. Our results revealed a pattern of global hypermethylation in miRNA and lncRNA genes of TLE patients. Bioinformatics analyses have found that aberrantly methylated miRNAs and lncRNAs are related to ion channel activity, drug metabolism, the mitogen-activated protein kinase (MAPK) signalling pathway, and the neurotrophin signalling pathway [15,16]. To gain a better understanding of noncoding RNAs in epilepsy, we performed ncRNA expression profiles in an animal model of epilepsy.

In this study, we analysed genome-wide lncRNA, mRNA and miRNA expression profiles based on microchips, comprehensive miRNA-target interactions, and functional analyses, demonstrating that the lncRNA TCONS_00129582 might be a typical lncRNA involved in ion transporter activity. We also investigated a subset of dysregulated miRNAs and their target genes, revealing that some of them play an important role in the cation channel complex. Furthermore, we constructed a map of the ceRNA analysis among these lncRNAs, miRNAs and their target genes. These analyses demonstrate that lncRNA-mediated ceRNA networks could be used to accelerate the discovery of molecular biomarkers and treatment for TLE.

2. Materials and methods

2.1. Animal model

Male Sprague-Dawley (SD) rats weighing 230–250 g were obtained from the Animal Experimental Centre of Central South University, China. Rats were randomly divided into a control group (n = 6) and an experimental group (n = 6) and were maintained in controlled conditions (12-h light/dark cycle, 18–25 °C, 50%–60% humidity) with food and water. All experiments were performed at the same time in the morning to minimize possible effects of circadian variation. All study protocols were approved by the Care and use of Laboratory Animals and the guidelines of the Animal Care Committee of Central South University Xiangya School of Medicine and Xiangya Hospital (NO: 201412438). The pilocarpine epilepsy rat model was performed as previously described [17]. Rats were administered lithium chloride (127 mg/kg, Sigma-Aldrich, Saint Louis, MO, USA) 24 h prior to pilocarpine injection and were injected intraperitoneally (i.p.) with a low dose of methylscopolamine (1 mg/kg, i.p.) 15 min prior to pilocarpine injection to reduce peripheral cholinergic effects and improve survival rate. Temporal lobe epilepsy animals were classified by using Racine's scale, which is divided into 5 stages of intensity: "mouth and facial movements" (stage 1); "head nodding" (stage 2); "forelimb clonus" (stage 3); seizures characterized by rearing, (stage 4) and seizures characterized by rearing and falling (stage 5). According to Racine's classification, SE was defined as continuous seizures lasting at least 30 min, and animals classified as Racine stage IV that fulfilled the SE criterion were used in this study. Following pilocarpine injection, rats spent approximately 30 min in the SE phase in Racine stage IV or V. Matched controls were injected i.p. with the same volume of normal saline instead of lithium chloride and pilocarpine. All rats were administered chloral hydrate injection (10%, 3 ml/kg, i.p.) to terminate behavioural seizures. At the same time, the behaviour of seizure animals was confirmed by video. In addition, some of the animals were confirmed to be in the acute epileptic stage (AE) (2 h after SE onset, Supplemental Fig. 1 A) and chronic epileptic stage (CE) (2 months after SE onset, presenting as chronic spontaneous seizures with a frequency of 5–12 seizures in 24 h, Supplemental Fig. 1B) by EEG. For this study, we chose only chronic epileptic group rats for subsequent experiments.

2.2. RNA extraction and lncRNA chip array

Total RNA was extracted from the hippocampus using TRIzol® reagent (Thermo Fisher, USA). Total RNA was quantified by using the NanoDrop ND-2000 (Thermo Fisher, USA), and RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). Sample labelling, microarray hybridization and washing were performed based on the manufacturer's standard protocols (OEbiotech Corporation NO: OE2014510, Shanghai China). Briefly, total RNA was transcribed to double stranded cDNA, synthesized into cRNA and labelled with Cyanine-3-CTP. Labelled cRNAs were hybridized onto a microarray. After washing, the arrays were scanned by using the Agilent Scanner G2505C (Agilent Technologies). MiRNA chip data were obtained from our previous work [17].

2.3. Bioinformatics analysis

2.3.1. Screening for differentially expressed lncRNAs and mRNAs

Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyse array images to obtain raw data. GeneSpring was employed to complete the basic analysis of raw data. First, raw data were normalized using the quantile algorithm and were subsequently analysed using an unpaired t-test, with a p-value cut-off of 0.01 and a fold-change cut-off of 1.5.

2.3.2. Differentially expressed lncRNA and mRNA clustering analysis

Different lncRNAs and mRNAs were analysed using Cluster 3.0 software as previously described [18], and data were used to examine a series of parameters, such as log transformed data, normalized genes and arrays, and hierarchical parameters of genes and arrays. Results were further analysed using Tree View software. Green and yellow indicate low expression, while red indicates high expression.

2.3.3. LncRNA co-expression analysis and gene function annotation

Expression of different lncRNAs and mRNAs was analysed using Pearson's correlation coefficient. An absolute value of 0.8 was considered relevant, a value less than 0.8 represented a negative correlation, and a value greater than 0.8 represented a positive correlation. A p-value < 0.01 was considered statistically significant. Expression of genes encoding each differentially expressed lncRNA, ontology classification of co-expressed genes based on gene annotation, and summary information are available through DAVID (Database for Annotation, Visualization and Integrated Discovery). Predicted target genes were assigned to functional groups based on molecular function, biological processes and specific pathways. The lncRNA gene function was predicted based on the Gene Ontology (GO) functional annotation of co-expressed genes. Statistical function annotation generated additional GO terms, the most enriched of which might reflect potential lncRNA functions.

2.3.4. Putative miRNA target genes and GO analysis

MiRNA target genes were downloaded from the microrna.org database (http://www.microrna.org/microrna/getDownloads.do); we selected differentially expressed miRNAs and their target genes from the data. Target genes were put into the DAVID website for GO analysis as mentioned above. The relationships between miRNAs and their target genes were built using Cytoscape software.

2.3.5. The ceRNA relation analysis between lncRNAs and miRNAs

We used the StarBase v2.0 website (http://starbase.sysu.edu.cn/mrnaCeRNA.php) to download sequences of lncRNAs with dysregulated expression levels from the chip company and then searched for miRNAs that contained a ceRNA relationship with lncRNAs. Then, we selected only miRNAs from the chip that were abnormally expressed. The ceRNA relationship between lncRNAs and miRNAs was built using Cytoscape software.

2.4. Quantitative reverse transcription-polymerase chain reaction analysis

To confirm lncRNA and miRNA microarray results, real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to detect lncRNAs and miRNAs expression. Primers for RT-PCR to detect miRNA, lncRNA and mRNA were designed based on sequences provided by the Sanger Centre Registry and were synthesized and purified by BGI.tech Co. (Shenzhen, China, supplement 10). Total RNAs were isolated from TLE rat hippocampus tissues as described above. RT reactions were performed using the iScript cDNA synthesis kit (Clontech, US). Real-time PCR was performed on an ABI, Steponeplus Multicolor Real-Time PCR Detection System. U6 RNA and GAPDH were used as endogenous controls for miRNA, mRNA and lncRNA detection. The real-time PCR cycle was 98 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s A final melting curve analysis (60–95 °C) was included. A standard curve was produced with slopes at approximately -3.32 (~100% efficiency). Real-time PCR results were quantified using the $2^{\Delta\Delta ct}$ method against GAPDH or U6 for normalization. Data represent means from three experiments.

3. Results

3.1. MiRNA, lncRNA and mRNA expression profile in the rat model of TLE

To evaluate the lncRNA-mediated ceRNA network (LMCN), we constructed an LMCN using a multi-step approach (Supplement 2). First, lncRNAs and mRNA microarray analyses were performed on hippocampal tissues from pilocarpine-induced status epilepticus groups compared to normal control groups. After separating the signal from noise and performing a t-test, 54 lncRNAs and 122 mRNAs were identified as significantly differentially expressed (fold change > 1.5 and FDR < 0.01). Among these lncRNA molecules, information for 6 lncRNAs was obtained from the Ensembl gene database, information for 9 lncRNAs was obtained from the UCSC gene database, and information for the other 39 lncRNAs was obtained from RNA sequencing results of the chip company. Then, lncRNA and mRNA expression data were clustered using Cluster 3.0 with dendrogram-based methods for clustering. Samples were further separated into two subgroups through hierarchical clustering based on similar expression patterns. Thus, expression of these lncRNAs and mRNAs potentially distinguished the epilepsy group from the control group (Fig. 1 and Supplement 3). Fourteen lncRNAs exhibited upregulated expression, and 40 lncRNAs exhibited downregulated expression (Fig. 1A). In contrast, in the two groups, 85 mRNAs were upregulated, and 37 mRNAs were downregulated (Fig. 1B). Notably, miRNA expression profiles were obtained from our previous work on miRNA array chips. After separating the signal from noise and performing a non-parametric test, 36 significantly differentially expressed miRNAs were observed in rat hippocampus (fold change > 1.5, and FDR < 0.01). Of these, 10 miRNAs were upregulated, and 26 miRNAs were downregulated (Fig. 1C).

3.2. LncRNA functional enrichment analysis

Based on the “guilt by association” strategy for characterizing lncRNA function, results of lncRNA and mRNA chip analyses were subjected to Pearson’s correlation coefficient analysis to further explore the function of lncRNAs in epilepsy. A Pearson’s correlation coefficient with p > 0.8 was considered as co-expression (Supplement 4). Each lncRNA’s function in epilepsy was annotated by its co-expressed genes. The lncRNA TCONS_00129582 was downregulated most significantly, showing a representative result. One hundred six genes (e.g., *KCTD8, KCTD19, KCNE1, KCTD18*) were related to lncRNA TCONS_00129582 and exhibited P > 0.8 (Supplement 5). Enrichment analysis showed significant modulation of 66 Gene Ontology (GO) terms (Fig. 2A and B, Supplement 6). The most significantly modulated GO terms were related to “potassium channel activity” (p = 3.28E-04), “metal ion transmembrane transporter activity” (p = 6.51E-04), and “voltage-gated potassium channel activity” (p = 8.93E-04). Most of these significantly modulated GO terms were related to ion activity, complex and transport, implying that lncRNA TCONS_00129582 may play a critical role in ion channel activity.

Furthermore, all other lncRNA co-expressed genes were also analysed by DAVID GO, and pathways similar to the lncRNA TCONS_00129582 were identified. Among the 20 most significantly modulated GO terms, we observed the following: “structural constituent of myelin sheath” (p = 4.84E-05), “extracellular region” (p = 4.96E-05), “MHC class I protein complex” (p = 1.16E-04), and “ion homeostasis” (p = 3.98E-04). These GO terms had the lowest p-values (Fig. 2C and Supplement 6). The GO term “ion binding” exhibited the most enrichment (count = 66) and included the genes *STAT5A, GATA2, CYP2J10, GABRR2, ALOX12, ADAMTS4, KCNE1, KCNJ5, MAPK12,* and *AOX1*. The molecular functions of these genes are reportedly related to the epileptic pathway.

3.3. MiRNA functional enrichment analysis

Putative target genes of 36 differentially expressed miRNAs were explored with online algorithms for their target prediction using microrna.org. Several thousand target genes were predicted for the 36 differentially expressed miRNAs. We merged the selected miRNA target genes that were differentially expressed in the mRNA microarray chips (p < 0.01 and fold change > 1.5) for further analysis. Then, we compared the gene expression profiles for mRNA and miRNA. We found that 10 miRNAs were dysregulated, including miR-124-5p, miR-203a-3p, miR-211-5p, miR-214-3p, miR-301a-3p, miR-34c-5p, miR-369-3p, miR-494-3p, miR-7a-5p and miR-7b, and their target genes were also inversely expressed in epilepsy, including *Kctd8, Kctd18, Zkscan1, Klhl4, RGD1359508, Lpar1, Akr7a3, Agmo, Fbxo48, Mtus1, Klf4, Olig3, Gulp1,* and *Apold1* (Supplement 7). This strategic method narrows the scope of miRNA target genes and will aid with future research on miRNAs (Fig. 3A).

Statistical enrichment tests of target genes were performed for GO, and pathway annotations are provided in Fig. 3B and Supplement 8. Enrichment and p-value analysis showed significant modulation of GO annotations in the direct target genes of miRNAs. We observed that “GO: 0005886~plasma membrane” (p = 7.56E-03), “GO: 0008076~voltage-gated potassium channel complex” (p = 3.64E-02), and “GO: 0034705~potassium channel complex” (p = 3.64E-02) were among the most significantly modulated GO terms. Of these, 10 GO terms were associated with the voltage-gated potassium channel complex or ion transport. This finding may imply that these differentially expressed miRNAs negatively regulate their target genes. These target genes play a significant role in ion transport, which mediates axon guidance.

3.4. LncRNA-miRNA-mRNA ceRNA network

According to the ceRNA hypothesis, mRNAs and lncRNAs “talk” to each other using microRNA response elements (MREs) as letters of a new language [13]. We explored the relationship between lncRNAs and miRNA ceRNAs as described in the methods section (Supplement 9). Eighteen lncRNAs and 17 miRNAs comprised the ceRNA relationship (Table 1). The lncRNA TCONS_00006847 had the largest number of miRNAs (miR-7b/ miR-153-5p/ miR-301a-5p/ miR-7a-5p), containing the MREs in its ORF length. The lncRNAs TCONS_00016070 and ENSRNOT00000075021 contained 3 miRNAs, but TCONS_00016070 contained 5 MREs (AAACATT) with miR-33-3p. However, according to the StarBase v2.0 web site score, TCONS_00016070 was not the highest scoring; miR-124 had 2 MREs with lncRNA ENSRNOT00000075021, making it the highest scoring among these molecules. The remaining lncRNAs only contained 1 miRNA MRE, except for ENSRNOT00000038157, which contained 2 miRNAs. These data were

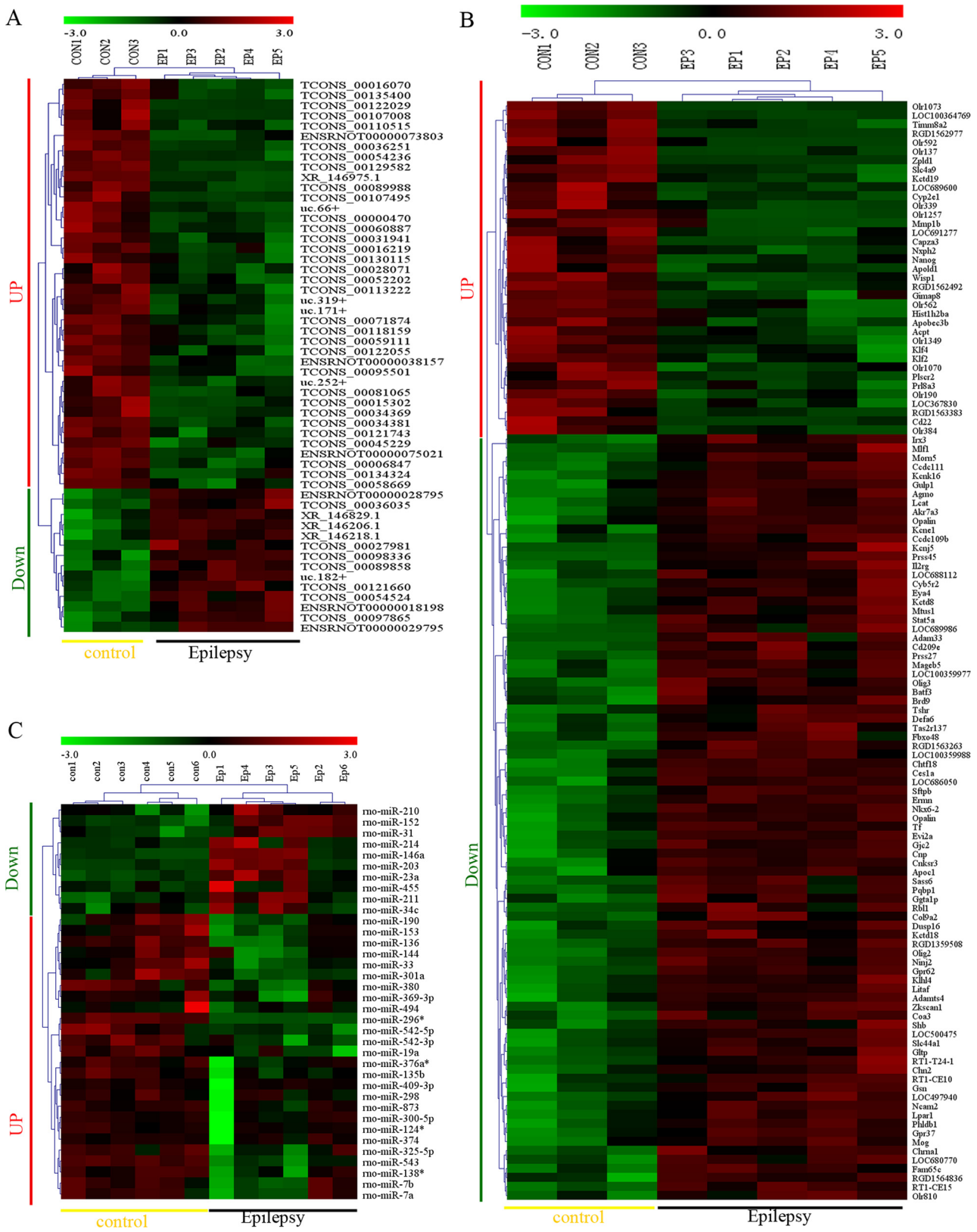

Fig. 1. Differentially expressed lncRNAs, mRNAs and miRNA cluster heatmaps in epilepsy.
A: Differentially expressed lncRNA cluster heatmap in epilepsy and control groups. B: Differentially expressed mRNA cluster heatmap in epilepsy and control groups. C: Differentially expressed miRNA cluster heatmap in epilepsy and control groups. Con1-6 represents the control group, and EP1-6 represents the epilepsy group. Probes are in rows; samples are in columns. Red colour indicates upregulated expression, and green colour indicates downregulated expression. Both upregulated and downregulated lncRNAs are observed in epilepsy compared to normal controls.

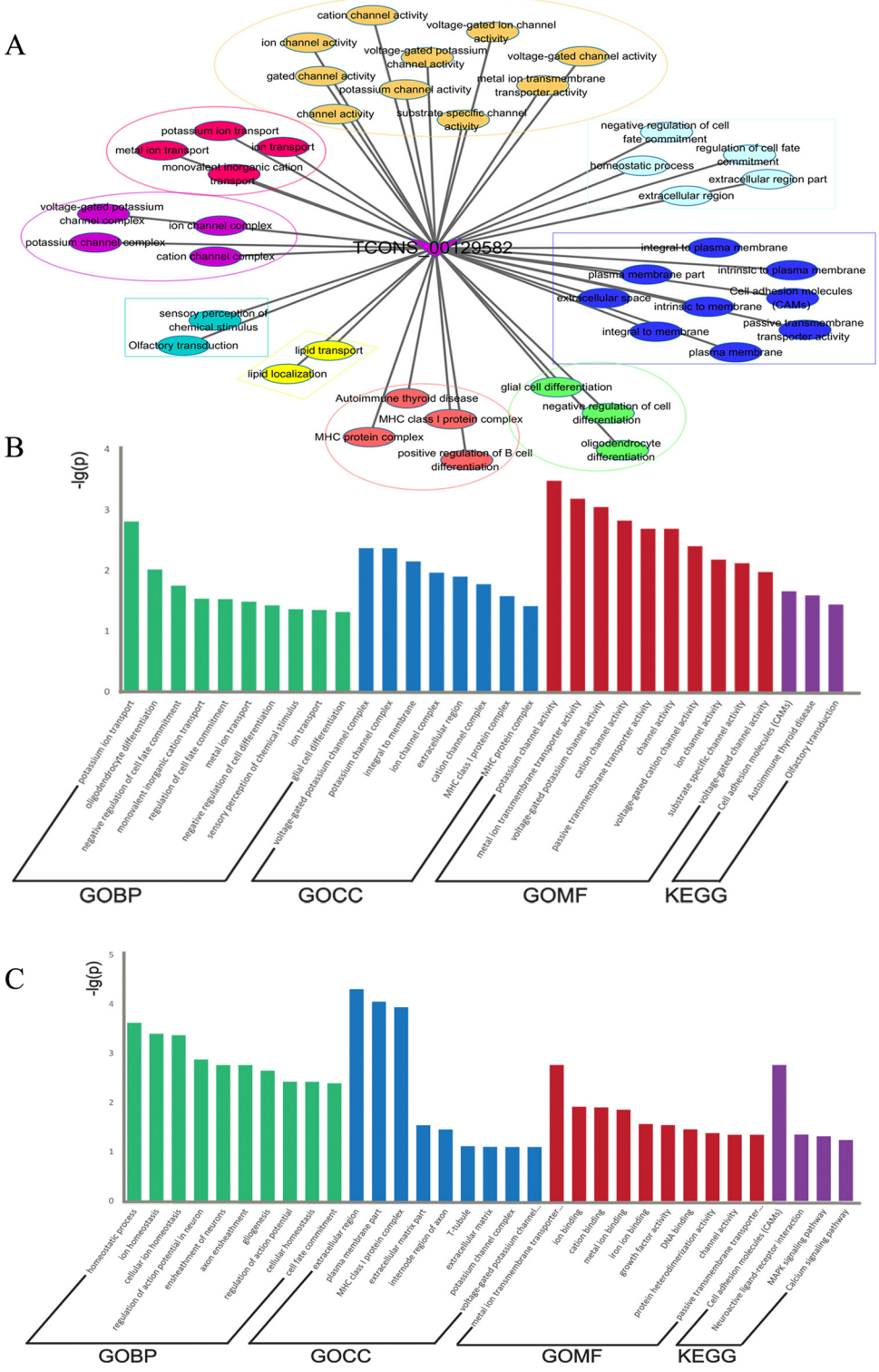

Fig. 2. GO analysis of differentially expressed lncRNA co-expressed genes.
A: The lncRNA TCONS_00129582 co-expressed genes GO cluster; 10 saffron yellow ellipse shapes represent the channel activity groups, 4 purple ellipse shapes represent the channel complex, 4 red ellipse shapes represent the ion transport, 9 blue ellipse shapes represent the ion transport, 9 blue ellipse shapes represent the membranes, 4 ellipse shapes represent the immunity complex, 3 green ellipse shapes represent cell differentiation, two light yellow ellipse shapes are associated with lipid localization and transport, and 2 blue-green ellipse shapes are associated with sensory perception and olfactory transduction. B: The top 10 p-values of lncRNA TCONS_00129582 co-expressed genes using GO analysis; C: The top 10 p-values of differentially expressed lncRNAs co-expressed genes using GO analysis.

then merged with the dysregulated miRNA array chip data. The relationship between the lncRNAs and miRNAs is shown in Fig. 4A. Depending on the miRNAs, target gene expression can be regulated (Fig. 3A) according to the ceRNA relationship and their MREs (Table 1). LncRNA-miRNA-mRNA ceRNA relationships were constructed among the 13 mRNAs, 10 miRNAs and 11 lncRNAs in epilepsy (Supplement 10). As shown in Fig. 4B, miR-124 not only downregulated levels of its target genes *kctd8* and *Zkscan1* but also affected expression of the lncRNAs TCONS_00130115 and ENSRNOT00000075021 through its MRE (TGAACAC). According to the ceRNA hypothesis, TCONS_00130115 and ENSRNOT00000075021 regulate expression of *kctd8* and *Zkscan1* by miR-124. This entire network provides new insight for future research on the relationship between lncRNAs and miRNAs in epilepsy.

3.5. Validation of the expression of disrupted molecules

To confirm our microarray data results, 4 lncRNAs (ENSRNOT00000028795, TCONS_00129582, TCONS_00122055, and TCONS_00006847), 4 miRNAs (miR-211-5p, miR-7a-5p, miR-34c, and

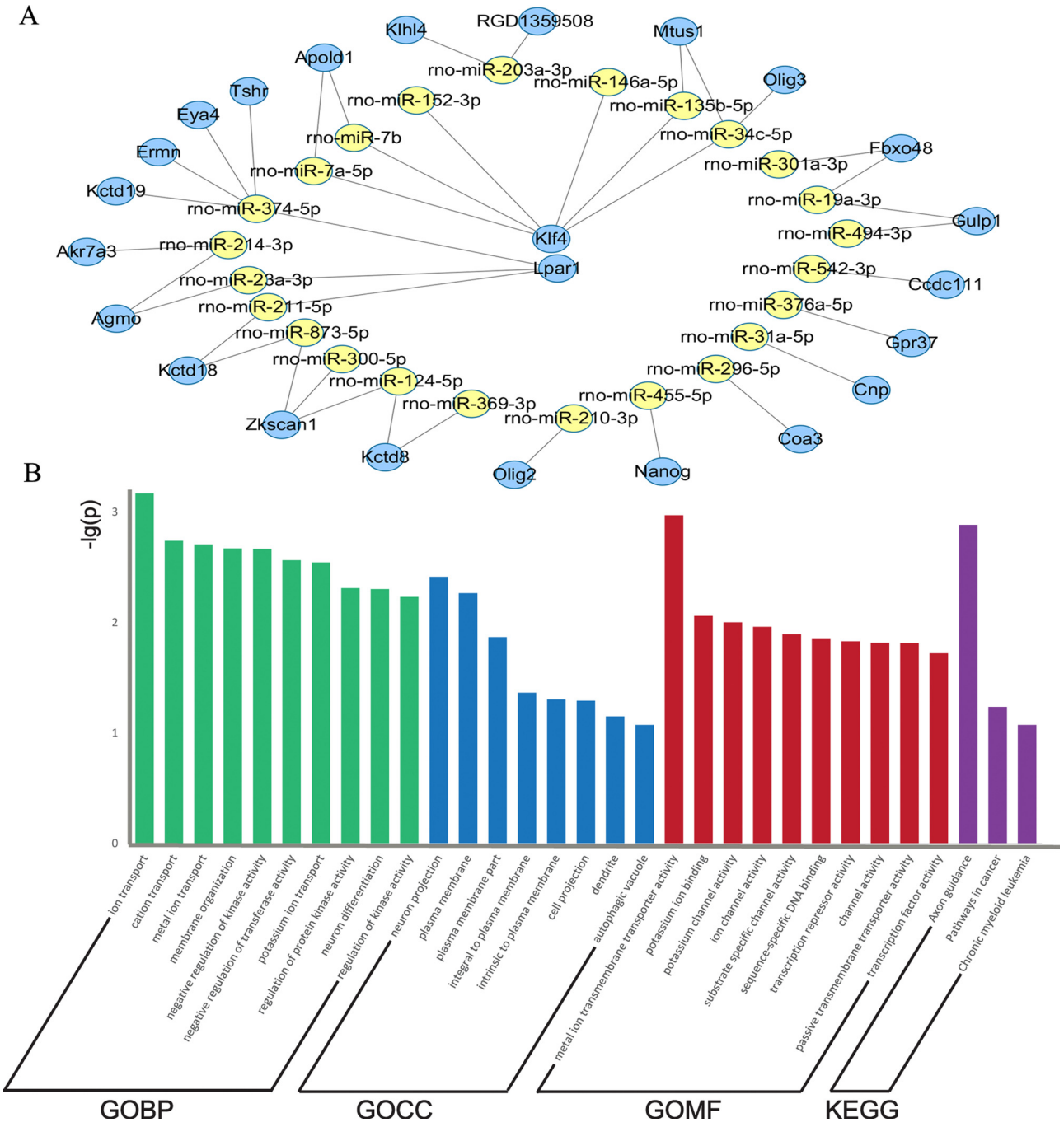

Fig. 3. MiRNAs and their target genes in the epilepsy network.
A: The simplified and matched network between miRNAs and their target genes. The ellipse shape represents miRNAs that were differentially expressed in the epilepsy chip, and the hexagon shape represents the target gene. B: The top 10 p-values of miRNA target genes using GO analysis.

miR-124-5p) and 4 target genes (*Kctd8, Mtus1, Apold1,* and *Klf4*) were randomly selected for investigation using real-time PCR. Specifically, real-time PCR was performed in 3 epilepsy hippocampal neuron cases and 3 normal hippocampal neuron tissue cases to verify the microarray results. The real-time PCR results were consistent with microarray results (Fig. 5).

4. Discussion

The study of epilepsy-associated genomes has rapidly expanded in recent years [19]. It has been reported that abnormal gene expression in epilepsy plays a key role in neural network reconstruction, synaptic transmission, and multidrug resistance. However, expression of lncRNAs in epilepsy remains unknown. Herein, we found that subgroups of lncRNAs were abnormally expressed in epilepsy. In this group, 14 lncRNAs were upregulated and 40 were downregulated, 85 mRNAs were upregulated and 37 were downregulated, and 10 miRNAs were upregulated and 26 were downregulated (Fig. 1 and Supplement 3). This outcome implies that these lncRNAs, miRNAs and mRNAs may represent potential biomarkers in epilepsy. Notably, out of the 54 disrupted lncRNAs from the chips, only 15 had been previously reported but not in epilepsy. These newly discovered dysregulated lncRNAs provide guidance for further research.

Thus far, the functions of lncRNAs remain obscure. Based on the

Table 1
The ceRNA information between the differentially expressed lncRNAs and miRNAs in TLE.

Primary Accession	Score	miRNA	miRNA sequence	Seed sequence	Binding position in lncRNAs
TCONS_00122055	63	miR-146a-3p	ACCUGUGAAGUUCAGUUCUUU	**T CACAGG**	1480
TCONS_00016219	56	miR-211-5p	UUCCCUUUGUCAUCCUUUGCCU	**AAAGGG A**	805, 1028
TCONS_00122055	84	miR-211-5p	UUCCCUUUGUCAUCCUUUGCCU	**A AAGGGA**	2330, 5292
TCONS_00028071	52	miR-203a-3p	GUGAAAUGUUUAGGACCACUAG	**CATTTCA**	223
TCONS_00122055	64	miR-203a-5p	AGUGGUUCUUAACAGUUCAAC	**G AACCAC**	1290, 4932
TCONS_00121660	60	miR-203b-5p	AGUGGUCCUAAACAUUUCAC	**GGACCAC**	524
ENSRNOT00000028795	65	miR-214-3p	ACAGCAGGCACAGACAGGCAG	**CCTGCTG**	273, 442
ENSRNOT00000038157	57	miR-214-5p	AGAGUUGUCAUGUGUCU	**ACAA CTC**	1267
ENSRNOT00000038157	57	miR-214-3p	ACAGCAGGCACAGACAGGCAG	**CCTGCT G**	525, 773, 1216
TCONS_00122055	78	miR-214-5p	AGAGUUGUCAUGUGUCU	**ACAACTC**	863
TCONS_00118159	56	miR-214-5p	AGAGUUGUCAUGUGUCU	**ACAACTC**	186
TCONS_00122055	60	miR-34c-5p	AGGCAGUGUAGUUAGCUGAUUGC	**CACTGCC**	4824
TCONS_00016070	70	miR-33-3p	CAAUGUUUCCACAGUGCAUCA	**AAAC ATT**	317, 1290, 2841, 3381, 4659
TCONS_00110515	79	miR-33-5p	GUGCAUUGUAGUUGCAUUGCA	**CAATGCA**	24
TCONS_00016070	59	miR-873-3p	GAGACUGACAAGUUCCCGGGA	**TCAGTCT**	103, 602, 1112
TCONS_00130115	58	miR-124-5p	CGUGUUCACAGCGGACCUUGAU	**TG AACAC**	29
ENSRNOT00000075021	80	miR-124-3p	UAAGGCACGCGGUGAAUGCC	**GTGCCTT**	481, 760
TCONS_00045229	64	miR-135b-3p	AUGUAGGGCUAAAAGCCAUGGG	**CCCTACA**	191
TCONS_00031941	63	miR-369-3p	AAUAAUACAUGGUUGAUCUUU	**GTATTAT**	884
TCONS_00006847	71	miR-7b	UGGAAGACUUGUGAUUUUGUUGU	**GTCTTCC**	84
TCONS_00095501	52	miR-298-5p	GGCAGAGGAGGGCUGUUCUUCCC	**CCTCTGC**	577, 2843, 4391
TCONS_00036035	51	miR-153-3p	UUGCAUAGUCACAAAAGUGAUC	**CTATG CA**	1536
TCONS_00006847	65	miR-153-5p	GUCAUUUUUGUGAUGUUGCAGCU	**AAAATG A**	325, 1065, 1489, 1736
TCONS_00060887	78	miR-374-3p	CUUAGCACGUUGUAUUAUUAUU	**GTGCTAA**	1794
ENSRNOT00000075021	59	miR-374-3p	CUUAGCACGUUGUAUUAUUAUU	**GTGCTAA**	859
TCONS_00006847	70	miR-301a-5p	GCUCUGACUAGGUUGCACUACU	**GTCAGAG**	3192
ENSRNOT00000075021	53	miR-301a-3p	CAGUGCAAUAGUAUUGUCAAAGC	**TTGCACT**	231
TCONS_00052202	59	miR-494-3p	UGAAACAUACACGGGAAACCUCU	**ATGT TTC**	1147, 1639
TCONS_00016070	57	miR-7a-2-3p	CAACAAGUCCCAGUCUGCCACA	**ACTTGTT**	3101, 3675
TCONS_00006847	71	miR-7a-5p	UGGAAGACUUGUGAUUUUGUUGU	**GTCTTCC**	84

"guilt by association" strategy, each lncRNA co-expressed gene was screened by Pearson's correlation coefficient if $P > 0.8$, and the function of the lncRNA may be interpreted by the co-expressed gene GO term and pathway. Our data (Fig. 2A, and B) demonstrate that the lncRNA TCONS_00129582 is associated with the activity of voltage-gated ion channels, including potassium channels, metal ion transmembrane transporters, and voltage-gated potassium channels. Neuronal voltage-gated ion channels and ligand-gated synaptic receptors play a critical role in maintaining the delicate balance within the neuronal networks in the brain. In particular, hyper-polarization activates cyclic nucleotide-gated (HCN) sodium channels, calcium channels, and ligand-gated synaptic receptors, such as *GABAB* receptors (*GABABRs*), which are considered promising drug targets for the treatment of mental health disorders. *GABABRs* are obligate heteromers of principal *GABAB1* and *GABAB2* subunits [20]. *GABABRs* can additionally associate with auxiliary *KCTD8*, 12, 12b and 16 subunits, and they bind G-protein and differentially regulate G-protein signaling [20]. These auxiliary subunits constitute receptor subtypes with distinct functional properties. *KCTDs* are modular proteins comprising a T1 tetramerization domain that binds to *GABA(B2)* and an H1 homology domain. *KCTD8* and -16 contain additional C-terminal H1 domains [21]. In this study, we discovered that *KCTD8, KCTD18* and *KCTD19* genes are dysregulated in epilepsy, and these genes are associated with 15 lncRNAs (Supplement 4) or 8 microRNAs (Supplement 7), such as ENSRNOT00000028795, ENSRNOT00000073803, TCONS_00016070, TCONS_00016219, TCONS_00129582, miR-135, miR-144, and miR-31, among others. Other potassium channel molecules (*KCNJ5, KCNK16, KCNE1, CHRNA1*) were also discovered in this study [22], which were all molecular associates of epilepsy. In particular, Barro et al. [22] reported that *KCNQ1* channels are highly dependent on associated KCNE-beta subunits. *KCNQ1/KCNE1* channel activation occurs in two steps: first, mutually independent voltage sensor movements in the four *KCNQ1* subunits generate the main gating charge movement and underlie the initial delay in the activation time course of *KCNQ1/KCNE1* currents. Second, slower and concerted conformational changes of all four voltage sensors and gates may open the *KCNQ1/KCNE1* channel. The results obtained in the present study suggest that lncRNA TCONS_00129582 plays an important role in the voltage-gated potassium channel activity pathogenesis of epilepsy.

Epileptogenesis is a complex dynamic biological process that involves multiple steps of genetic and regulatory alterations. However, our understanding of this complex network intertwined by coding genes and lncRNAs in epilepsy biology is still at an early stage. We found that either aberrant lncRNAs or miRNAs perturb specific common pathways, such as ion homeostasis and potassium channel activity pathways, indicating that lncRNAs mediate the dysregulation of these pathways in a coordinated manner. In the dysregulated lncRNA co-expressed gene GO and KEGG pathway, we revealed that "MHC class I protein complex" and "antigen processing and presentation of peptide antigen via MHC class I" were related to immunity. Over the past 10 years, an increasing body of clinical and experimental evidence has provided strong support for the hypothesis that inflammatory processes within the brain might constitute a common and crucial mechanism in the pathophysiology of seizures and epilepsy [23]. Inflammation is characterized by the production of an array of inflammatory mediators, from tissue-resident cells to blood-circulating immunocompetent cells, and involves the activation of innate and adaptive immunity. Both innate and adaptive immunity have been implicated in epilepsy [24], and microglia, astrocytes and neurons are believed to contribute to the innate immunity-type processes that cause brain inflammation. *GABA* regulates the inhibition of the T-lymphocyte response and macrophage production of *IL-6* and *IL-12* [25]. In this paper, we observed that RT1-CE families and IL2RG are dysregulated in epilepsy, and these genes are associated with the MHC class I protein complex and inflammation. Roos [26] and Naper [27] et al. reported that RT1-CE families are a region of the rat major histocompatibility complex and are associated with the Ly49 family of lectin-like receptors. These MHC class I protein complexes and inflammatory genes are co-expressed with the 16 lncRNAs (ENSRNOT00000028795, ENSRNOT00000073803, TCONS_00129582, etc., Supplement 4–6).

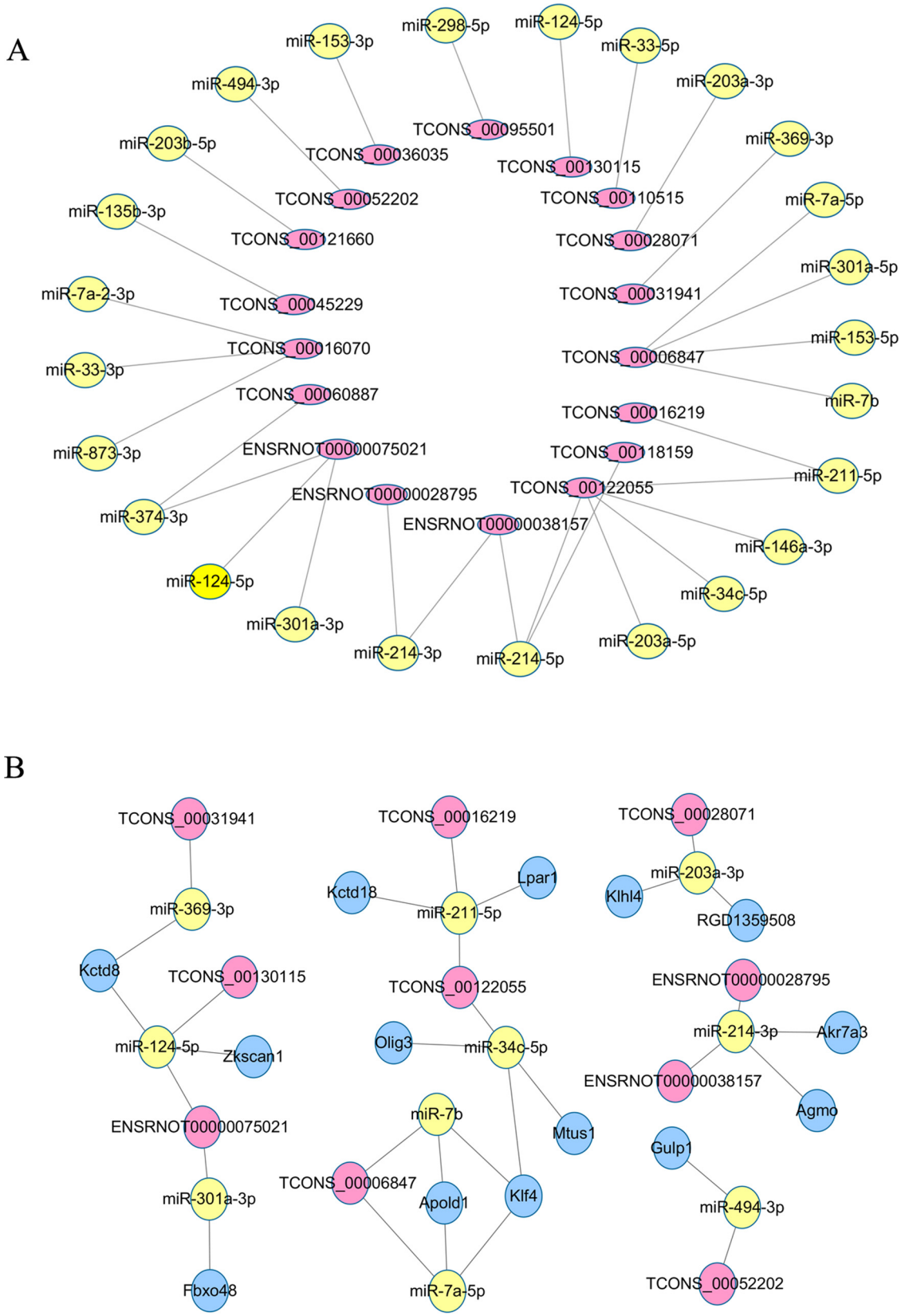

Fig. 4. LncRNA-miRNA-mRNA ceRNA regulation core network map in three groups. A: LncRNA-miRNA-mRNA regulation network map of the disturbed expression of lncRNAs in epilepsy; B: Core lncRNA-miRNA-mRNA ceRNA regulation network map.

Molecular regulation through lncRNAs remains unknown because the functions of lncRNAs vary [28]. Indeed, lncRNAs have been identified to have a variety of methods, and the number of lncRNAs is increasing. Their function also varies in any pattern, including roles in imprinting [29], enhancer function [30], X chromosome inactivation [31], chromatin structure [32] and genomic rearrangements during the

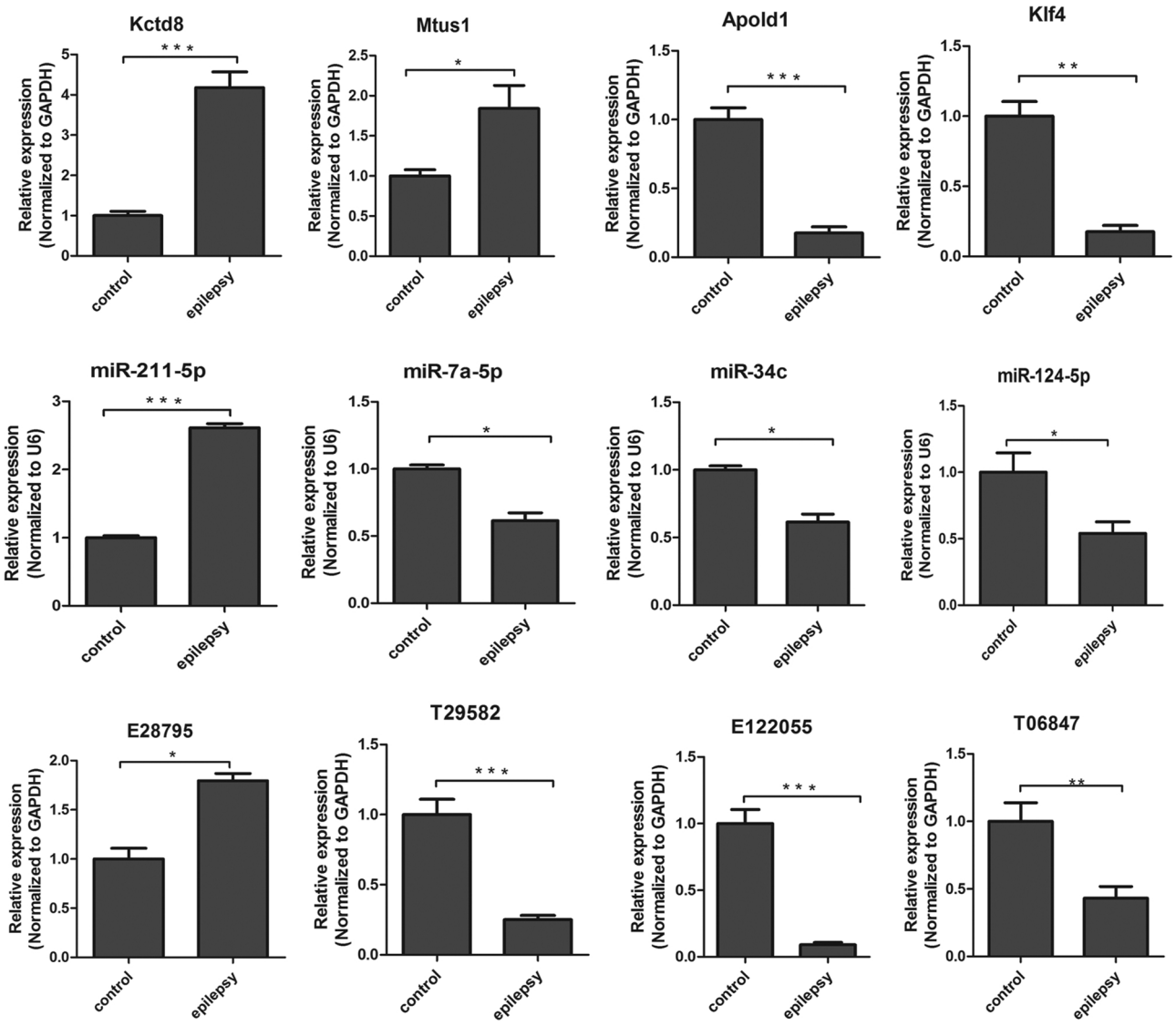

Fig. 5. Validation of differentially expressed lncRNAs, miRNAs and mRNAs in epilepsy. * P < 0.05, ** P < 0.01, ***P < 0.001.

generation of antibody diversity [33]. CeRNAs have been identified in many diseases [34], indicating a new and intricate regulatory network. Tay illuminated the multi-layered complexity of ceRNA crosstalk and competition [35]. Competition between various molecular species to bind to a specific molecular target has been described in multiple contexts and includes DNA–protein, RNA–protein, RNA–RNA and protein–protein crosstalk. Herein, we utilized ceRNA regulatory mechanisms to obtain further information for dysregulated lncRNAs and miRNAs. In the present study, the MRE site of each miRNA in the body of lncRNAs was explored, and dysregulated miRNAs were selected (Supplement 9). The outstanding lncRNAs are shown in Fig. 4A. There were 17 lncRNAs and 18 miRNAs comprising the network. For example, lncRNA TCONS_00016070 contained 5 MRE sites (AAAC ATT) that were the seed sequence of miR-33-3p; this lncRNA was also connected to miR-124-5p, which not only regulates the target genes *Kctd8* and *Zkscan1* but also regulates the lncRNA TCONS_00130115 and ENSRNOT00000075021. These three elements may affect other expression levels through the MRE site. Aronica et al. [36] reported that miR-146a in reactive astrocytes suggest the possible involvement of miRNAs in the modulation of the astroglial inflammatory responses in TLE. Brennan et al. [37] found that miR-124 attenuates epileptogenesis via NRSF while promoting epilepsy via inflammation. In this work, we also identified miR-124 and miR-146 dysregulation in a rat model of TLE. Furthermore, we analysed miR-124 and miR-146 potential target genes associated with lncRNA. Thus, the ceRNA analysis provides another method to interpret lncRNA function and biological processes in the pathogenesis of epilepsy.

5. Conclusion

This paper provides new evidence of non-coding RNAs implicated in the pathogenesis of epilepsy and may facilitate the development of new therapeutics and prognostic markers in epilepsy.

Conflict of interests

There are no competing interests to be declared.

Ethics approval and consent to participate

All procedures followed were in accordance with the ethical standards of the responsible committee on animal and human experimentation (the Ethics Review Committee of Xiangya Hospital, number: 201412438) and with the Helsinki Declaration of 1964 and later versions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.seizure.2019.04.010.

References

[1] Rossignol E, et al. WONOEP appraisal: new genetic approaches to study epilepsy. Epilepsia 2014;55(8):1170–86.
[2] Pefanis E, et al. RNA exosome-regulated long non-coding RNA transcription controls super-enhancer activity. Cell 2015;161(4):774–89.
[3] Qureshi IA, Mattick JS, Mehler MF. Long non-coding RNAs in nervous system function and disease. Brain Res 2010;1338:20–35.
[4] Martens JA, Laprade L, Winston F. Intergenic transcription is required to repress the Saccharomyces cerevisiae SER3 gene. Nature 2004;429(6991):571–4.
[5] Kia SK, et al. SWI/SNF mediates polycomb eviction and epigenetic reprogramming of the INK4b-ARF-INK4a locus. Mol Cell Biol 2008;28(10):3457–64.
[6] Lee ES, et al. The consensus 5' splice site motif inhibits mRNA nuclear export. PLoS One 2015;10(3). p. e0122743.
[7] Li CH, Chen Y. Targeting long non-coding RNAs in cancers: progress and prospects. Int J Biochem Cell Biol 2013;45(8):1895–910.
[8] Guo X, et al. Long non-coding RNAs function annotation: a global prediction method based on bi-colored networks. Nucleic Acids Res 2013;41(2). p. e35.
[9] Guo Q, et al. Comprehensive analysis of lncRNA-mRNA co-expression patterns identifies immune-associated lncRNA biomarkers in ovarian cancer malignant progression. Sci Rep 2015;5. p. 17683.
[10] Salmena L, et al. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell 2011;146(3):353–8.
[11] Tay Y, et al. Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. Cell 2011;147(2):344–57.
[12] Kumar MS, et al. HMGA2 functions as a competing endogenous RNA to promote lung cancer progression. Nature 2014;505(7482):212–7.
[13] Cesana M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 2011;147(2):358–69.
[14] Cao Y, et al. Identification of prognostic biomarkers in glioblastoma using a long non-coding RNA-mediated, competitive endogenous RNA network. Oncotarget 2016;7(27):41737–47.
[15] Xiao W, et al. Genome-wide DNA methylation patterns analysis of noncoding RNAs in temporal lobe epilepsy patients. Mol Neurobiol 2018;55(1):793–803.
[16] Long HY, et al. Blood DNA methylation pattern is altered in mesial temporal lobe epilepsy. Sci Rep 2017;7. p. 43810.
[17] Hu K, et al. MicroRNA expression profile of the hippocampus in a rat model of temporal lobe epilepsy and miR-34a-targeted neuroprotection against hippocampal neurone cell apoptosis post-status epilepticus. BMC Neurosci 2012;13. p. 115.
[18] Luo Z, et al. Systems biology of myasthenia gravis, integration of aberrant lncRNA and mRNA expression changes. BMC Med Genomics 2015;8. p. 13.
[19] Noebels J. Pathway-driven discovery of epilepsy genes. Nat Neurosci 2015;18(3):344–50.
[20] Rajalu M, et al. Pharmacological characterization of GABAB receptor subtypes assembled with auxiliary KCTD subunits. Neuropharmacology 2015;88:145–54.
[21] Seddik R, et al. Opposite effects of KCTD subunit domains on GABA(B) receptor-mediated desensitization. J Biol Chem 2012;287(47):39869–77.
[22] Barro-Soria R, et al. KCNE1 divides the voltage sensor movement in KCNQ1/KCNE1 channels into two steps. Nat Commun 2014;5. p. 3750.
[23] Pollak TA, et al. Epilepsy-related psychosis: a role for autoimmunity? Epilepsy Behav 2014;36:33–8.
[24] Vezzani A, et al. The role of inflammation in epilepsy. Nat Rev Neurol 2011;7(1):31–40.
[25] Prud'homme GJ, et al. GABA protects human islet cells against the deleterious effects of immunosuppressive drugs and exerts immunoinhibitory effects alone. Transplantation 2013;96(7):616–23.
[26] Roos C, Walter L. Considerable haplotypic diversity in the RT1-CE class I gene region of the rat major histocompatibility complex. Immunogenetics 2005;56(10):773–7.
[27] Naper C, et al. Two structurally related rat Ly49 receptors with opposing functions (Ly49 stimulatory receptor 5 and Ly49 inhibitory receptor 5) recognize nonclassical MHC class Ib-encoded target ligands. J Immunol 2005;174(5):2702–11.
[28] Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature 2012;482(7385):339–46.
[29] Nagano T, Fraser P. Emerging similarities in epigenetic gene silencing by long noncoding RNAs. Mamm Genome 2009;20(9-10):557–62.
[30] Kim A, et al. Beta-globin intergenic transcription and histone acetylation dependent on an enhancer. Mol Cell Biol 2007;27(8):2980–6.
[31] Lee JT. Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome. Genes Dev 2009;23(16):1831–42.
[32] Rinn JL, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007;129(7):1311–23.
[33] Krangel MS. T cell development: better living through chromatin. Nat Immunol 2007;8(7):687–94.
[34] Sanchez-Mejias A, Tay Y. Competing endogenous RNA networks: tying the essential knots for cancer biology and therapeutics. J Hematol Oncol 2015;8. p. 30.
[35] Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. Nature 2014;505(7483):344–52.
[36] Aronica E, et al. Expression pattern of miR-146a, an inflammation-associated microRNA, in experimental and human temporal lobe epilepsy. Eur J Neurosci 2010;31(6):1100–7.
[37] Brennan GP, et al. Dual and opposing roles of MicroRNA-124 in epilepsy are mediated through inflammatory and NRSF-Dependent gene networks. Cell Rep 2016;14(10):2402–12.