Circular RNAs regulate its parental genes transcription in the AD mouse model using two methods of library construction

Nana Ma1 | Changrui Tie1 | Bo Yu2,3 | Wei Zhang1,4 | Jun Wan1,4

1Shenzhen Key Laboratory for Neuronal Structural Biology, Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, Shenzhen, China
2Shenzhen Key Laboratory for Translational Medicine of Dermatology, Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, Shenzhen, China
3Department of Dermatology, Peking University Shenzhen Hospital, Shenzhen, China
4Greater Bay Biomedical Innocenter, Shenzhen Bay Laboratory, Shenzhen, China

Abstract
Circular RNA (circRNA) is an important class of noncoding RNA. Current, protocols to detected circRNAs have utilized nonpolyadenylated RNAs, ribosomal RNA-depleted RNA samples (rRNA− library), and rRNA-depleted RNA that has been treated with RNase R to digest linear RNA (rRNA− RNase R+ library). Accumulating evidence suggests the participation of circRNAs in Alzheimer’s disease (AD)-associated pathophysiology, but the details remain largely unknown. Here, we elucidated the brain circRNAs profiles of AD-model and WT mice using two methods of library construction (rRNA− RNase R+ libraries and rRNA− libraries). We focused on the construction of libraries that best allow the identification of circRNAs from next-generation RNA sequencing data. We obtained a significantly higher abundance of circRNAs in the rRNA− RNase R+ libraries than in the rRNA− libraries. Additionally, the rRNA− RNase R+ libraries more clearly revealed differentially expressed circRNAs. We performed a correlation analysis between differentially expressed circRNAs and their parental genes and performed KEGG analysis of the parental genes to explore the role of circRNA in AD. Our results identified significantly dysregulated circRNAs and KEGG analysis revealed that the identified circRNAs are involved in regulating AD development from distinct origins, including cAMP signaling, MAPK signaling, insulin secretion, Axon guidance, Long-term potentiation, dopaminergic synapse, and ErBb signaling pathways. Following rigorous selection, we identified several important circRNAs and mRNAs and propose the circRNAs regulate parental gene transcription or affect variable splicing, which

Abbreviations: AD, Alzheimer’s disease; APP/PS1, (APPswe, PSEN1dE9); circRNA, circular RNA; GABA, gamma aminobutyric acid; KEGG, Kyoto encyclopedia of genes and genomes; NGS, high-throughput next-generation sequencing; Pol II, RNA polymerase II; RNA-seq, RNA-sequencing; rRNA, ribosomal RNA; RNase R, ribonuclease R; UTR, untranslated region; WT, wild-type.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020 The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.
were discovered to be mainly involved in Aβ production and cognitive performance (TRPC6) and neuronal differentiation and development (NME7). These newly identified circRNAs should serve as a valuable resource for the future development of potential biomarkers and therapeutic targets for AD.

**KEYWORDS**
Alzheimer’s disease (AD), APP/PS1 mouse, circRNA, library construction, RNA-sequencing

## 1 | INTRODUCTION

Circular RNAs (circRNAs) are a recently discovered kind of noncoding RNA\(^1\); are abundant,\(^2\) stable,\(^3\) and ubiquitous in diverse eukaryotic organisms\(^4\); and are highly conserved across evolution.\(^5\) CircRNAs are RNA molecules in which an upstream 50 splice site is coaxially linked to a downstream splice site to form a circle (in a process known as back-splicing),\(^5\) and the closed-loop structure is more resistant to degradation than linear structure.\(^6\) CircRNAs usually lack poly(A) tails and may contain complicated structures with one or multiple exons, as well as introns, intergenic, and UTR sequences.\(^5,7\) RNA-sequencing (RNA-Seq technology has allowed the identification and validation of more and more circRNAs, and accumulated studies have reported their involvement in many physiological processes\(^1,4\) and association with various diseases.\(^8-11\) The functions of some circRNAs have been described, including suppressing the activity of specific microRNAs\(^12\) and upregulating/downregulating the expression levels of specific genes.\(^13\) Recently, several studies have shown the potential effects of circRNAs on multiple translation processes.\(^14,15\) Further explorations on circRNAs have suggested contributions to disease occurrence and development, with potential use in precision diagnosis and treatments.\(^16,17\)

CircRNAs are markedly enriched particularly in neurons and at synaptosomes.\(^18\) Interestingly, brain circRNAs are regulated during aging in both the fly and the mouse, which suggests potential roles of circRNA in aging-associated neurodegenerative diseases.\(^19,20\) For example, CDR1as (also known as CiRS-7, a circRNA sponge that binds to miR-7) and miR-7 are associated with nervous system development and disease. CDR1as dysfunction can result in miR-7 upregulation, which might lead to the downregulation of AD-related targets, including ubiquitin-protein ligase A.\(^21-23\) Another circRNA derived from SRY acts as a natural miRNA sponge to inhibit the activity of miR-138,\(^24\) which can affect learning and memory by regulating acyl protein thioesterase 1.\(^25,26\)

Alzheimer’s disease (AD) is the most common form of dementia and is associated with the progressive loss of memory, cognition, and behavioral capabilities.\(^27-30\) AD is a significant focus of research, but the molecular mechanisms underlying AD pathogenesis remain largely unknown,\(^31\) and there are currently no effective prognostic biomarkers. Intriguingly, numerous noncoding genes, such as those that encode circRNA, have potential roles in the initiation and progression of AD in humans.\(^27,32-35\) However, there has been no comprehensive analysis of circRNAs and their potential activities in AD.

The introduction of high-throughput next-generation sequencing (NGS) technology provides millions of short RNA sequence reads with single-nucleotide level accuracy, providing an accurate and robust method to measure the levels of circular RNAs. Novel protocols based on nonpolyadenylated RNAs, ribosomal RNA-depleted RNA (rRNA− library), and rRNA-depleted and RNase R-treated to digest linear RNA (rRNA− RNase R+ library) have improved detection of circRNAs, but the relative efficacy and accuracy of these methods to enrich for circRNA has not been assessed in AD.

Thus, in this study, we used RNA-seq of an rRNA− library and an rRNA− RNase R+ library, to examine circRNAs in the brain of AD-model mice (APP/PS1 mice) and wild-type (WT) control mice at the 6- and 9-month-old stages. This study is the first to identify circRNAs in the APP/PS1 mouse model of AD, and our data provide a useful resource to assess the use of these two kinds of RNA-seq libraries. It has been shown circRNAs might regulate gene transcription and alternative splicing (AS). Here, we performed a correlation analysis between differentially expressed circRNAs and their parental genes to explore the role of circRNA in AD. Our results identified several important circRNAs and mRNAs and propose the circRNAs regulate parental gene transcription or affect variable splicing, which were discovered to be mainly involved in Aβ production and cognitive performance (TRPC6) and neuronal differentiation and development (NME7). KEGG analysis revealed that the identified circRNAs are involved in regulating AD development from distinct origins, including cAMP signaling, MAPK signaling, insulin secretion, Axon guidance, Long-term potentiation, dopaminergic synapse, and ErBb signaling pathways. These newly identified circRNAs should serve as a valuable resource to facilitate the future development of AD therapeutic targets or novel diagnostics.

## 2 | MATERIALS AND METHODS

### 2.1 | Tissue preparation

WT and APP/PS1 mice [originally from The Jackson Laboratory; strain B6.Cg-Tg (APPSwe, PSEN1dE9) 85Dbo/
Mmjax[46]) were purchased from the Model Animal Research Center of Nanjing University. The mice were housed one per cage under standard specific conditions (25°C, 50% humidity, 12/12-hours light/dark cycle, and pathogen-free environment). The mice were provided free access to the standard diet until they met the age requirements (6 and 9 months), and then, three male mice were randomly selected from each group and cerebral cortex samples were collected for RNA-seq. All animal experiments were performed in accordance with animal use protocols approved by the Committee for the Ethics of Animal Experiments, Shenzhen Peking University, The Hong Kong University of Science and Technology Medical Center (SPHMC) (protocol number 2011-004).

### 2.2 RNA extraction and qualification

Total RNA from each sample was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and samples were separated on 1% of agarose gels to assess RNA degradation and contamination. RNA purity was measured using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA), RNA concentration was measured using a Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA), and RNA integrity was evaluated using the RNA Nano 6000 Assay Kit of a Bioanalyzer 2100 System (Agilent Technologies, CA, USA).

### 2.3 RNA-sequencing

A total amount of 5 μg RNA per sample was used as input material for the RNA sample preparations. Firstly, ribosomal RNA was removed by Epicentre Ribo-zero rRNA Removal Kit (Epicentre, USA), and rRNA free residue was cleaned up by ethanol precipitation (rRNA− library). Subsequently, the linear RNA was digested with 3 U of RNase R (Epicentre, USA) per μg of RNA (rRNA− RNase R+ library). The sequencing libraries were generated by NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer’s recommendations. Briefly, fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH−). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. In the reaction buffer, dNTPs with dTTP were replaced by dUTP. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends 2 of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150-200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3 μL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 minutes followed by 5 minutes at 95°C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system polymerase, Universal PCR primers and Index (X) Primer. At last, products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Raw data (raw reads) of fastq format were firstly processed through in-house perlscripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads on containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

In conclusion, the complete circRNA study involved the following steps: RNA isolation, quantification, and qualification; library preparation for circRNA sequencing; sequencing and clustering of reads; quality control; mapping to the reference genome; circRNA identification; quantification of gene-expression level; and differential-expression analysis. Normalized circRNA levels (estimated based on TPM values) were calculated as listed below, and transcripts were considered to be differentially expressed between APP/PS1 and WT mice when their \( P \) value was <.05.

\[
\text{Normalized expression} = \frac{(\text{mapped reads})}{(\text{total reads})} \times 1,000,000.
\]

### 2.4 KEGG pathway analyses

The DAVID (https://david.ncifcrf.gov/summary.jsp) database was used to analyze genes related to pathways. KEGG terms with \( q \) values of <.05 were considered significantly enriched.

### 2.5 Real-time qPCR validation

Total RNA was extracted using TRIzol reagent (Sigma) according to the manufacturer’s protocol. RNA quantity was measured using a NanoDrop 2000 (Thermo Fisher Scientific). Quantitative RT-PCR was performed using the GoScript Reverse Transcription System (Promega), in a C1000 Thermal Cycler (Bio-Rad). The glyceraldehyde-3-phosphate dehydrogenase gene (\textit{Gadph}) is expressed with a high level in almost all tissues. The protein expression level in the same kind of cells or tissues is generally constant, and it is kept constant by the influence of inducing substances. Therefore,
it is widely used as a standard internal reference for extracting total RNA, poly (A) + RNA, Western blot, and other experimental operations. In our study, Gaphh was selected as the internal control of total RNA. Relative gene-expression levels were calculated using the 2−∆CT method (n = 3). The list of primers used in this research is shown in Table 1

2.6 | Statistical analysis

Two normally distributed groups were compared using t tests. Parameters for the high-throughput sequencing-related data were calculated and statistical computing was performed using R software. All data are expressed as means ± SD; a value of P < .05 was considered statistically significant.

2.7 | Data access

All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE132177.

3 | RESULTS

3.1 | The workflow of RNA-seq

The workflow of two libraries was showed in Figure 1 and the details of the methods used for rRNA− library and rRNA− RNase R+ library preparation are described in Materials and Methods.

3.2 | Profiling circRNAs in the brain of WT and APP/PS1 mice

We characterized circRNA transcripts by performing RNA-seq analyses through isolating RNA from the brain of 6- and 9-month-old WT and APP/PS1 mice. The prepared rRNA− RNase R+ libraries generated a total of 739,349,016 raw reads (WT and APP/PS1 reads: 6 months, 177,433,438 and 191,552,170; 9 months, 185,516,382 and 184,847,026). After removal of low-quality, and adapter-containing reads from the raw data, 724,361,150 clean reads remained (WT and APP/PS1: 6 months, 173,777,328 and 186,369,844; 9 months, 181,497,660 and 182,716,318). The rRNA − libraries generated a total of 1,145,853,722 raw reads (WT and APP/PS1 reads: 6 months, 317,603,558 and 276,260,804; 9 months, 286,203,120 and 265,786,240). After the removal of low-quality, and adapter-containing reads from the raw data, 1,129,413,874 clean reads remained (WT and APP/PS1: 6 months, 294,445,468 and 303,115,112; 9 months, 304,767,684 and 300,815,710). The list of primers used in this research is shown in Table 1.

Table 1: The list of primers used in this research

| Name               | Primer                      |
|--------------------|-----------------------------|
| novel_circ_004900-F | TCGGGGCTCTGGATAGTAGA        |
| novel_circ_004900-R | CTGTGAGGGAGCTTGTAGGT        |
| novel_circ_0010578-F | AGTCGTCATACAGCCTTTGCTG     |
| novel_circ_0010578-R | AGTTGCCACAGTGTCCTTGGA       |
| novel_circ_0058120-F | AGACTCCCTAAACACACTGC        |
| novel_circ_0058120-R | ACTCCATCCAGGCTTCTTGGA       |
| novel_circ_0021634-F | AGAGAGCAGTGCGTTTACCAT      |
| novel_circ_0021634-R | AGAAGAGACAGAGGGCCCATAC     |
| novel_circ_0015639-F | AGATGGGAGGCCAAGGAACT       |
| novel_circ_0015639-R | CTCACTCTCATGGCCAGAGCG      |
| novel_circ_0009334-F | TGCACCTCAGACCTGAGCTG       |
| novel_circ_0009334-R | CATGCCGCGACGCTAGG           |
| novel_circ_0010615-F | CTTGCTCTGGATAGGAGCTG       |
| novel_circ_0010615-R | GTCCTCCAATCCACCTACA         |
| novel_circ_0022084-F | CTGACACCAATCCCCAGCTAGG     |
| novel_circ_0022084-R | GTTTCGTGCTAAGGCGCTG        |
| novel_circ_0015659-F | CAGACCTTCGACGAGAGG         |
| novel_circ_0015659-R | CGACCTTGTCAGAATGTCGA        |
| novel_circ_0006322-F | CCGACGCGATTTCCAGAGAC       |
| novel_circ_0006322-R | CACACTTGAGACCTACG          |
| novel_circ_0003994-F | TTGAGTGTCTTCAGTAC          |
| novel_circ_0003994-R | TCCAGACCCCCAAGATAC         |
| novel_circ_0016550-F | GGGAGTGTTCTCACG            |
| novel_circ_0016550-R | ATGCCGCTGAGAATTGGGC         |
| novel_circ_0014492-F | GACAGAGCCCTTGGAGAC         |
| novel_circ_0014492-R | GACACTCTGAGAAACCCAGA       |
| novel_circ_0014085-F | GGTGAGCGGGCTGGATAGA        |
| novel_circ_0014085-R | ATGTGGAGCGTCTGAGTCTG       |
| novel_circ_0009694-F | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-R | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-F | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-R | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-F | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-R | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-F | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-R | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-F | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-R | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-F | GCCATTTGAGGCCAGAGA         |
| novel_circ_0009694-R | GCCATTTGAGGCCAGAGA         |
The reference genome and gene model annotation files were downloaded directly from the genome website ftp://ftp.ensembl.org/pub/release-79/fasta/mus_musculus/dna/ and ftp://ftp.ensembl.org/pub/release-79/gtf/mus_musculus/. An index of the reference genome was built using bowtie2 v2.2.8 and paired-end clean reads were aligned to the reference genome by using Bowtie. By using find_circ software and CIRI2, we detected and identified 15,713 circRNAs in the rRNA− RNase R+ libraries and 4821 circRNAs in rRNA− libraries (Figure 2A and Tables S1-S2).

We found most of all circRNAs were detected only in rRNA− RNase R+ libraries (10,970) or co-detected by both rRNA− RNase R+ libraries and rRNA− libraries (4743). Only 78 circRNAs were detected only in the rRNA− libraries (Figure 2A). We annotated the circRNA candidates by using the RefSeq database. Most of the circRNAs included sequence of protein-coding exons, and fewer aligned with introns and intergenic regions of the genome (Figure 2B-D). Most circRNAs were <5000 nucleotides (nt) long, with a median length of 1000 nt (Figure 2E,G). The circRNAs detected in the rRNA− libraries had relatively short overall length (Figure 2F). In this analysis, the circRNA was evenly distributed on different chromosomes and the number of circRNAs detected only by rRNA− libraries was relatively small (Figure 2H-J).

3.3 Abundances of circRNAs in rRNA− RNase R+ libraries and rRNA− libraries

We normalized the junction reads (support for circRNAs) by read counts. The profiles differed for circRNAs in rRNA− RNase R+ libraries and in rRNA− libraries (Figure 3 and Table S3), with a significantly higher abundance of circRNAs in the rRNA− RNase R+ libraries than that in rRNA− libraries.

3.4 Differential expression analysis of rRNA− RNase R+ libraries and rRNA− libraries: APP/PS1 vs WT

We normalized the junction reads (support for circRNAs) by read length and the total number of mapped reads (spliced reads per billion mapped reads, denoted as TPM). This permitted quantitative comparisons between junction reads from different RNA-seq data. Expression analysis of the circRNA transcripts revealed expression of numerous circRNAs across various groups. The profile of circRNAs in APP/PS1 differed from that in WT in both the rRNA− RNase R+ libraries and rRNA− libraries. The differences in circRNA expression were calculated using Wilcoxon rank-sum test.

For the 6-month-old mice, circRNAs detected only in rRNA− RNase R+ libraries: 166 transcripts that were differentially regulated in APP/PS1 mice relative to the levels in WT mice, with 98 and 68 transcripts significantly ($P < .05$) upregulated and downregulated, respectively. For the circRNAs detected only in rRNA− libraries: only eight circRNA transcripts showed differential expression: with three and five transcripts significantly ($P < .05$) upregulated and downregulated, respectively, in APP/PS1 mice relative to the levels in WT mice (Figure 4A-B and Tables S4-S5).

In the 9-month-old mice, circRNAs detected only in rRNA− RNase R+ libraries, 167 circRNA transcripts were differentially regulated in APP/PS1 mice relative to the levels in WT mice, with 96 and 71 transcripts significantly ($P < .05$) upregulated and downregulated, respectively. For
the circRNAs detected only in rRNA− libraries: only five circRNA transcripts showed differential expression: with three and two transcripts significantly ($P < .05$) upregulated and downregulated, respectively, in APP/PS1 mice relative to the levels in WT mice (Figure 4C-D and Tables S6-S7).

CircRNAs co-detected in rRNA− RNase R+ and rRNA− libraries: for the 6-month-old mice, 104 circRNA transcripts showed differential expression: with 56 and 48 transcripts significantly ($P < .05$) upregulated and downregulated, respectively, in the rRNA− RNase R+ data sets; 163 circRNA transcripts showed differential expression: with 82 and 81 transcripts significantly ($P < .05$) upregulated and downregulated, respectively, in the rRNA− data sets (Figure 4E-F and Tables S4-S5). For the 9-month-old mice, identified 63 circRNA transcripts showed differential expression: 39 and 24 transcripts were significantly ($P < .05$) upregulated and downregulated, respectively, in the rRNA− RNase R+ data sets; 111 circRNA transcripts showed differential expression: 55 and 56 transcripts were significantly ($P < .05$) upregulated and downregulated, respectively, in the rRNA− data sets (Figure 4G,H and Tables S6-S7).

In order to see whether the up-and-down regulation of the circRNAs co-detected in the two libraries is the same, we performed a cluster analysis. We can see that the up-and-down is highly consistent, but the circRNA expression detected in the rRNA− RNase R+ libraries are higher (Figure 5).

Next, Validation of differential expression of circRNAs detected only by rRNA− RNase R+ library and detected only by rRNA− library by using qPCR. We found that the validation results of the rRNA− RNase R+ library are highly consistent with the sequencing results, while the rRNA− library is relatively low in consistency (Figure 6).

### 3.5 circRNAs and their parental genes

Most identified circRNAs are derived from exons, with fewer intron-containing circular RNAs (named ciRNAs). The ciRNAs are characterized by constrained expression in the nucleus, consistent with the presence in the nucleus of most linear RNAs with retained introns. Several pieces of evidence suggest that these ciRNAs can transcriptionally regulate parental genes. We next performed a correlation analysis for differentially expressed ciRNAs and their parental genes to explore the role of circRNA in AD through effects on parental gene transcription and splicing (Figure 7A and Table S8). These are potentially important circRNAs and mRNAs that can be studied in the future.

KEGG pathway analysis was conducted to determine the signaling cascades related to the identified parental genes. By using $q < .05$ as the threshold value, the following significantly enriched pathways potentially related to AD were identified (Figure 7B and Table S9): glutamatergic synapse, long-term potentiation, dopaminergic synapse, GABAergic synapse, cAMP signaling pathway, cGMP-PKG signaling pathway, and axon guidance.
**FIGURE 2** CircRNA profiling in the brain of WT and APP/PS1 mice in rRNA− RNase R+ libraries and rRNA− libraries. A. Grouping: Venn diagrams show circRNAs detected only in the rRNA− RNase R+ library, circRNAs detected only in the rRNA− library, and co-detected circRNAs. B-D. Number of circRNAs derived from distinct genomic regions; numbers are listed as log2N, where N is the number of exon, intron, or intergenic reads. From the origin, for example, the number of exons is ~2^14. B: circRNAs were detected only in the rRNA− RNase R+ library; C: circRNAs were detected only in the rRNA− library; D: co-detected circRNAs. E-G. Length distribution of identified circRNAs; x-axis: lengths of circRNAs detected in this study; y-axis: abundance of circRNAs classified by different lengths; z-axis: sample information. E: circRNAs were detected only in the rRNA− RNase R+ library; F: circRNAs were detected only in the rRNA− library; G: co-detected circRNAs. H-J. Distribution of circRNAs in different chromosomes. Count the reads of all the chromosomes that were compared to the chromosomes in each sample and use circos to plot the reads on each chromosome. Select the top 10 contigs or scaffolds to display. H: circRNAs were detected only in the rRNA− RNase R+ library; I: circRNAs were detected only in the rRNA− library; J: co-detected circRNAs
To confirm the correlation analysis between circRNA and parental genes by RNA-seq experiments, we used qPCR and analyzed four pairs transcripts that were Pearson correlation > .90 and $P < .0001$ selected. All selected transcripts were detected in the brain of 2-9-month-old APP/PS1 and WT mice and exhibited statistically significant differential expression between the two groups (Figure 8). Overall, the qPCR results were highly consistent with the RNA-seq data.

**4 | DISCUSSION**

Circular RNAs are difficult to detect and distinguish from other small RNAs and miRNAs based on size or mobility. Some techniques, such as “rapid amplification of cDNA ends” (RACE) or poly(A) enrichment of samples for transcriptome studies are not effective because circRNAs lack a defined end and lack free 3′ or 5′ ends that could be modified to allow detection. Additionally, one of the main features of circRNAs, their generation by a “backsplicing” process, is not exclusive of these species of small RNAs, and initial RNA-seq alignment tools eliminated those sequences.42

Recently, new methodology has been developed that improves the selection of circRNAs during sample processing, such as exonuclease-enrichment approaches, the sequencing of ribosomal RNA (rRNA)-depleted libraries instead of poly(A)-enriched libraries with longer reads and higher coverage, and the generation of novel bioinformatic tools. Jeck et al43 introduced a new biochemical protocol called CircleSeq. This methodology treats RNA samples with an exonuclease enzyme (RNase R) to degrade linear RNAs, leaving the circRNAs intact, which is rRNA− RNase R+ library in our research.

In our study, we adopted two methods of library construction to prepare rRNA− RNase R+ libraries and rRNA− libraries. We found some circRNAs were not detected in the rRNA− RNase R+ libraries but were detected in the rRNA− libraries, which might suggest some circRNAs are sensitive to RNase R. Some circRNAs were not detected in the rRNA-depleted libraries but were detected in the rRNA− RNase R+ libraries, possibly due to the presence of other similar RNA species or a bias based on the elimination of rRNA. The highly abundant circRNAs may have important functions. There were higher abundances of circRNAs in the rRNA− RNase R+ libraries compared to the rRNA− libraries, potentially reflecting the effect of the RNase R treatment. Most of the circRNAs consisted of protein-coding exons, with fewer containing sequences of introns and intergenic regions of the genome and no obvious difference was seen in the relative amounts of intron- or exon-containing circRNAs for the two methods of libraries construction.

Differential expression analysis between rRNA− RNase R+ libraries and rRNA− libraries revealed that the method of library construction affected the detection of differentially expressed circRNAs. We can see the circRNAs co-detected in two libraries, rRNA− RNase R+ libraries are more sensitive to detecting differential circRNAs. Further, the differential expression circRNAs co-detected through rRNA− RNase R+ and rRNA− libraries at 6 months and 9 months are highly consistent in rRNA− RNase R+ data sets and rRNA− data sets. However, Validation of differential expression of circRNAs detected only by rRNA− RNase R+ library and detected only by rRNA− library by using qPCR. We found that the validation results of the rRNA− RNase R+ library are highly consistent with the sequencing results, while the rRNA− library is relatively low in consistency, which might be due to the lower abundance in the rRNA− R libraries will affect the detection of differential circRNAs.

So when our goal is to find a lot of different circRNAs, it might be better to use rRNA− RNase R+ library, and when
FIGURE 4  Differential expression analysis of rRNA− RNase R+ libraries and rRNA− libraries: APP/PS1 vs WT. Volcano plots show circRNAs that were downregulated (green points), upregulated (red points), or showed no statistically significant difference (dark green points) in WT and APP/PS1 mice; x-axis: log2 ratio of circRNA expression levels between WT and APP/PS1 mice; y-axis: false-discovery rate value (-log10 transformed) of circRNAs. A and B, Differential expression analysis of circRNAs detected only by rRNA− RNase R+ library (rRNA− RNase R+ data sets) or detected only by rRNA− library (rRNA− data sets) at 6 months. C and D, Differential expression analysis of the circRNAs detected only by rRNA− RNase R+ library (rRNA− RNase R+ data sets), or detected only by rRNA− library (rRNA− data sets) at 9 months. E and F, Differential expression analysis of co-detected circRNAs at 6 months in rRNA− RNase R+ data sets and rRNA− data sets. G and H, Differential expression analysis of co-detected circRNAs at 9 months in rRNA− RNase R+ data sets and rRNA− data sets. Data are presented as P < .05, |log2 fold-change| > .5.
we choose subsequent experimental research, we should choose circRNAs with high abundance. At present, these two types of libraries construction are the most widely used, but the difference between the selected circRNAs is different, which must increase the attention of researchers.

Several pieces of evidence suggest that circRNAs can alter transcription of genes in cis by promoting transcription by RNA polymerase II (Pol II) of their parental genes. Nevertheless, the direct causal mechanism remains unclear.5,18 The generation of circRNAs via circularization of exons has been suggested to be a process that competes with the splicing machinery acting on the same splice-sequence sites. This competition was observed in neural tissue.18 In brain tissue during the aging process, there is increased expression of circRNAs and reduced levels of parental RNAs.44 This high level of circRNAs in certain tissues suggests that RNA circularization can control gene expression by displacing the machinery required for the canonical splicing of parental RNAs.45 We performed a correlation analysis between differentially expressed circRNAs and their parental genes to explore the role of circRNA in AD to regulate parental gene transcription and alter splicing. We consider these circRNAs and their parental genes to be associated with the pathogenesis of AD. For instance, CircTrpc6-Trpc6, CircKif1b-Kif1b, CircAdgrl3-Adgrl3.

**Figure 5** Differential expression analysis of co-detected circRNAs in rRNA− RNase R+ data sets and rRNA− data sets. Heatmap of readcount of differential expression of circRNAs co-detected in rRNA− RNase R+ libraries and rRNA− libraries, heat map made by log2N processing, where N is the readcount. A, co-detected differential expression of circRNAs at 6 months in rRNA− RNase R+ data sets. B, co-detected differential expression of circRNAs at 6 months in rRNA− data sets. C, co-detected differential expression of circRNAs at 9 months in rRNA− RNase R+ data sets. D, co-detected differential expression of circRNAs at 9 months in rRNA− data sets.
Figure 6 Validation of differential expression of circRNAs detected only by rRNA− RNase R+ library and detected only by rRNA− library by using qPCR. The bar graph shows the final result. The black bars are the wild-type control group, the white bars and the gray bars are the APP/PS1 group. The white bar indicates the same as the sequencing result, and the gray bar indicates the inconsistency. A, Differential expression of circRNAs detected only by rRNA− RNase R+ library. B, Differential expression of circRNAs detected only by rRNA− library. The transcripts expression was quantified relative to Gapdh expression level by using the comparative cycle threshold (∆CT) method. Data are presented as means ± SD (n = 3, *P < .05, **P < .01)
Transient receptor potential canonical 6 (TRPC6), which specifically interacts with APP leading to inhibition of its cleavage by g-secretase and reduction in Ab production. Furthermore, TRPC6 mRNA levels in the blood cells are specifically reduced in AD and MCI patients, and TRPC6 might be a biomarker for the early diagnosis of AD. KIF1B is a kinesin-like, microtubule-based molecular motor protein involved in anterograde axonal vesicular transport in vertebrate and invertebrate neurons. Certain KIF1B isoforms have been implicated in different forms of human neurodegenerative disease, with characterization of their functional integration and regulation in the context of synaptic signaling still ongoing. ADGRL3/LPHN3 regulates the number of synapses formed by L2/3 neurons in L5 and the strength of synaptic drive from the L2/3-L5 pathway. The olfactomedin domain of LPHN3 is required for this effect on synapse number and binding to its postsynaptic ligand FLRT3 and LPHN3 is important in determining the connectivity rates between principal neurons in the cortex.
KEGG pathway analysis was conducted to determine the signaling cascades related to the identified differentially expressed circRNA parental genes, and several pathways potentially related to AD were identified: glutamatergic synapse, long-term potentiation, dopaminergic synapse, GABAergic synapse, cAMP signaling pathway, cGMP-PKG signaling pathway, and axon guidance. Thus, we have discovered some potentially important circRNAs and mRNAs that can be studied in the future.

In conclusion, we elucidated the brain circRNAs profiles of APP/PS1 and WT mice by using two methods of library construction (rRNA− RNase R+ libraries and rRNA− libraries) for next-generation RNA sequencing data. The abundances of circRNAs in the rRNA− RNase R+ libraries were significantly higher than the abundances in rRNA− libraries and allow detection of a greater number of differentially expressed circRNAs. We performed a correlation analysis between differentially expressed circRNAs and their parental genes and KEGG analysis of the parental genes to explore the role of circRNA in AD. These circRNAs may regulate parental gene transcription and affect variable splicing. These newly identified circRNAs should serve as a valuable resource for the development of future potential biomarkers and as therapeutic targets for AD.

ACKNOWLEDGEMENTS
We would like to thank the Shenzhen Biomedical Research Support Platform and the Shenzhen Molecular Diagnostic Platform of Dermatology for technical help. This work was supported by National Key R&D Program of China Grant 2016YFA0501903, National Natural Scientific Foundation of China (Grant No. 81673053), Natural Scientific Foundation of Guangdong Province (2016A030312016) and Shenzhen Basic Research Grants (JCYJ20160229153100269, JCYJ20170411090739316, JCYJ20170815153617033, JCYJ20180507182657867, JCYJ20170306161450254 and JCYJ20170306161807726).
CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS CONTRIBUTIONS

J. Wan, W. Zhang, and N. Ma designed the study; N. Ma, C. Tie, and B. Yu participated in the animal experiments including tissue collection and RNA/protein extraction; N. Ma performed the experiments and analyzed the data; N. Ma, J. Wan, and W. Zhang analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Qu S, Yang X, Li X, et al. Circular RNA: a new star of noncoding RNAs. Cancer Lett. 2015;365(2):141-148.
2. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA. 2013;19(2):141-157.
3. Tay ML, Pek JW. Maternally inherited stable intronic sequence RNA triggers a self-reinforcing feedback loop during development. Curr Biol. 2017;27(7):1062-1067.
4. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular intronic long non-coding RNAs are a large class of animal RNAs with regulatory potency. Nature. 2013;495(7441):333-338.
5. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. PLoS Genet. 2013;9(9):e1003777.
6. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO. Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. PLoS One. 2012;7(2):e30733.
7. Zhang Y, Zhang X-O, Chen T, et al. Circular intronic long noncoding RNAs. Mol Cell. 2013;51(6):792-806.
8. Zeng Y, Du WW, Wu Y, et al. A circular RNA binds to and activates AKT phosphorylation and nuclear localization reducing apoptosis and enhancing cardiac repair. Theranostics. 2017;7(16):3842-3855.
9. Wang K, Long BO, Liu F, et al. A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. Eur Heart J. 2016;37(33):2602-2611.
10. Du WW, Yang W, Chen Y, et al. Foxo3 circular RNA promotes cardiac senescence by modulating multiple factors associated with stress and senescence responses. Eur Heart J. 2016;38(18):1402-1412.
11. Werfel S, Notzhue S, Schwarzmayr T, Strom TM, Meiting T, Engelhardt S. Characterization of circular RNAs in human, mouse and rat hearts. J Mol Cell Cardiol. 2016;98:103-107.
12. Guarniero J, Bezzi M, Jeong JC, et al. Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. Cell. 2016;166(4):1055-1056.
13. Holdt LM, Kohlmaier A, Teupser D. Molecular functions and specific roles of circRNAs in the cardiovascular system. Noncoding RNA Res. 2018;3(2):75-98.
14. Legnini I, Di Timoteo G, Rossi F, et al. Circ-ZNF609 Is a circular RNA that can be translated and functions in myogenesis. Mol Cell. 2017;66(1):22-37.
15. Yang Y, Fan X, Mao M, et al. Extensive translation of circular RNAs driven by N(6)-methyladenosine. Cell Res. 2017;27(5):626-641.
16. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of cRNA crosstalk and competition. Nature. 2014;505(7483):344-352.
17. Salmena L, Poliseno L, Tay Y, Katz L, Pandolfi, PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 2011;146(3):353-358.
18. Rybak-Wolf A, Stottmeister C, Glazár P, et al. Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. Mol Cell. 2015;58(5):870-885.
19. Gruner H, Cortés-López M, Cooper DA, Bauer M, Miura P. CircRNA accumulation in the aging mouse brain. Sci Rep. 2016;6(1):38907.
20. Westholm J, Miura P, Olson S, et al. Genome-wide analysis of drosophila circular RNAs reveals their structural and sequence properties and age-dependent neural accumulation. Cell Rep. 2014;9(5):1966-1980.
21. Lukiw WJ. Circular RNA, circRNA) in Alzheimer's disease (AD. Front Genet. 2013;4(4):307–317.
22. Bingol B, Sheng M. Deconstruction for reconstruction: the role of proteolysis in neural plasticity and disease. Neuron. 2011;69(1):22-32.
23. Irina L, Shekoyan AR, Hebron ML, Nicole D, Algarzae NK, Charbel E-HM. Diminished parkin solubility and co-localization with intraneuronal amyloid-β are associated with autophagic defects in Alzheimer’s disease. J Alzheimers Dis. 2013;33(1):231-247.
24. Hansen TB, Jensen TI, Clausen BH, et al. Natural RNA circles function as efficient microRNA sponges. Nature. 2013;495(7441):384-388.
25. Schröder J, Ansaloni S, Schilling M, et al. MicroRNA-138 is a potential regulator of memory performance in humans. Front Hum Neurosci. 2014;8:501–510.
26. Tatro ET, Rishbough V, Soontorniyomkij B, et al. Short-term recognition memory correlates with regional CNS expression of microRNA-138 in mice. Am J Geriatric Psych. 2013;21(5):461-473.
27. Okazaki Y, Furuno M, Kasukawa T, et al. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature. 2002;420(6915):73–563.
28. Bishop NA, Tao L, Yankner BA. Neural mechanisms of ageing and cognitive decline. Nature. 2010;464(7288):529–535.
29. Destrooper B, Karran E. The cellular phase of Alzheimer's disease. Cell. 2016;164(4):603-615.
30. Canter RG, Penney J, Tsai LH. The road to restoring neural circuits for the treatment of Alzheimer's disease. Nature. 2016;539(7628):187-196.
31. Zädori D, Veres G, Szalárdy L, Klivényi P, Vécsei L. Alzheimer's disease: recent concepts on the relation of mitochondrial disturbances, excitotoxicity, neuroinflammation, and kynurenes. J Alzheimers Dis. 2018;62(2):521-547.
32. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals a potential regulator of memory performance in humans. Front Hum Neurosci. 2014;8:501–510.
33. Tatro ET, Rishbough V, Soontorniyomkij B, et al. Short-term recognition memory correlates with regional CNS expression of microRNA-138 in mice. Am J Geriatric Psych. 2013;21(5):461-473.
34. Okazaki Y, Furuno M, Kasukawa T, et al. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature. 2002;420(6915):73–563.
35. Bishop NA, Tao L, Yankner BA. Neural mechanisms of ageing and cognitive decline. Nature. 2010;464(7288):529–535.
36. Mercier TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009;10(3):155-159.
37. Necsulea A, Soumillon M, Warnefors M, et al. The evolution of lncRNA repertoires and expression patterns in tetrapods. Nature. 2014;505(7485):635-640.
38. Yvonne T, John R, Pier PP. The multilayered complexity of ceRNA crosstalk and competition. Nature. 2014;505(7483):344-352.
39. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-359.
40. Memczak S, Jens M, Elefsinioti A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature. 2013;495(7441):333-338.
38. Gao Y, Zhang J, Zhao F. Circular RNA identification based on multiple seed matching. Brief Bioinform. 2018;19(5):803-810.
39. Pruitt KD, Tatiana T, Brown GR, Maglott DRNBI. Reference sequences (RefSeq): current status, new features and genome annotation policy. Nucleic Acids Res. 2012;40(Database issue):D130-D135.
40. Li Z, Huang C, Bao C, et al. Exon-intron circular RNAs regulate transcription in the nucleus. Nat Struct Mol Biol. 2015;22(3):256-264.
41. Braunschweig U, Barbosa-Morais NL, Pan Q, et al. Widespread intron retention in mammals functionally tunes transcriptomes. Genome Res. 2014;24(11):1774-1786.
42. Zhang X, Wang Y, Zhao Z, Wang J. An efficient algorithm for sensitively detecting circular RNA from RNA-seq data. Int J Mol Sci. 2018;19(10).
43. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol. 2014;32(5):453-461.
44. Gruner H, Cortes-Lopez M, Cooper DA, Bauer M, Miura P. CircRNA accumulation in the aging mouse brain. Sci Rep. 2016;6:38907.
45. Huang S, Yang B, Chen BJ, et al. The emerging role of circular RNAs in transcriptome regulation. Genomics. 2017;109(5-6):401-407.
46. Wang J, Lu R, Yang J, Li H, He Z, Jing N, et al. TRPC6 specifically interacts with APP to inhibit its cleavage by γ-secretase and reduce Aβ production. Nat Commun. 2015;6:8876–8920.
47. Lu R, Wang J, Tao R, et al. Reduced TRPC6 mRNA levels in the blood cells of patients with Alzheimer’s disease and mild cognitive impairment. Mol Psychiatr. 2018;23(3):767-776.
48. Charalambous DC, Pasciuto E. KIF1Bβ transports dendritically localized mRNPs in neurons and is recruited to synapses in an activity-dependent manner. Cell Mol Life Sci. 2013;70(2):335-356.
49. Sando R, Jiang X, Südhof TC. Latrophilin GPCRs direct synapse specificity by coincident binding of FLRTs and teneurins. Science. 2019;363(6429):837–837.

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Ma N, Tie C, Yu B, Zhang W, Wan J. Circular RNAs regulate its parental genes transcription in the AD mouse model using two methods of library construction. The FASEB Journal. 2020;34:10342–10356. https://doi.org/10.1096/fj.201903157R