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Original Paper

The Differentially Expressed Circular RNAs in the Substantia Nigra and Corpus Striatum of Nrf2-Knockout Mice

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Key Words
Circular RNAs • Nrf2 • Oxidative stress • Microarray • Neuroprotection

Abstract
Background/Aims: The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway plays a protective role in both acute neuronal damage and chronic neurodegeneration-related oxidative stress. Circular RNAs (circRNAs) are involved with various diseases in the central nervous system (CNS). This study aimed to identify the key circRNAs involved in Nrf2-neuroprotection against oxidative stress. ***Methods:*** The differentially expressed circRNAs (DEcircRNAs) in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice were identified by microarray analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) was then used to validate the expression of selected DEcircRNAs in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice. Based on our previous microarray analysis of the differentially expressed mRNAs (DEmRNAs) in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice, the DEcircRNA-miRNA-DEmRNA interaction network was constructed. Functional annotation of DEmRNAs that shared the same binding miRNAs with DEcircRNAs was performed using gene ontology (GO) and pathway analyses. ***Results:*** A total of 65 and 150 significant DEcircRNAs were obtained in the substantia nigra and corpus striatum of Nrf2 (-/-) mice, respectively, and seventeen shared DEcircRNAs were found in both these two tissues. The qRT-PCR results were generally consistent with the microarray results. The DEcircRNA-miRNA-DEmRNA interaction network and pathway analysis indicated that mmu_circRNA_34132, mmu_circRNA_017077 and mmu-circRNA-015216 might be involved with Nrf2-mediated neuroprotection against oxidative stress. Mmu_circRNA_015216 and mmu_circRNA_017077 might play roles in the Nrf2-related transcriptional misregulation and Nrf2-

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mediated processes of rheumatoid arthritis, respectively. In addition to these two processes, mmu_circRNA_34132 may be a potential regulator of Nrf2-mediated protection for diabetes mellitus and Nrf2-mediated defence against ROS in hearts. ***Conclusion****:* In conclusion, our study identified the key DEcircRNAs in the substantia nigra and corpus striatum of Nrf2 (-/-) mice, which might provide new clues for further exploring the mechanism of Nrf2-mediated neuroprotection against oxidative stress and other Nrf2-mediated processes.

Introduction

Maintaining redox homeostasis in the brain is essential for survival. Oxidative stress is induced by a persistent imbalance between production and clearance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. Neural tissues are indicated to be particularly sensitive to oxidative stress due to their high oxygen consumption and high lipid content [2].

Oxidative stress serves as one of the major mechanisms involved in neuronal damage and death after acute traumatic brain injury and acute ischaemic stroke [3]. Moreover, accumulated evidences have demonstrated that oxidative stress is a shared causative agent of various chronic neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (HD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) [4-6].

Nuclear factor erythroid 2-related factor 2 (Nrf2), also named Nfe2l2, is a transcription factor that exerts regulated roles in the transcription of antioxidant-response elements (AREs)[5]. The Nrf2-ARE pathway drives expression of various detoxifying and antioxidant genes such as superoxide dismutase 1 (SOD1), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate cysteine ligase regulatory subunit (GCLM), glutathione peroxidase 2 (GPX2), glutathione peroxidase 3 (GPX3), glutathione-disulphide reductase (GSR) and others [6, 7], which serves as an intrinsic mechanism of defence against oxidative stress [8].

The Nrf2 pathway was reported to be highly inducible in astrocytes [9], and its neuroprotective effects on astrocytes have been observed in models of PD, ALS, HD, ischaemia and Alexander's disease [10-14]. Deficiency of Nrf2 in mice could replicate transcriptomic changes in patients with AD [15]. Exacerbation of degeneration of nigral dopaminergic neurons and increased expression of α-synuclein were observed in Nrf2-knockout mice, which cooperated to aggravate neuronal death and inflammation in early-stage PD [16]. In addition, the protective effect of the Nrf2 pathway on ischaemic damage was found to be lacking in Nrf2-knockout mice as well [17]. Hence, Nrf2-knockout mice were used to explore the exact mechanisms of Nrf2-mediated neuroprotection.

As an evolutionarily conserved type of non-coding RNAs, circular RNAs (circRNAs) are formed by exon skipping or back-splicing events that are expressed abundantly in mammalian cells [15]. Moreover, circRNAs are preferentially expressed in neural tissues along neural genes [18] that are associated with various diseases in the central nervous system (CNS), such as AD, PD and ischaemia [19-21]. CircRNA CDR1as, an antisense transcript of CDR1, was reported to be involved with the pathogenesis of PD [20]. Altered circRNA expression profiles were found in mouse brain after transient focal ischaemia [21]. However, biological functions of the majority of circRNAs remain unclear and whether circRNAs are involved in Nrf2-mediated neuroprotection against oxidative stress needs to be explored.

In this study, differentially expressed circRNAs (DEcircRNAs) in the substantia nigra and corpus striatum tissue between Nrf2(-/-) and Nrf2(+/+) mice were obtained by microarray analysis. Differentially expressed mRNAs (DEmRNAs) in the substantia nigra and corpus striatum tissue between Nrf2(-/-) and Nrf2(+/+) mice were also obtained by our microarray analysis. Then, construction of the DEcircRNA-miRNA-DEmRNA interaction network and functional annotation of DEmRNAs were performed to predict potential functions of DEcircRNAs. Hopefully, our study could identify the key circRNAs correlated with Nrf2 and provide new clues for understanding the mechanism of Nrf2-mediated neuroprotection against oxidative stress.

Materials and Methods

Nrf2-knockout mice and ethics

Academician Chun-Yan Li (Department of Neurology, Second Hospital of Hebei Medical University, Shijiazhuang, China) kindly supplied three adult male Nrf2 (+/+) mice (25-30 g, 3-4 months, n = 3) and three Nrf2 (-/-) mice (25-30 g, 3-4 months, n = 3) for this study. None of these mice underwent perfusion. Polymerase chain reaction (PCR) amplification of genomic DNA from tails was used to determine the genotypes [(Nrf2 (+/+) and Nrf2 (-/-)] of the mice. All the mice were sacrificed using an overdose of an isoflurane/oxygen mixture. We obtained both the substantia nigra and corpus striatum tissues of each mouse from surgery, and they were then immediately homogenized for the extraction of total RNA (Trizol, Invitrogen, Carlsbad, CA, USA).

The animal experiments in this study complied with the regulations of the Animal Welfare Act of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised 1996) and were approved by the ethics committee of Hebei Medical University.

CircRNA Microarrays

The concentration of total RNA was determined by OD260 by using a NanoDrop ND-1000 instrument (Nanodrop Technologies Inc). The integrity of total RNA was assessed by electrophoresis on a denaturing agarose gel. Sample labelling and array hybridization were performed according to the manufacturer's protocol. Briefly, total RNA was digested with RNase R (Epicentre, Inc.) to remove linear RNAs and enrich circular RNAs. By utilizing a random priming method with a Super RNA Labelling Kit (Arraystar, Inc), the enriched circular RNAs were amplified and transcribed into fluorescent cRNA. The fluorophore-labelled cRNA was purified with a RNeasy Mini Kit (Qiagen). The concentration and specific activity of labelled cRNAs (pmol Cy3/μg cRNA) were measured using a NanoDrop ND-1000 (Nanodrop Technologies Inc). Then, 1 μg of each labelled cRNA was fragmented by adding 5 μl 10 × Blocking Agent and 1 μl of 25 × Fragmentation Buffer, then heating the mixture at 60°C for 30 min, and finally, 25 μl 2 × Hybridization buffer was added to dilute the labelled cRNA. Hybridization solution (50 μl) was dispensed into the gasket slide and assembled to a mouse circRNA microarray (8x15K, Arraystar) slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven.

Differentially expressed CircRNAs

The hybridized arrays were washed, fixed and scanned using the Agilent Scanner G2505C and scanned images were imported into Agilent Feature Extraction software for raw data extraction. By using the R software limma package, the raw data were quantile normalized and then low intensity filtering was performed. The expression profiles of circRNAs in the substantia nigra and corpus striatum tissues of Nrf2 (+/+) and Nrf2 (-/-) mice were obtained. To compare the profile differences in the substantia nigra and corpus striatum tissues between Nrf2 (+/+) and Nrf2 (-/-) mice, the fold change between these groups for each circRNA was computed. The T-test was used to estimate the statistical significance of the difference. CircRNAs with fold changes ≥1.5 and *p*-values ≤ 0.05 were selected as the significantly DEcircRNAs. The shared DEcircRNAs in both the substantia nigra and corpus striatum of Nrf2 (-/-) mice compared to Nrf2 (+/+) mice were obtained using Venny version 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/). Hierarchical cluster analysis of group samples based on the expression values of circRNAs was visualized through a "pheatmap" package in R language.

Real-Time PCR Validation

Expression changes of circRNAs identified by microarray analysis were validated by quantitative real-time PCR for two up-regulated (mmu_circRNA_32463 and mmu_circRNA_34132) and two down-regulated DEcircRNAs (mmu_circRNA_34106 and mmu_circRNA_015216) in the substantia nigra, and four down-regulated (mmu_circRNA_33836, mmu_circRNA_34106, mmu_circRNA_017077 and mmu_circRNA_003119) and one up-regulated DEcircRNA (mmu_circRNA_34132) in the corpus striatum tissues of Nrf2 (-/-) mice.

Total RNA of the substantia nigra and corpus striatum tissues in 3 Nrf2 (-/-) and 3 Nrf2 (+/+) mice were extracted using Trizol (Thermo Fisher Scientific Inc.) according to the manual instructions. cDNA was generated by 1 μg RNA with an M-MLV First Strand Kit (Thermo Fisher Scientific Inc). Quantitative real-time

polymerase chain reaction (qRT-PCR) was performed using SuperReal PreMix Plus (SYBR Green) (TIANGEN) in an Illumina Eco PCR machine (Illumina). Cycling conditions were as follows: an initial denaturation step of 15 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 53°C, and 20 s at 72°C. β-actin served as an endogenous control for normalization. All experiments were performed in triplicate. Relative expression of circRNAs was analysed with the $2^{-\Delta\Delta Ct}$ method. Table 1 displays the primers used in real-time PCR validation.

Table 1. The primers used in qRT-PCR experiments

CircRNA	Primers
mmu_circRNA_32463	Forward: 5′ AAGAAAAGAGAGAGTCCATCAGCAA 3′ Reverse: 5′ TTCAAAGTAAACCAGCTTAACCAGG 3′
mmu_circRNA_34132	Forward: 5′ TGAAGAACTCTGTCTACCGAAGCC 3′ Reverse: 5′ TAGTCAAAGCCTTGCACGGGAT 3′
mmu_circRNA_34106	Forward: 5′ GGCTGCTGAAGAGTGAACTTGGAT 3′ Reverse: 5′ AGGTGAGGATGGAGCTGTGTCTTC 3′
mmu_circRNA_015216	Forward: 5′ AAAGTCAGATGTGTGGTCATTTGGAA 3′ Reverse: 5′ TTCCACTCCATAATGACTGGCACTT 3′
mmu_circRNA_33836	Forward: 5′ GAACCTTACTCAAAAGCATCCCACT 3′ Reverse: 5′ TTGGGCCAACATGTATTATTCTTCC 3′
mmu_circRNA_017077	Forward: 5′ ACGGGGAAGACCAATGACTTTACCA 3′ Reverse: 5′ GCTGGAGCCAAAGGCAATTGTACT 3′
mmu_circRNA_003119	Forward: 5′ AGACTTACGAACGCCTTGTTGCCCA 3′ Reverse: 5′ TTGTCTATAGGTTGATTCTGTGCCTCTCGA 3′
β-actin	Forward: 5′ TCATCACTATTGGCAACGAGCGGT 3′ Reverse: 5′ GTGTTGGCATAGAGGTCTTTACG 3′

Construction of DEcircRNA-miRNA-DEmRNA interaction networks

CircRNAs were speculated to serve as miRNA sponges, which may play regulatory roles by competing with mRNAs for shared binding miRNAs [22]. Hence, various studies have used construction of a circRNA-miRNA-mRNA interaction network to explore the functions of circRNAs [23-25]. The DEcircRNA-miRNA-mRNA interactions including seven DEcircRNAs (mmu_circRNA_32463, mmu_circRNA_34132 mmu_circRNA_34106, mmu_circRNA_015216, mmu_circRNA_33836, mmu_circRNA_017077 and mmu_circRNA_003119) were predicted with Arraystar's homemade miRNA target prediction software based on TargetScan & MiRanda [26, 27]. In these DEcircRNA-miRNA-mRNA interactions, circRNA and mRNA were predicted to be targeted by shared miRNAs. Our previous work has identified the differentially expressed mRNAs (DEmRNAs) in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice, respectively [22]. DEmRNAs that targeted shared miRNAs with those seven DEcircRNAs (mmu_circRNA_32463, mmu_circRNA_34132 mmu_circRNA_34106, mmu_circRNA_015216, mmu_circRNA_33836, mmu_circRNA_017077 and mmu_circRNA_003119) were further identified. The DEcircRNA-miRNA-mRNA interactions that contained these obtained DEmRNAs were retained for the following research. Cytoscape (http://www.cytoscape.org/) was applied to build the DEcircRNA-miRNA-DEmRNA interaction networks.

Functional annotation of DEmRNAs of miRNAs targeted by DEcircRNAs

By using the online software GeneCodis (http://genecodis.cnb.csic.es/analysis), Gene Ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were conducted for target DEmRNAs of miRNAs targeted by these seven DEcircRNAs in the substantia nigra and corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice, respectively. FDR<0.05 was defined as the criterion of statistical significance.

Statistical analysis

Mean ± standard deviation and independent samples t-test were used in the statistical analysis. A value of $p < 0.05$ was considered significant.

Results

DEcircRNAs in the substantia nigra between Nrf2 (-/-) and Nrf2 (+/+) mice.

Compared to Nrf2 (+/+) mice, a total of 65 DEcircRNAs, including 36 up-regulated circRNAs and 29 down-regulated circRNAs, were obtained in the substantia nigra of Nrf2 (-/-) mice with a ≥ 1.5-fold change and p-value ≤ 0.05. The scatter and volcano diagrams of circRNA expression in the substantia nigra between Nrf2 (-/-) and Nrf2 (+/+) are displayed in

Table 2. Top 10 up- and down-regulated DEcircRNAs in the substantia nigra of Nrf2 (-/-) mice

circRNA	circRNA-type	chrom	p-value	Regulation	Parental gene
mmu_circRNA_34107	sense overlapping	chr2	0.001831	up	Commd9
mmu_circRNA_003949	intronic	chrX	0.002287	up	Gm15155
mmu_circRNA_32463	exonic	chr19	0.002462	up	Pank1
mmu_circRNA_33657	exonic	chr2	0.00367	up	Rbms1
mmu_circRNA_33827	exonic	chr2	0.004075	up	Mettl8
mmu_circRNA_016771	exonic	chr2	0.004279	up	Rbms1
mmu_circRNA_33580	exonic	chr2	0.006098	up	Galnt13
mmu_circRNA_34109	sense overlapping	chr2	0.007533	up	Ldlrad3
mmu_circRNA_29746	sense overlapping	chr16	0.008427	up	Gramd1c
mmu_circRNA_33826	exonic	chr2	0.009312	up	Mettl8
mmu_circRNA_33836	exonic	chr2	0.000101	down	Dcaf17
mmu_circRNA_34137	exonic	chr2	0.000678	down	D430041D05Rik
mmu_circRNA_34106	exonic	chr2	0.001076	down	Prr5l
mmu_circRNA_34718	exonic	chr2	0.001083	down	Rin2
mmu_circRNA_018654	antisense	chr17	0.001654	down	Rn45s
mmu_circRNA_33549	sense overlapping	chr2	0.002515	down	XLOC_012859
mmu_circRNA_19263	sense overlapping	chr2	0.003784	down	Dtd1
mmu_circRNA_19266	sense overlapping	chr2	0.005943	down	Itch
mmu_circRNA_38403	exonic	chr5	0.008852	down	Yes1
mmu_circRNA_015216	exonic	chr5	0.009892	down	Yes1

Table 3. Top 10 up- and down-regulated DEcircRNAs in the corpus striatum of Nrf2 (-/-) mice

circRNA	circRNA-type	chrom	p-value	Regulation	Parental gene
mmu_circRNA_33679	exonic	chr2	0.000592	up	Slc4a10
mmu_circRNA_34132	exonic	chr2	0.000658	up	D430041D05Rik
mmu_circRNA_40962	sense overlapping	chr6	0.000862	up	Grin2b
mmu_circRNA_31348	exonic	chr18	0.001182	up	Taf4b
mmu_circRNA_000661	exonic	chr1	0.001392	up	Unc80
mmu_circRNA_34109	sense overlapping	chr2	0.00159	up	Ldlrad3
mmu_circRNA_43478	exonic	chr8	0.00178	up	Pdpr
mmu_circRNA_40182	exonic	chr6	0.001861	up	Hipk2
mmu_circRNA_002520	sense overlapping	chr7	0.001881	up	Gm25647
mmu_circRNA_37354	exonic	chr4	0.002	up	Zyg11b
mmu_circRNA_33836	exonic	chr2	0.000177	down	Dcaf17
mmu_circRNA_34137	exonic	chr2	0.000292	down	D430041D05Rik
mmu_circRNA_33831	exonic	chr2	0.000486	down	Dcaf17
mmu_circRNA_45602	exonic	chrX	0.000745	down	Pola1
mmu_circRNA_29699	exonic	chr16	0.000852	down	Gsk3b
mmu_circRNA_33542	intronic	chr2	0.00087	down	
mmu_circRNA_003119	exonic	chr2	0.000933	down	Cstf3
mmu_circRNA_29578	exonic	chr16	0.001164	down	Osbpl11
mmu_circRNA_19255	sense overlapping	chr2	0.001404	down	Cstf3
mmu_circRNA_33548	sense overlapping	chr2	0.003167	down	Gm13483

Fig. 1A and 1B, respectively. Compared to Nrf2 (+/+) mice, mmu_circRNA_33836 and mmu_circRNA_34107 were the most down-regulated and up-regulated circRNAs in the substantia nigra of Nrf2 (-/-) mice, respectively (Table 2). Hierarchical clustering of the expression of these DEcircRNAs indicated that there was an obvious discrimination in the substantia nigra between Nrf2 (-/-) and Nrf2 (+/+) mice (Fig. 1C).

These 65 DEcircRNAs were classified into 5 types. Three (4.62%) were antisense, 3 (4.62%) were intronic, 2 (3.08%) were intergenic, 41 (63.08%) were exonic and 16 (24.62%) were sense-overlapping (Fig. 2A).

DEcircRNAs in the corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice.

Compared to Nrf2 (+/+) mice, a total of 150 DEcircRNAs including 106 up-regulated and 44 down-regulated circRNAs in the corpus striatum of Nrf2 (-/-) mice were obtained with ≥1.5-fold change and *p*-value ≤ 0.05. The scatter and volcano diagrams of circRNA expression in the corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) are displayed in Fig. 1D and 1E, respectively. Compared to Nrf2 (+/+) mice, mmu_circRNA_33836 and mmu_circRNA_33679 were the most down-regulated and up-regulated circRNAs in the corpus striatum of Nrf2 (-/-) mice, respectively (Table 3). Hierarchical clustering of the expression of these DEcircRNAs indicated that there was an obvious discrimination in the corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice (Fig. 1F). These 150 DEcircRNAs were classified into 4 types. Six (4.00%) were intronic, 1 (0.67%) was intergenic, 118 (78.67%) were exonic and 25 (16.67%) were sense overlapping (Fig. 2B).

Shared DEcircRNAs in both the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice.

A total of 17 DEcircRNAs shared in both the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice were obtained. Among them, 7 DEcircRNAs (mmu_circRNA_009773, mmu_circRNA_33826, mmu_circRNA_34835, mmu_circRNA_34132, mmu_circRNA_34109, mmu_circRNA_44531 and mmu_circRNA_32463) were significantly up-regulated while 10 DEcircRNAs (mmu_circRNA_33836, mmu_circRNA_33549, mmu_circRNA_33548, mmu_circRNA_33542, mmu_circRNA_34137, mmu_circRNA_19263, mmu_circRNA_34106, mmu_circRNA_34794, mmu_circRNA_34718 and mmu_circRNA_19266) were significantly down-regulated in both the substantia nigra and corpus striatum of Nrf2 (-/-) mice compared to Nrf2 (+/+) mice.

qRT-PCR validation of the expression levels of candidate circRNAs

Compared to Nrf2 (-/-) mice, two DEcircRNAs (mmu_circRNA_32463 and mmu_circRNA_34132) were significantly up-regulated, while two (mmu_circRNA_34106 and mmu_circRNA_015216) were significantly down-regulated in the substantia nigra of Nrf2 (-/-) mice, and four DEcircRNAs (mmu_circRNA_33836, mmu_circRNA_34106, mmu_circRNA_017077 and mmu_circRNA_003119) were significantly down-regulated, while one (mmu_circRNA_34132) was significantly up-regulated in the corpus striatum tissues of Nrf2 (-/-) mice (Fig. 3). Moreover, mmu_circRNA_34132 was significantly up-regulated in both the substantia nigra and corpus striatum of Nrf2 (-/-) mice, and mmu_circRNA_34106 was significantly down-regulated in both the substantia nigra and corpus striatum of Nrf2 (-/-) mice compared to Nrf2 (+/+) mice. Generally, the expression of these selected circRNAs in qRT-PCR validation results was totally consistent with those in our microarray results.

DEcircRNA-miRNA-DEmRNA interaction networks

Based on the obtained circRNA-miRNA-mRNA interactions, mmu_circRNA_34132, mmu_circRNA_015216, mmu_circRNA_32463, mmu_circRNA_34106, mmu_circRNA_017077, mmu_circRNA_33836, and mmu_circRNA_003119 were predicted to compete with 4640, 3672, 3342, 1993, 2315, 770 and 556 genes for binding miRNAs, respectively. Our previous study has identified 96 and 643 DEmRNAs in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice, respectively [28]. After these DEmRNAs were retained to construct the DEcircRNA-miRNA-DEmRNA interaction network, mmu_circRNA_015216, mmu_circRNA_32463, mmu_circRNA_34106 and mmu_circRNA_34132 were predicted to compete with 10, 11, 8 and 17 DEmRNAs in the substantia nigra between Nrf2 (-/-) and Nrf2 (+/+) mice, respectively; and mmu_circRNA_33836, mmu_circRNA_017077 and mmu_circRNA_003119, mmu_circRNA_34132 and mmu_circRNA_34106 were predicted to compete with 22, 80 and 18, 169 and 78 DEmRNAs, respectively, in the corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice. The DEcircRNA-miRNA-DEmRNA interaction networks are displayed in Fig. S1 (For all supplemental material see www.karger.com/10.1159/000494478/).

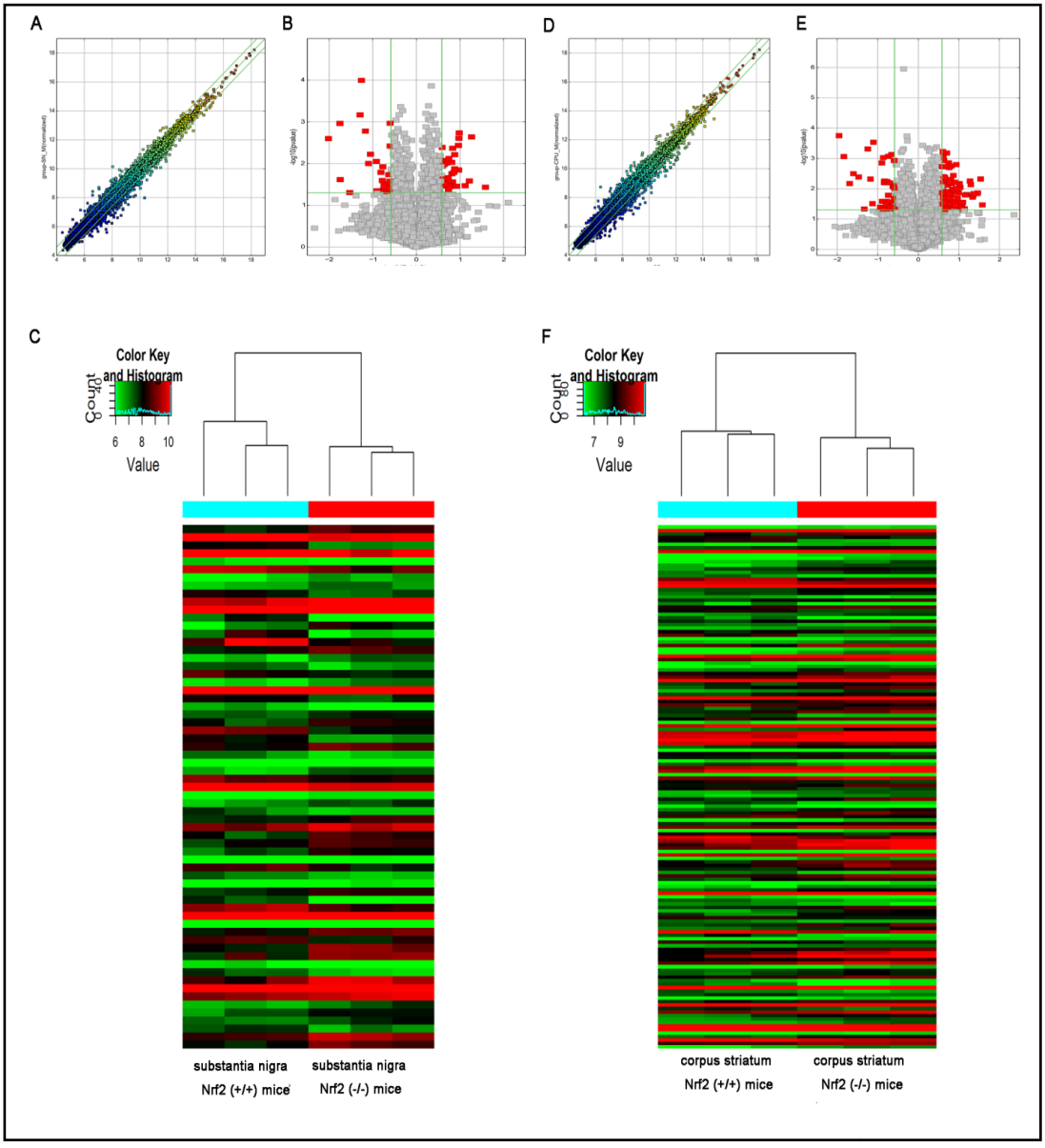


Fig. 1. Scatter diagram, volcano diagram and hierarchical clustering analysis of relative expression signal of circRNAs in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice. (A). Scatter diagram of DElncRNAs expression in Nrf2 (-/-) substantia nigra. (B). Volcano diagram of DElncRNAs expression in Nrf2 (-/-) substantia nigra. (C). Hierarchical clustering analysis based on the expression profile of the DElncRNAs in Nrf2 (-/-) substantia nigra. (D). Scatter diagram of DElncRNAs expression in Nrf2 (-/-) corpus striatum. (E). Volcano diagram of DElncRNAs expression in Nrf2 (-/-) corpus striatum. (F). Hierarchical clustering analysis based on the expression profile of the DElncRNAs in Nrf2 (-/-) corpus striatum.

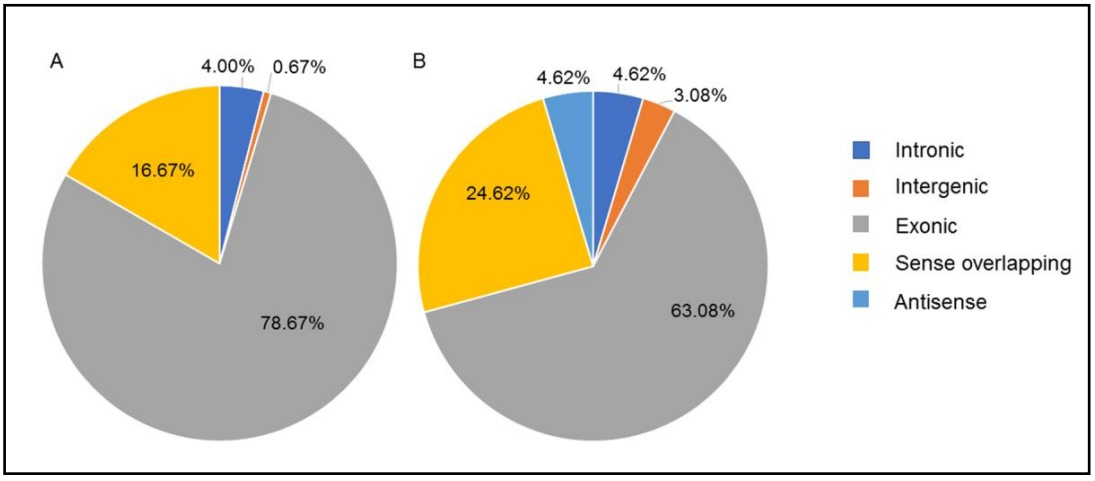
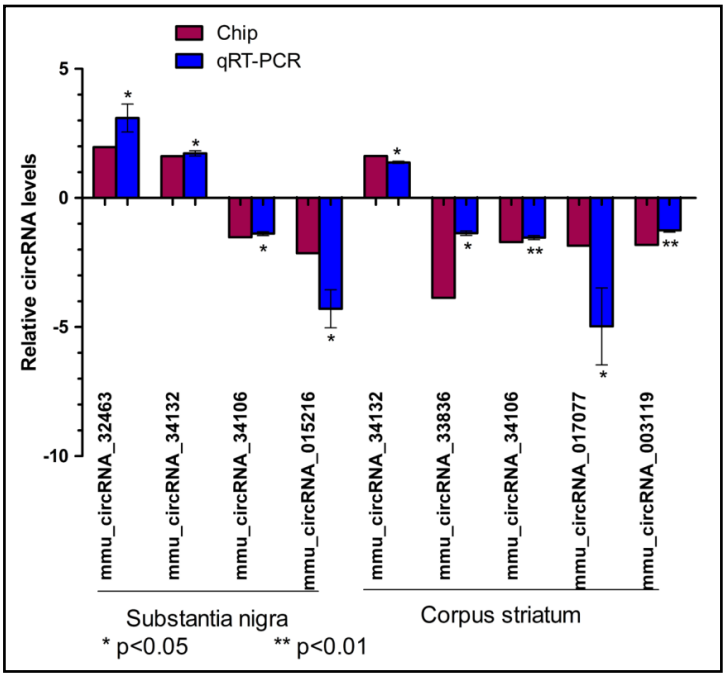


Fig. 2. The type of DEcircRNAs in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice.

Fig. 3. qRT-PCR validation of expression levels of DEcircRNAs in Nrf2 (-/-) substantia nigra and corpus striatum tissues. * indicates P<0.05 and ** indicates that P<0.01. Deep red column indicates the expression status of DEcircRNAs through microarray analyses; blue column indicates the expression status of DEcircRNAs through qRT-PCR experiments.



Functional annotation of DEmRNAs of miRNAs targeted by DEcircRNAs in the corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice

Functional annotation of DEmRNAs that shared the same binding miRNAs with DEcircRNAs in the corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice was conducted. According to the KEGG enrichment analysis (Fig. 4), Alzheimer's disease (KEGG: mmu05010), cardiac muscle contraction (KEGG: mmu04260), rheumatoid arthritis (KEGG: mmu05323), and insulin secretion (KEGG: mmu04911) were significantly enriched pathways. Six DEmRNAs (Apbb1, Fas, Gnaq, Grin1, Grin2b, and Ncstn) were enriched in Alzheimer's disease. Among them, Gnaq, Grin2b, and Ncstn were targets of mmu_circRNA_34132; Fas, Grin1 and Ncstn were targets of mmu_circRNA_017077; and Ncstn and Apbb1 were targets of mmu_circRNA_33836 and mmu_circRNA_34106, respectively. DEcircRNAs that shared the same binding miRNAs with these six AD-related genes are displayed in Table 4.

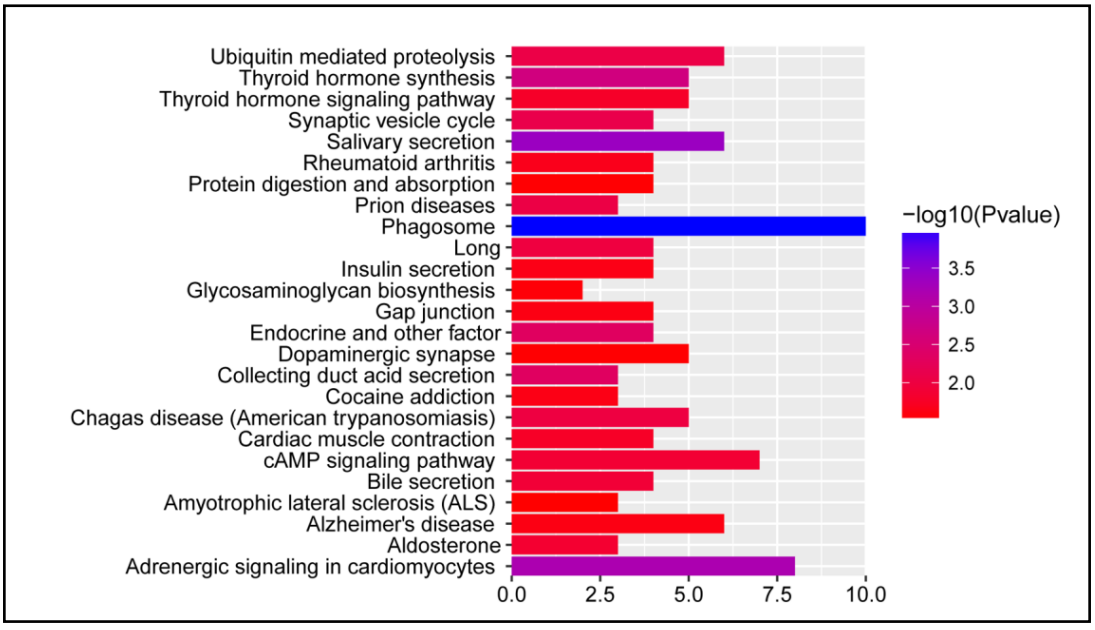


Fig. 4. The top 25 most significantly enriched KEGG pathways of DEceRNAs of miRNAs targeted by DEcircRNAs between Nrf2 (-/-) and Nrf2 (+/+) corpus striatum. The x-axis shows counts of genes that enriched in KEGG pathways and y-axis shows the KEGG pathways. Color scale depicted -log (p-value).

Table 4. The DEcircRNAs bound by shared miRNAs of Alzheimer's disease -related genes in corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice

mRNA	DEcircRNA	Shared miRNA
Fas	mmu_circRNA_017077	mmu-miR-7649-3p, mmu-miR-3473c, mmu-miR-5127, mmu-miR-150-5p
Grin1	mmu_circRNA_017077	mmu-miR-664-5p, mmu-miR-5133, mmu-miR-3473e, mmu-miR-3473b, mmu-miR-505-5p, mmu-miR-7032-3p, mmu-miR-1953, mmu-miR-7662-3p
Ncstn	mmu_circRNA_017077	mmu-miR-1964-5p, mmu-miR-7649-3p, mmu-miR-3473c, mmu-miR-3473e, mmu-miR-3473b, mmu-miR-1953, mmu-miR-6343
Ncstn	mmu_circRNA_33836	mmu-miR-542-3p, mmu-miR-194-1-3p, mmu-miR-6898-3p, mmu-miR-216a-3p, mmu-miR-6963-5p, mmu-miR-6974-3p, mmu-miR-7656-5p
Ncstn	mmu_circRNA_34132	mmu-miR-6974-3p, mmu-miR-1953, mmu-miR-599, mmu-miR-7075-5p, mmu-miR-194-1-3p, mmu-miR-7030-5p, mmu-miR-5107-5p, mmu-miR-7076-5p, mmu-miR-6941-5p, mmu-miR-7024-5p, mmu-miR-8093, mmu-miR-7081-5p, mmu-miR-7055-5p, mmu-miR-7119-5p, mmu-miR-7023-5p, mmu-miR-7117-5p, mmu-miR-145a-3p
Grin2b	mmu_circRNA_34132	mmu-miR-351-5p, mmu-miR-216c-3p, mmu-miR-29b-1-5p, mmu-miR-6384, mmu-miR-7074-3p, mmu-miR-7001-5p, mmu-miR-7075-5p, mmu-miR-6929-5p, mmu-miR-6957-5p, mmu-miR-7016-3p, mmu-miR-6964-5p, mmu-miR-7073-5p, mmu-miR-743a-5p, mmu-miR-346-3p, mmu-miR-7030-5p, mmu-miR-672-3p, mmu-miR-125a-5p, mmu-miR-7076-5p, mmu-miR-6941-5p, mmu-miR-6943-5p, mmu-miR-7024-5p, mmu-miR-7228-5p, mmu-miR-6367, mmu-miR-7119-5p, mmu-miR-7023-5p, mmu-miR-125b-5p, mmu-miR-145a-3p
Gnaq	mmu_circRNA_34132	mmu-miR-1904, mmu-miR-6962-3p, mmu-miR-7053-5p, mmu-miR-3082-5p, mmu-miR-7075-5p, mmu-miR-6929-5p, mmu-miR-6957-5p, mmu-miR-493-3p, mmu-miR-7016-3p, mmu-miR-6918-5p, mmu-miR-3059-5p, mmu-miR-7073-5p, mmu-miR-7030-5p, mmu-miR-7033-3p, mmu-miR-3074-2-3p, mmu-miR-7076-5p, mmu-miR-6941-5p, mmu-miR-1966-5p, mmu-miR-7024-5p, mmu-miR-7081-5p, mmu-miR-7069-5p, mmu-miR-683, mmu-miR-302c-3p, mmu-miR-7119-5p, mmu-miR-7023-5p, mmu-miR-6978-3p
Apbb1	mmu_circRNA_34106	mmu-miR-7072-5p, mmu-miR-6924-5p, mmu-miR-3076-5p, mmu-miR-7030-3p, mmu-miR-5623-3p

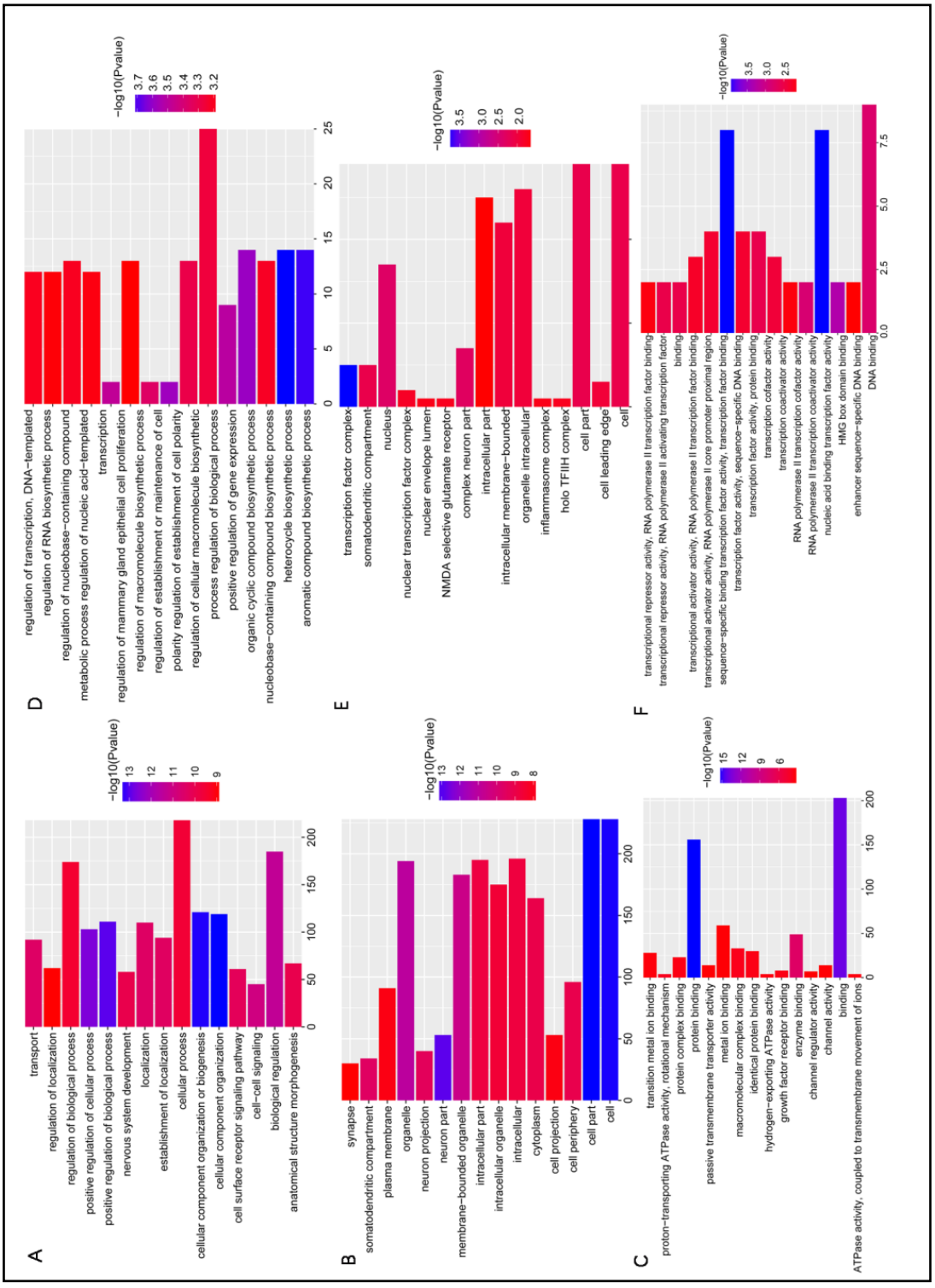


Fig. 5. The top 15 most significantly enriched GO terms of DEceRNAs of miRNAs targeted by DEcircRNAs between Nrf2 (-/-) and Nrf2 (+/+) mice. (A) Biological progress in the corpus striatum; (B) Cellular component in the corpus striatum; (C) Molecular function in the corpus striatum; (D) Biological progress in the substantia nigra; (E) Cellular component in the substantia nigra; (F) Molecular function in the substantia nigra. The x-axis shows counts of genes that enriched in GO terms and y-axis shows the GO terms. Color scale depicted -log (p-value).

Four genes were enriched in the pathways of cardiac muscle contraction (Atp1b2, Cacnb2, Fxyd2 and Tpm1), rheumatoid arthritis (Atp6v0a1, Atp6v0b, Atp6v0c and Atp6v0e2) and insulin secretion (Atp1b2, Fxyd2, Gnaq And Prkaca). Three insulin secretion-related genes (Gnaq, Atp1b2 and Prkaca) and mmu_circRNA_34132 could bind to two shared miRNAs (mmu-miR-7024-5p and mmu-miR-7081-5p). Moreover, two rheumatoid arthritis-related genes (Atp6v0c, Atp6v0e2) and mmu_circRNA_017077 shared the same binding miRNA, mmu-miR-346-3p; two other rheumatoid arthritis-related genes (Atp6v0e2 and Atp6v0a1) could compete with mmu_circRNA_34132 for binding to mmu-miR-346-3p as well. Mmu_circRNA_34132 and two cardiac muscle contraction-related genes (Cacnb2 and Atp1b2) were targeted by seven shared miRNAs including mmu-miR-6929-5p, mmu-miR-6918-5p, mmu-miR-6941-5p, mmu-miR-7024-5p, mmu-miR-7055-5p, mmu-miR-7119-5p and mmu-miR-7023-5p. GO enrichment analysis (Fig. 5) indicated that nervous system development (biological process: 0007399), neuron differentiation (biological process: 0030182), neuron part (cellular component: 0097458), somato-dendritic compartment (cellular component: 0036477) and neuron projection (cellular component: 0043005) were significantly enriched GO terms.

Functional annotation of DEmRNAs of miRNAs targeted by DEcircRNAs in the substantia nigra between Nrf2 (-/-) and Nrf2 (+/+) mice

Functional annotation of DEmRNAs, which shared the same binding miRNAs with DEcircRNAs in the substantia nigra between Nrf2 (-/-) and Nrf2 (+/+) mice, was conducted. Transcriptional misregulation in cancer (KEGG: mmu05202) was the only significantly enriched pathway. KLF3 and TFE3 were two genes that were enriched in this pathway, which were predicted to compete with mmu_circRNA_34132 and mmu_circRNA_015216 for shared binding miRNAs, respectively. Heterocycle biosynthetic process (biological process: 0018130), neuron part (cellular component: 0097458), somatodendritic compartment (cellular component: 0036477) and neuron projection (cellular component: 0043005) were significantly enriched GO terms (for all. 5).

Discussion

Accumulated evidences have demonstrated that activation of Nrf2 in astrocytes plays a neuroprotective role in both acute neuronal damage- and chronic neurodegeneration-related oxidative stress. CircRNAs are an evolutionarily conserved class of non-coding RNAs [29] which were reported to be remarkably enriched in the nervous system [30] and play roles in brain function [21, 31, 32]. To identify key circRNAs that correlated with Nrf2-mediated neuroprotection in the brain, DEcircRNAs in the substantia nigra and corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice were identified by microarray and bioinformatics analysis.

In this present study, a total of 65 and 150 DEcircRNAs were obtained in the substantia nigra and corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice, respectively. Moreover, qRT-PCR validation of selected DEcircRNAs was completely consistent with that of our microarray data, which indicated that our study was convincing.

Recent studies indicated that circRNAs may play regulatory roles as “sponges” of miRNAs to regulate the transcription or translation of mRNAs [33]. Previous *in silico* analysis predicted 85 NRF2–miRNA interactions, with 63 miRNAs that can regulate NRF2 directly or indirectly [34]. Nfe2l2 proved to be a critical player in homeostasis and several organs in mammals, such as brain, liver and kidney [35].

Various miRNAs including miR-7, miR27a, miR-34a and miR142-5p were reported to regulate the Nrf2-ARE pathway and glutathione homeostasis in the brain [36-39]. MiR-7 could increase the activity of Nrf2 and its downstream ARE, haeme oxygenase-1 (HO-1) and glutamate-cysteine ligase modifier subunit (GCLM), by repressing the inhibitor of Nrf2, Kelch-like ECH-associated protein 1 (Keap1) to exert cytoprotective effects in the brain [38].

In addition, up-regulated miR-7 was found in sporadic AD and serves as a key mechanistic contributor to the sporadic AD processes [40]. In this study, three DEcircRNAs (mmu-circRNA-018899, mmu-circRNA-38709 and mmu-circRNA-008691) in the corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice, were targets of mmu-miR-7a-5p, mmu-miR-7a-2-3p and mmu-miR-7b-5p, respectively, which suggested that these three circRNAs may be involved in neuroprotection through regulation of the Nrf2-ARE pathway. MiR-27a was up-regulated in neuroepithelial cells with maternal diabetes-induced oxidative stress and played an inhibitory role on the expression of Nrf2 and Nrf2-controlled antioxidant genes [35]. Moreover, dysregulated miR-27a was found in neurodegenerative diseases including AD, HD and PD, as well [41-43]. MiR-34a was found to exert an inhibitory role on the Nrf2 pathway in a PD model and to be involved in the hepatoprotective effect of hydrogen sulphide on ischaemia/reperfusion injury [44, 45] . As an up-regulated DEcircRNA in the substantia nigra tissues of Nrf2 (-/-) mice, mmu-circRNA-32463 was a target of mmu-miR-34a. A down-regulated DEcircRNA in the substantia nigra tissues of Nrf2 (-/-) mice, mmu-circRNA-015216 was a target of both mmu-miR-27a and mmu-miR-34a, which suggested that both mmu-circRNA-32463 and mmu-circRNA-015216 might be potential regulators in Nrf2-mediated neuroprotection against oxidative stress.

Moreover, DEmRNAs that competed with DEcircRNAs between Nrf2 (-/-) and Nrf2 (+/+) mice for the binding site of the same miRNAs were identified, and functional annotation of these DEmRNAs was performed. Nervous system development and Alzheimer's disease were significantly enriched biological processes, and the pathway of DEmRNAs of miRNAs targeted by DEcircRNAs in the corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice. Six DEceRNAs (Apbb1, Fas, Gnaq, Grin1, Grin2b, Ncstn) were enriched in the pathway of Alzheimer's disease.

Moreover, Fas-induced apoptosis was involved with the apoptotic cell death of substantia nigra dopaminergic neurones in PD, motor neuron apoptosis in ALS and ischaemic brain damage induced by cerebral ischaemia [46-48]. Nrf2 could induce the production of glutathione (GSH) with a precursor to GSH, N-acetyl L-cysteine (NAC), which protects cells from Fas-mediated killing [49]. Based on our analysis, mmu_circRNA_017077 could compete with Fas for 4 binding miRNAs (mmu-miR-7649-3p, mmu-miR-3473c, mmu-miR-5127 and mmu-miR-150-5p), which might be involved with the Nrf2-mediated Fas-induced apoptosis in various nervous system diseases related to oxidative stress.

Human gene GRIN2B was mainly expressed in the central nervous system (CNS) which encodes the glutamate ionotropic receptor N-methyl-D-aspartate (NMDA) type subunit 2B [50] . As the agonist binding site for glutamate, GRIN2B acts as the major excitatory neurotransmitter receptor in the mammalian brain and variants of GRIN2B play roles in neurodegenerative diseases including AD, PD and HD [50-52]. Grin2b could compete with mmu_circRNA_34132 for binding to mmu-miR-29b-1-5p based on this study. Moreover, Nrf2 could downregulate miR-29b-1 through binding to specific ARE sites [53]. These findings suggested that mmu_circRNA_34132-mmu-miR-29b-1-5p-Grin2b interaction might play a role in neurodegenerative diseases that are regulated by Nrf2.

Two AD-regulated genes (Grin1 and Ncstn) and another two AD-regulated genes (Gnaq and Ncstn) could compete for binding miRNAs with mmu_circRNA_017077 and mmu_circRNA_34132, respectively. These findings emphasized the importance of mmu_circRNA_017077 and mmu_circRNA_34132 in the Nrf2-mediated processes of AD.

In addition to Nrf2-mediated neuroprotection, transcriptional misregulation in cancer (KEGG: mmu05202) was a significantly enriched pathway for DEmRNAs of miRNAs targeted by DEcircRNAs in the substantia nigra tissues between Nrf2 (-/-) and Nrf2 (+/+) mice. KLF3 and TFE3 were two genes that were enriched in this pathway, which were predicted to compete with mmu_circRNA_34132 and mmu_circRNA_015216, respectively, for miRNA bind sites. Moreover, several miRNAs (miR-507, -634, -450a, and -129-5p) were found to negatively regulate the NRF2-mediated oncogenic pathway by directly targeting NRF2, which highlighted the importance of circRNAs that bind to these miRNAs [32].

In addition, insulin secretion, cardiac muscle contraction and rheumatoid arthritis were

three significantly enriched pathways for the DEmRNAs of miRNAs targeted by DEcircRNAs in the corpus striatum tissues between Nrf2 (-/-) and Nrf2 (+/+) mice.

Nrf2 has been found to play a crucial role in diabetic model mice by inducing glutathione-related genes and suppressing pancreatic ?-cell apoptosis mediated via nitric oxide [54]. Moreover, miR-200a, miR-21 and miR-424 were found to regulate Nrf2 in type 2 diabetes mellitus. In this study, three insulin secretion-related genes (Gnaq, Atp1b2 and Prkaca) and mmu_circRNA_34132 could bind to two shared miRNAs (mmu-miR-7024-5p and mmu-miR-7081-5p). In addition, mmu_circRNA_34132 and two cardiac muscle contraction-related genes (Cacnb2 and Atp1b2) were targeted by seven shared miRNAs. Nrf2 is critical in antioxidative response in heart muscle cells, which could defend against high glucose-induced oxidative damage in cardiomyocytes [54]. These findings suggested that mmu_circRNA_34132 might play a role in Nrf2-mediated protection for diabetes mellitus and be involved in defence against ROS in hearts, regulated by Nrf2 as well.

Oxidative stress has been found to play a role in the cartilage degradation activated in experimental arthritis, and Nrf2 serves as a major requirement for limiting cartilage destruction by downregulating endogenous antioxidants [54]. Additionally, activation of Nrf2 could inhibit the overproduction of proinflammatory cytokines to attenuate the progression of rheumatoid arthritis [35]. Moreover, two rheumatoid arthritis-related genes (Atp6v0c, Atp6v0e2) and mmu_circRNA_017077 shared the same binding miRNA, mmu-miR-346-3p; and two other rheumatoid arthritis-related genes (Atp6v0e2 and Atp6v0a1) could compete with mmu_circRNA_34132 for binding to mmu-miR-346-3p, as well. These findings suggested that both mmu_circRNA_017077 and mmu_circRNA_34132 might be involved in the Nrf2-mediated process of rheumatoid arthritis by regulating mmu-miR-346-3p-Atp6v0a1/Atp6v0c/Atp6v0e2 interactions.

Conclusion

In conclusion, we identified the expression profiling of abnormally expressed circRNAs in the substantia nigra and corpus striatum between Nrf2 (-/-) and Nrf2 (+/+) mice. Mmu-circRNA-015216 might play a role in Nrf2 neuroprotection regulated by Nrf2-related miRNAs (mmu-miR-34a and mmu-miR-27a). Mmu_circRNA_017077-mmu-miR-7649-3p/mmu-miR-3473c/mmu-miR-5127/mmu-miR-150-5p-Fas interactions and mmu_circRNA_34132-mmu-miR-29b-1-5p-Grin2b interactions were predicted to participate in AD. In addition, mmu_circRNA_015216 and mmu_circRNA_017077 were speculated to be involved with Nrf2-related transcriptional misregulation and Nrf2-mediated processes of rheumatoid arthritis, respectively. In addition to these two processes, mmu_circRNA_34132 might play a role in Nrf2-mediated protection for diabetes mellitus and be involved in defence against ROS in hearts, regulated by Nrf2 as well. Our study might be the foundation for future investigations to shed light on the molecular mechanisms of Nrf2-mediated neuroprotection against oxidative stress and other Nrf2-mediated processes.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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