

Circular RNA expression profiling in dorsal root ganglion of rats with peripheral nerve injury-induced neuropathic pain

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Short report

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Abstract

Background : Circular RNAs (circRNAs) comprise a class of endogenous species of RNA consisting of a covalently closed loop structure that is crucial for genetic and epigenetic regulation. The significance of circRNA in neuropathic pain remains to be investigated.

Methods : The sciatic nerve chronic constriction injury (CCI) model was established to induce neuropathic pain. We performed genome-wide circRNA analysis of 4 paired DRG sample from CCI and NC rats via next generation sequencing technology. The differentially expressed circRNAs (DEcircRNAs) were identified by differential expression analysis and the expression profile of circRNAs was validated by quantitative real-time PCR (qPCR). Functional annotation analysis was performed to predict the function of DEcircRNAs.

Results : A total of 374 DEcircRNAs were identified between CCI and NC rats using circRNA High-throughput sequencing (HTS). Expression levels of 9 DEcircRNAs were validated by qPCR. Functional annotation analysis showed that DEcircRNAs were mainly enriched in pathways and functions such as 'dopaminergic synapse', 'renin secretion', 'MAPK signaling pathway' and 'neurogenesis'. Competing endogenous RNAs analysis showed that top 50 circRNAs exhibited interactions with four pain related miRNAs. Circ:chr2:33950934-33955969 is the largest node in the circRNA-miRNA interaction network.

Conclusion : DEcircRNAs may advance our understanding of the molecular mechanisms underlying neuropathic pain. **Key words :** neuropathic pain, circRNA, CCI, differential expression analysis

Background

Neuropathic pain develops after injury of the somatosensory system [1, 2]. It is characterized by spontaneous continuous or paroxysmal pain and stimuli-evoked pain [1, 3]. It has been emerged as one of the most difficult pain syndromes, affecting 7–10% of the population [1]. Moreover, the incidence of neuropathic pain is increasing with the aging of the global population [1]. Despite recent improvements, the management of neuropathic pain remains unsatisfactory, mainly due to insufficient understanding of the molecular mechanisms underlying the syndrome [2, 4]. Therefore, there is a need to reveal the underlying mechanisms to benefit the clinical management of neuropathic pain.

Dorsal root ganglion (DRG) contains a great proportion of body's sensory neurons, which are critical for transducing sensory information from the periphery to the central nervous system [5, 6]. Accumulating evidence has highlighted that the development of neuropathic pain is associated with changes in DRG neurons, such as release of cytokines and dysregulation of ion channels[5]. In addition to changes in the expression of individual genes, recent studies have highlighted gene expression alterations in DRG of neuropathic pain. After examining gene expression in DRG of normal rats and peripheral axotomy operated rats, Xiao et al., identified significant differences in the expression of 173 genes, including neuropeptides, ion channels and synaptic vesicle proteins [10]. Reinhold et al., have confirmed differential expression patterns between damaged DRG neurons and adjacent neurons, using fluorescent neuronal

tracers [11]. Furthermore, universal alterations of miRNA expression in DRG have been demonstrated in neuropathic pain. Li et al., have identified a total of 114 differentially expressed miRNAs in DRG of neuropathic pain rats[12]. Besides, in another study, the expression of a total of 42 miRNAs was shown to be significantly altered in DRG of streptozotocin (STZ)-induced diabetic neuropathic pain mice [13].

Circular RNAs (circRNAs) are a large family of covalently closed RNA molecules with regulatory roles in gene transcription, miRNA function and protein production [14–16]. CircRNAs are expressed in diverse cell types and preferentially expressed in neural tissues [16]. The expression levels of circRNAs are dynamically modulated in neurons, which are essential for synaptic plasticity and neuronal function [17]. Alterations in the expression of circRNAs may play roles in neuropathic pain[18]. In a recent study, 469 circRNAs were identified to be differentially expressed in spinal dorsal horn of CCI rats [18]. However, our knowledge about the alterations in the expression of circRNAs in DRG of neuropathic pain is limited.

In order to gain a global view of aberrant circRNA expression in DRG of CCI rats, we established CCI model and evaluated circRNA expression levels in DRG using next generation sequencing technology. Differentially expressed circRNAs (DEcircRNAs) between CCI and NC rats were identified and subsequently validated by quantitative real-time PCR (qPCR). Functional annotation was implemented to explore the biological function of DEcircRNAs.

Methods

Animals

Sprague Dawley (SD) rats (adult, male, weighed 200–250 g) were acquired from the Experimental Animal Center of Fudan University (Shanghai, China). Rats were maintained in a pathogen-free environment under a standard 12 h light/dark cycle and were provided with rodent diet and water. Rats were randomly assigned to CCI or negative control groups of 4 rats each. The CCI rat model was produced under sodium pentobarbital anesthesia (40 mg/kg, intraperitoneal injection), based on the procedure established by Bennett and Xie [19]. The sciatic nerves on left side were first exposed and ligatures were then tied loosely around the nerve. Rats in negative control (NC) group were subjected to sciatic nerve exposures without ligation. Animal experiments were approved by the Institutional Animal Care and Use Committee in Fudan University.

Behavioral test

Paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTL) tests were implemented to assess mechanical allodynia and thermal hyperalgesia, respectively. PWMT in response to mechanical stimuli was carried out with a modified Von Freytest. Seven calibrated Von Frey filaments (Stoelting, Wood Dale, IL, USA) were applied to the plantar surface of one hindpaw in ascending order (2,4,6,8,10, 15, and 26 g). For one trail, each Von Frey filament was applied five times at 30-second intervals. Brisk withdrawal or flinching evoked by at least 3 of 5 applications was considered as a positive response. PWTL in response to radiant heat was carried out using a heat-mediated pain stimulator

(Model 336; IITC Life Science, Woodland Hill, CA, USA). The time from onset of application to hind paw withdrawal was measured, and each rat was tested 3 times with a 10-min interval. Both tests were performed 1 day before and 1, 3, 5, 7, 9 and 14 days after operation.

RNA preparation

L4-L5 DRGs were excised and rapidly frozen at -80 °C at the end of the behavior test. Total RNA of each DRG sample was isolated using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The quality and quantity of total RNA samples were measured with a ND-1000 NanoDrop spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The integrity of each RNA sample was excised by electrophoresis using denaturing agarose gel.

circRNA expression profiling

Total RNA from four matched DRG samples of CCI and NC rats were treated with Epicenter Ribo-Zero rRNA Removal Kit (Illumina, CA, USA) and RNase R (Epicenter, CA, USA) to remove ribosomal and linear RNA. Then the RNA-seq libraries were constructed using TruSeqStranded Total RNA HT/LT Sample Prep Kit (Illumina, CA, USA). Sequencing was performed on Illumina HiSeq 2500 instrument. All the sequencing procedures and analyses were performed in RiboBio (Guangzhou, China).

Data preprocessing

Raw data extraction and analysis of acquired array images were performed using Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies). Following quantile normalization of the raw data, adapter sequences and low quality reads were removed using Skewer [20] and FastX-Toolkit [21], respectively. The qualified data were aligned to ribosomal RNA (rRNA) sequences from RNA central database to further remove rRNA interference. The clean reads were then aligned to unfusion genes of UCSC rat genome reference (GRCh37/hg) using TopHat2 [22], and the resulting unmapped reads were aligned to fusion genes of the reference genome using TopHat-Fusion [23]. The mapped reads resulting from both alignments were considered as back-spliced junction reads. Most circRNAs are flanked by GT/AG splicing signals [24]. Therefore, back-spliced junction reads with a GT/AG splice signal were selected, whereas those with ambiguous breakpoints were discarded [25]. Subsequently, the ends of the circRNA splice site were located and the functional components of circRNA were annotated by comparing them to the genome using ANNOVAR [26]. The sequences of circRNAs were then inferred from annotated transcripts as previously described [25]. Finally, the expression level of each circRNA was calculated based on reads Per Million mapped reads using HTSeq [27].

Differential expression analysis

DEcircRNAs between CCI and NC groups were identified using fold change filtering. Q value was acquired by adjusting the p value using the Benjamini-Hochberg method. The selection criteria for significantly DEcircRNAs were set as $|\log_2 \text{fold change (FC)}| > 1.0$ and $q < 0.001$. The cluster profile of DEcircRNAs was revealed by two-way hierarchical clustering analysis based on their expression values.

Functional annotation

The possible functions of circRNA may be related to their host genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) [28] and Gene Ontology (GO) [29] analysis were performed for DEcircRNAs to reveal neuropathic pain associated biological pathways and functions. Fisher's exact test was used during the enrichment process. The selection criterion for significantly enriched terms was set at $p < 0.05$.

Quantitative real-time PCR (qPCR)

cDNA samples were prepared by reversely transcribing total RNAs using the PrimeScript RT reagent Kit (TaKaRa, Japan). DEcircRNAs that were expressed in all samples and showed significant expression differences were selected for qPCR analysis. The expression levels of the selected DEcircRNAs were validated by qPCR assays using the SYBR Green qPCR Master Mix (ABI, CA, USA) and specific primers sequences (Supplementary Table 1). The expression levels of circRNAs were normalized to GAPDH expression and analyzed by the $2^{-\Delta\Delta Ct}$ method.

Results

Chronic constriction injury induces genome-wide circRNAs expression in the DRG

In order to reveal circRNA expression changes induced by neuropathic pain, rat CCI models were established. Mechanical allodynia and thermal hyperalgesia were assessed to evaluate the models. As revealed by PWT and PWL tests, PWT and PWL were significantly decreased in the CCI group from postoperative day (POD) 3 to POD14, compared with the NC group (Supplementary Fig. 1). A total of 7,514 circRNAs were detected in the L4-L5 DRG samples from the CCI and NC group by circRNAs HTS. Following differential expression analysis, 374 DEcircRNAs were identified between the CCI and the NC group. Among the DEcircRNAs, 84 were down-regulated and 290 were up-regulated in the CCI group. Hierarchical clustering analysis was performed to group samples, based on the expression levels of DEcircRNAs across samples. As shown by the heatmap, the CCI and NC samples showed distinguishable circRNAs expression profiles (Fig. 1A-D, Supplementary Table 2 and Supplementary Table 3). The top 20 upregulated are listed in Table 1 and downregulated circRNAs are listed in Table 2.

Validation of circRNA expression using qPCR

In order to validate the reliability of circRNA array, qPCR analysis was performed to measure the expression of nine typical DEcircRNAs (chr1:166208849–166259335, chr2:26590643–26650618, chr3:112752502–112762808, chr4:132075419–132113888, chr7:64125692–64131186, chr10:8331904–8371302, chr13:84161876–84168811, chr15:51936366–51944899, and chr20:50725400–50764570). As detected by circRNAs HTS, these circRNAs expressed in all CCI and NC samples and showed significant expression differences between groups. According to our qPCR results, except for chr10:8331904–8371302, chr13:84161876–84168811, and chr20:50725400–50764570, the

expression level of all the remaining DEcircRNAs were consistent with those measured by sequencing analysis (Fig. 1E).

Functional annotation of DEcircRNAs

In order to predict the functions and pathways related to DEcircRNAs identified above, KEGG and GO analysis were performed for the host genes of DEcircRNAs. According to our results, the most significantly enriched KEGG pathways included 'dopaminergic synapse', 'renin secretion', 'long - term depression', 'metabolic pathways', 'MAPK signaling pathway', and 'cGMP - PKG signaling pathway'. The most significantly enriched GO terms included 'generation of neurons', 'neurogenesis', 'GTPase regulator activity', 'negative regulation of metabolic process', and 'cellular process'. The top 30 significantly enriched KEGG and GO terms are shown in (Fig. 2A-B).

CircRNA-microRNA competing endogenous RNAs network

MicroRNAs play an important role in the development of chronic pain. In our previous study, we found that miRNA-146a-5p which is a key molecule in the negative regulation of innate immunity was expression dysregulation in the DRG of chronic pain rats [2]. Potential connections between circRNAs and microRNAs which were involved in chronic pain were predicted by using TargetScan and miRanda, and the interaction results were displayed by Cytoscape. Four inflammation-related microRNAs exhibited interaction with the top 50 circRNAs (Fig. 2C).

Discussion

CircRNAs are mainly alternative splice variants of pre-mRNA [30]. Universal circRNAs expression alterations have been identified in various diseases, including cancer [31], Alzheimer's disease [32] and type 2 diabetes [33]. In our study, we revealed circRNAs expression changes at a genome-wide level by comparing circRNAs expression in DRG of CCI and NC rats.

CCI is one of the most frequently used model of neuropathic pain [34]. As shown by our PWT and PWL test, PWT and PWL were significantly decreased in the CCI group compared with that in the NC group, indicating that the CCI rat model is successfully established. CircRNAs samples were prepared from DRG of CCI and NC rats and were subjected to circRNAs array. Differential expression analysis identified remarkable expression changes in 374 circRNAs, which suggests universal changes in the expression of circRNAs in DRG of neuropathic pain rats. Besides, we also confirmed the reliability of our sequencing data, as a general consistency in expression levels was revealed between HTS and qPCR results.

The vast majority of circRNAs are generated from sequences of their host genes and some circRNAs also play roles in transcriptional or post-transcriptional regulation of their host genes [30]. Therefore, the functions of circRNAs may be relevant to their host genes. In our study, functional annotation analysis was performed for the host genes of DEcircRNAs to reveal the functions of DEcircRNAs. According to our results, a wide variety of KEGG and GO terms were identified, indicating a broad range of functional

modulations in DRG of neuropathic pain. The most significant functional terms included 'dopaminergic synapse', 'renin secretion', 'MAPK signaling pathway', and 'neurogenesis'. Dopaminergic functions are commonly modulated in neuropathic pain and administration of dopaminergic agents may relieve neuropathic pain[35]. Renin is a component of renin–angiotensin–aldosterone system (RAAS) and increased levels of renin are associated with neuropathic pain [36]. Increased phosphorylation of MAPK in DRG has also been shown to be an early event in neuropathic pain following peripheral nerve injury and the ERK/MAPK pathway has been considered as a novel therapeutic target for neuropathic pain [37]. Dysregulation of neuro-genes in the hippocampus has also been suggested to be involved in neuropathic pain [38]. Therefore, we hypothesized that the biological functions of DRG are widely modulated in neuropathic pain.

The main advantage of our study was that circRNAs expression in DRG was compared between CCI and NC rats, which provided a global picture about circRNAs expression alterations in DRG. However, the limitations of our study should also be noticed. The CCI model was the only neuropathic model included in our study. Additional neuropathic models are required in future studies to validate our results. Besides, functional studies should also be performed to elucidate the roles of DEcircRNAs in neuropathic pain.

Conclusions

We developed a CCI model and identified 374 significant DEcircRNAs in DRG of CCI and NC rats. These DEcircRNAs may be involved in various biological pathways and functions, which are potentially modulated in DRG by neuropathic pain. Further bioinformatics analyses suggested that these DEcircRNAs could be sponged with miRNAs which targeted mRNAs associated with chronic pain. Our study may provide new insights into the molecular mechanisms underlying neuropathic pain.

Abbreviations

CCI: Chronic constriction injury; NC: Negative control; cGMP: cyclic guanosine monophosphate; DRG: Dorsal root ganglion; ERK: Extracellular signal-regulated protein kinase MAPK: Mitogen-activated protein kinase; PKG: Protein Kinase G.

Declarations

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Availability of data materials

There is no data, software, databases and application/tool available apart from the reported in the present study. All data is provided in manuscript and supplementary data.

Authors' contributions

FL and ZW conceived of the study and participated in its design and coordination to draft the manuscript. ZW performed the CCI and behavioral assessment of pain. JW, ZW and WX acquired, analyzed and interpreted the data. All authors read and approved the final manuscript.

Competing Interests

The authors have declared that no competing interest exists.

Consent for publication

Not applicable.

Ethics approval

All animal procedures performed in this study were reviewed and approved by the Institutional Animal Care and Use Committee in Fudan University.

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Tables

Tables 1 and 2 were not provided with this version of the paper.

Figures

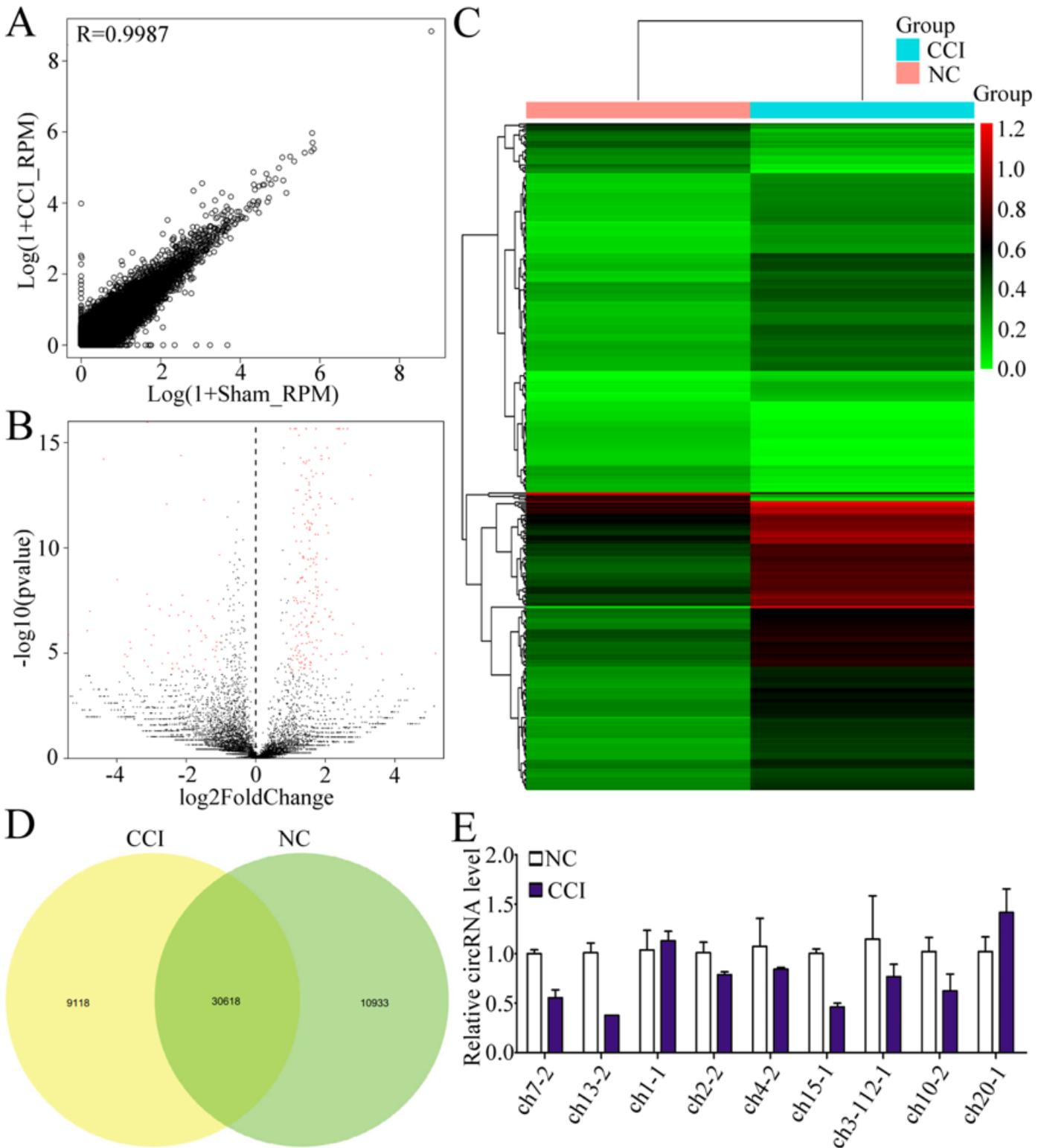


Figure 1

The differential expression of circRNAs (DEcircRNAs) in the DRG between negative control (NC) and chronic constriction injury (CCI) model. (A) Scatter plots showing the circRNA expression differences in the L4-5 DRGs of the CCI rats with respect to the NC group. (B) Volcano plot indicated DEcircRNAs in the in the L4-5 DRGs of CCI models. Red dots represent circRNAs with significantly expression, while black dots represent circRNAs with no significantly difference, respectively. (C) Heat map of DEcircRNAs

showing hierarchical clustering of changed DEcircRNAs of rats in CCI group compared with NC group. In clustering analysis, upregulated and downregulated genes are colored in red and green, respectively. (D) The Venn diagram indicated the number of overlap and distinct circRNAs between NC and CCI rats. (E) circRNAs expression changes validated by qPCR. qPCR analysis for differences in expression levels of DEcircRNAs in the DRG between CCI model and NC. Results were calculated by normalizing to GAPDH in the same sample with the $2^{-\Delta\Delta C_t}$ method. Changes in relative levels of circRNAs expressed as folds of controls. All values were mean \pm SEM. * $p < 0.05$ (n = 3)

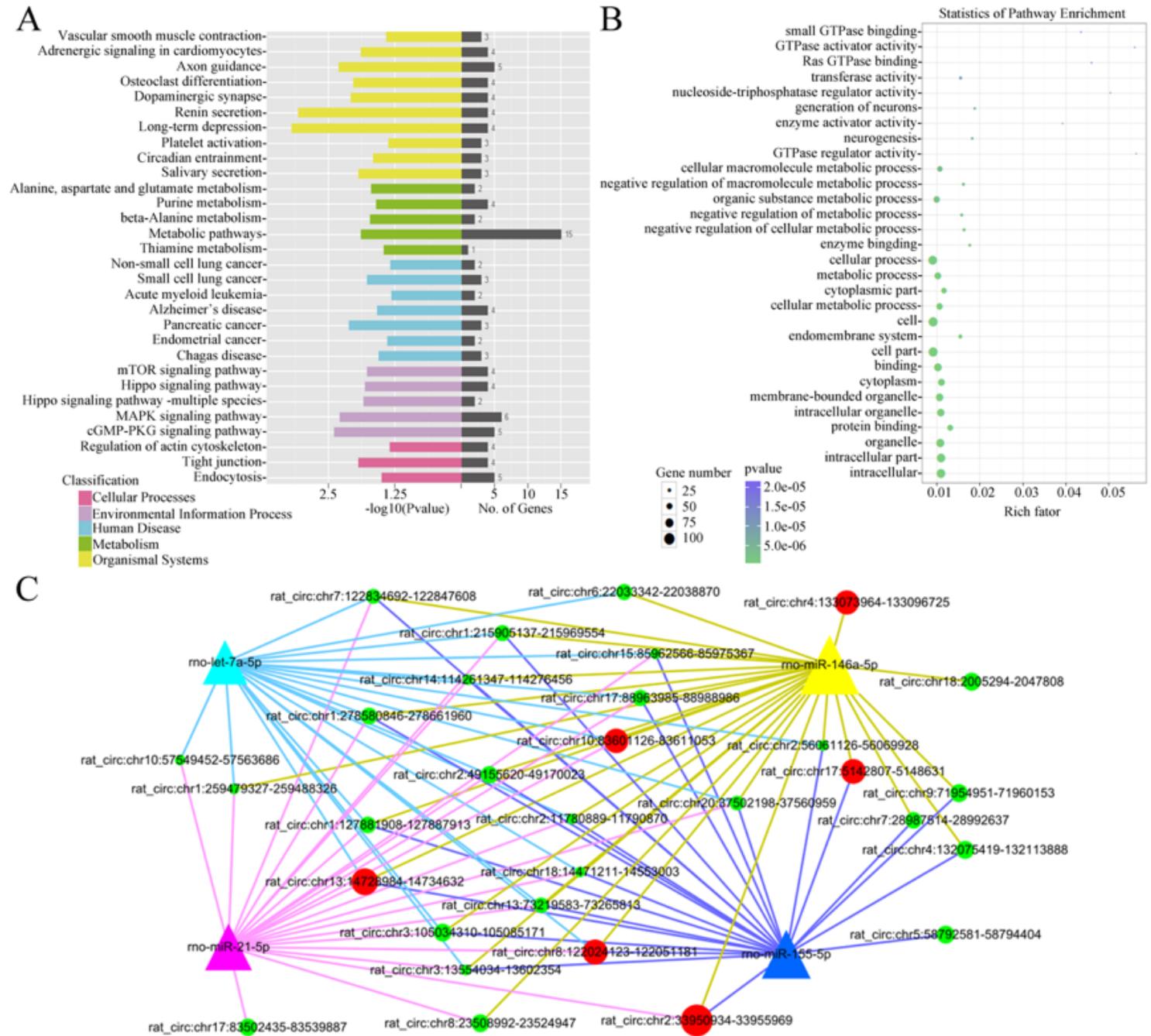


Figure 2

The DEcircRNAs functional analysis between NC and CCI rats. (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by DEcircRNAs. The vertical axis is the pathway category. The

horizontal axis is the $-\log_{10}$ (Pvalue) of the pathway and the number of DEcircRNAs; $p < 0.05$ was considered significantly. Different colors are used to distinguish Cellular Processes, Environmental Information Processing, Human Diseases, Metabolism and Organismal Systems. (B) The most enriched GO terms of DEcircRNAs. The vertical coordinate is the enriched GO terms, and the horizontal coordinate is the Rich factor. Rich factor value represents the enrichment degree. The size of points represents the number of DEcircRNAs in this term. The colors of the points correspond to different pvalue ranges. (C) CircRNA-microRNA competing endogenous RNAs network. The red nodes represent the up-regulated circRNAs, and the green nodes represent the down-regulated circRNAs. The size of each node represents the functional connectivity of each circRNA. The line color indicates potential interactions circRNAs and microRNAs which are predicted by using TargetScan and miRanda.

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