

Identification of key transcription factors associated with cerebral ischemia-reperfusion injury based on gene-set enrichment analysis

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Abstract. Cerebral ischemia-reperfusion injury (CIRI) usually causes detrimental complications following reperfusion therapy in stroke patients. The present study systematically investigated the regulatory mechanism involved in the pathogenesis of CIRI using gene set enrichment analysis of the transient middle cerebral artery occlusion mouse stroke model. The results revealed a total of 13 CIRI-related transcription factors (TFs), including CCAAT enhancer binding protein b (*Cebpb*), *Cebpa*, early growth response-1, *Fos*, *Rela*, *Jund*, signal transduction and activator of transcription 5a/b, transformation related protein 53, GLI family zinc finger 2 (*Gli2*), *Sp3*, TF AP-2 α (*Tfap2a*) and spleen focus forming virus proviral integration oncogene (*Spil*). To the best of our knowledge, five TFs (*Cebpa*, *Gli2*, *Sp3*, *Tfap2a* and *Spil*) were the first to be reported associated with CIRI in the present study. The five novel CIRI-related TFs were mainly associated with pathways of inflammation and responses to reperfusion, including the tumor necrosis factor signaling pathway (*Gli2*, *Spil* and *Tfap2a*, P=0.0035, 0.0035 and 0.048, respectively), interleukin-17 signaling pathway (*Cebpa*, *Gli2*, *Sp3*, *Spil* and *Tfap2a*, P=0.019, 0.047, 0.019, 0.035 and 0.005, respectively) and fluid shear stress and atherosclerosis (*Gli2*, *Sp3*, *Spil* and *Tfap2a*, P=0.047, 0.046, 0.013 and 0.003, respectively). These results may improve understanding of the potential molecular mechanism underlying the pathogenesis of CIRI at the genome-wide level.

Introduction

Cerebral ischemia-reperfusion injury (CIRI) is a complex pathophysiological process that occurs early in the restoration of blood supply following ischemia, and peaks at ~24 h after reperfusion (1,2). There are several factors that serve important roles in the pathogenesis of CIRI, including free radical formation, inflammatory cells infiltration, mitochondrial dysfunction and blood-brain-barrier disruption (3). These pathological events crucially rely on alterations in the transcription of various genes induced by ischemia and reperfusion. Numerous dysregulated genes associated with apoptosis, inflammation and metabolism were identified in a variety of ischemic stroke experimental models by high-throughput microarray or RNA-sequencing approaches (4,5); however, the underlying regulatory mechanism of gene dysregulation associated with CIRI requires further investigation.

Studies have reported that the activation of transcription factors (TFs) induced by different cellular signal transduction pathways after ischemia serves a pivotal role in controlling gene expression and is closely associated with ischemic injury and neuronal survival (6,7). Therefore, modulating the activity of CIRI-associated TFs, for example, via the recently reported RP105-phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (8), may be an effective strategy to disrupt CIRI. Studies have revealed that several TFs associated with neuronal injury are transcriptionally upregulated after focal ischemia, including interferon regulatory factor-1, nuclear factor- κ B (NF- κ B), early growth response-1 (Egr1), CCAAT enhancer binding protein- β (CEBP β), signal transduction and activator of transcription 3 (STAT3), and activating transcription factor-2 (9-13). However, additional TFs activities are thought to be regulated mainly by protein post-translational modifications, such as phosphorylation, which may not be investigated in traditional microarray expression analysis or experimental studies.

Considering that TFs regulate transcription by binding to specific regulatory elements upstream of the target genes, large-scale profiling of consensus TF binding sites (TFBS) from microarray expression data during ischemic brain injury can be another feasible option to comprehensively investigate

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the roles of TFs in cerebral ischemia. Several computational strategies were developed to predict TFs associated with cerebral ischemia, by detecting over-represented TFBS in the promoter regions of differentially expressed genes from microarray expression data (14-16). Although, to the best of our knowledge, few studies have focused on the TFs and their associated functions involved in CIRI after reperfusion via various pathways.

In the present study, we performed gene set enrichment analysis (GSEA) to determine the key TFs and their regulatory mechanisms of the biological pathways associated with CIRI. GSEA utilizes the whole-distribution testing model to identify the pre-defined TF-targeted gene-sets that are statistically enriched in CIRI group samples (17). In addition, the candidate TFs were re-calculated with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories associated with CIRI, using the 'Post-Analysis' feature of the EnrichmentMap software (18). Our findings could provide important insight into the various transcriptional mechanisms associated with CIRI during ischemic stroke.

Materials and methods

Microarray data. Microarray assays were performed based on a previous transcriptional study of mouse ischemic tolerance in transient middle cerebral artery occlusion (tMCAO) stroke model, which included 224 samples with 6 experimental conditions (19,20). In the present study, we investigated CIRI in mice suffering 45-min tMCAO or sham surgical procedure at 3 and 24 h after reperfusion ($n=4/\text{treatment/time}$), respectively (Table I). The original CEL files of the 45-min tMCAO group ($n=8$, I3 and I24 groups) and relevant sham surgery group ($n=8$, S3 and S24 groups) were downloaded from the National Center of Biotechnology Information Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) via the accession number GSE32529, which was based on the platform of the GPL1261 [Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array (Thermo Fisher Scientific, Inc.). Experimental analysis confirmed infarcts in tMCAO mice at 3 or 24 h after reperfusion, but not in sham-operated mice (19). It should be noted that we performed a bioinformatics study based on the public microarray data, and no mice were used for experimental analysis in the present study.

Data quality control and preprocessing. Data quality control and preprocessing were performed by the software packages of Bioconductor (v3.7, <https://www.bioconductor.org/>) based on the R statistical programming language (v3.5.0). Data quality was evaluated by RNA degradation plots using *AffyRNAdeg* algorithm in the *affy* package (v1.60.0) (21). The dataset showing a high slope of probe intensities from the 5' to 3' end, which serves as the quantitative indicator of the RNA degradation, was removed from our analysis prior to data preprocessing (Fig. S1A). For data preprocessing, we used the GC Robust Multi-array Average (gcRMA) algorithm in *gcRMA* package (v2.54.0) to normalize raw expression data and generate the normalized gene expression intensity of the two data-sets with logarithmic transformation. Following normalization, the distributions of the normalized probe set intensities were evaluated by density histograms and

boxplot (Fig. S1B and C). In the final step, the probe ID in normalized expression data was annotated to gene symbol based on the *mouse4302cdf* package (v2.18.0), and the data were exported to a formed text file ready for GSEA.

GSEA and leading-edge analysis. The gene set enrichment analysis and leading-edge analysis were performed using GSEA desktop application version 3.0 (Broad Institute), which employs predefined gene sets from the Gene Set Knowledgebase database (<http://ge-lab.org/#/data>). In the present study, GSEA was performed using the collected gene-sets of TF target genes (*mm_TF*), GO (*mm_GO*) and metabolic pathways (*mm_metabolic*), with the following parameters: 1,000 gene set permutations, weighted enrichment statistics, gene set size between 15-500, and signal-to-noise metrics for ranking genes. Regulated gene-sets were considered statistically significant if false discovery rate (FDR) ≤ 0.1 and nominal $P \leq 0.01$. The GSEA-derived normalized enrichment score was used to determine the magnitude of up- or down-regulation of enriched gene-sets. Leading-edge analysis was performed after each GSEA to determine the core genes that have the highest impact on the biological process of a given gene-set.

For each database of gene sets, GSEA was respectively performed across two expression datasets: i) S3I3 dataset, which contains the expression data from the sham and tMCAO groups 3 h after reperfusion; ii) S24I24 dataset, which contains the expression data from the sham and tMCAO groups 24 h after reperfusion; and iii) I3I24 dataset, which contains the expression data from the tMCAO groups of 3 and 24 h after reperfusion.

Functional enrichment visualization and annotation. The GSEA results were visualized using EnrichmentMap (v3.1) plugin based on Cytoscape Desktop program (v3.6.1, <https://cytoscape.org/>) (18). The enrichment results were mapped as a network of gene-sets (nodes) where the nodes represent statistically significant terms and the links (edges) represent the degree of gene-set similarity; combined Jaccard (50%) and Overlap (50%) metrics with the default cutoff of 0.375 were employed. For gene-sets annotation, the enriched gene-sets were grouped by AutoAnnotate software (v1.2, <http://apps.cytoscape.org/apps/autoannotate>) according to the Markov cluster (MCL) algorithm based on the edge weights of similarity coefficients and were automatically annotated using the WordCloud algorithm with the maximum of four words per label (22).

KEGG (<https://www.genome.jp/kegg/>) pathway enrichment analysis was performed using clusterProfiler package (v3.8.1) (23) and visualized via the *ggplot2* package (v3.0.0) in R (24). The significant KEGG categories were identified with BH adjusted $P < 0.01$ and FDR $q < 0.05$.

Signature gene-sets post-analysis. Using the 'Post-Analysis' feature of the EnrichmentMap software, we constructed the transcriptional regulatory network associated with CIRI. The CIRI-related signature gene-sets were primarily defined as the gene-sets specifically enriched in the I24 group by Venn analysis (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) of the GSEA results between S3I3 and S24I24 datasets. The *gmt* file of I24-specific TFs signature gene-sets (*mm_I24_TF*) was created from the *mm_TF* gene-set database using custom

Table I. Description of the tMCAO experimental groups.

Group	Sample no.	Treatment	Time point (h)
S3	GSM805706	Sham	3
S3	GSM805707	Sham	3
S3	GSM805708	Sham	3
S3	GSM805709	Sham	3
I3	GSM805730	tMCAO	3
I3	GSM805731	tMCAO	3
I3	GSM805732	tMCAO	3
I3	GSM805733	tMCAO	3
S24	GSM805714	Sham	24
S24	GSM805715	Sham	24
S24	GSM805716	Sham	24
S24	GSM805717	Sham	24
I24	GSM805741	tMCAO	24
I24	GSM805742	tMCAO	24
I24	GSM805743	tMCAO	24
I24	GSM805744	tMCAO	24

tMCAO, transient middle cerebral artery occlusion.

written R code. The transcriptional regulatory network was constructed based on the GSEA results of the GO or metabolic pathway network in EnrichmentMap, followed by post-analysis with CIRI-associated TFs signature gene-sets (mm_I24_TF), respectively. The edge weights, which represent the overlap between the signature gene-sets and enriched gene-sets in the network, were computed by Mann-Whitney U test with $P=0.05$, and were visualized as pink edges with thickness indicating the degree of significance.

Microarray gene-expression analysis. The gene expression analysis of CIRI-associated TFs and their targeted genes were performed using linear regression models implanted in limma package (v3.38.2); P-values were calculated using an empirical Bayesian method, adjusted by Bonferroni correction (25). The pheatmap package (v1.0.10) was used to show the expression pattern of CIRI-associated TFs in the S3I3 and S24I24 datasets, and the hierarchical clustering method was selected as 'complete'.

Results

Differentially enriched GO terms associated with CIRI in tMCAO. Cerebral ischemia/reperfusion induces a cascade of secondary delayed cell deaths that are partially mediated by alterations in molecular transcriptional activities during reperfusion (4,26). To improve understanding into the transcriptional mechanisms of CIRI, we performed a computational analysis workflow to identify candidate CIRI-associated TFs and their regulatory mechanisms, by combining a statistically principled GSEA with differentially expressed signature gene-sets post-analysis (Fig. 1).

GSEA of S3I3 and S24I24 datasets yielded a total of 423 and 953 GO terms that were significantly enriched at 3 and 24 h

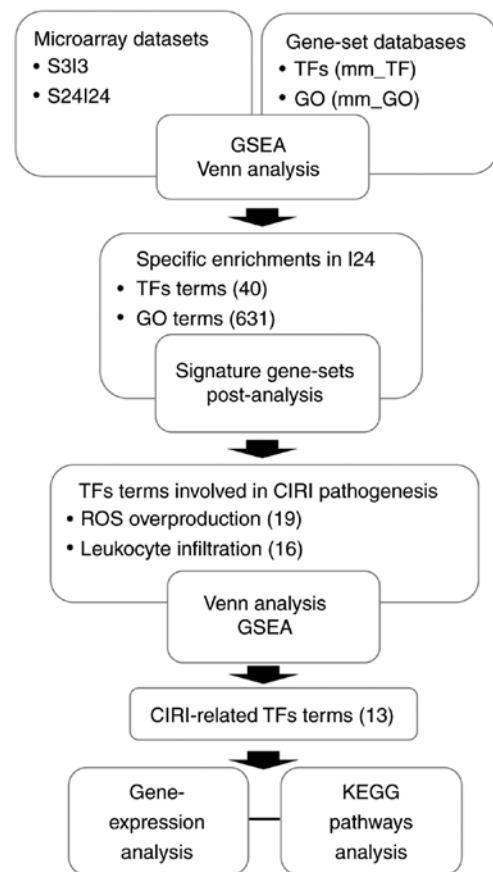


Figure 1. Workflow of analysis. The strategy was executed in a three-step manner. In the first step, gene set enrichment and Venn analyses were performed to identify the GO terms and TF-target gene-sets (TF terms) that were specifically enriched in the I24 group. In the second step, we calculated the gene-sets similarity of TF-target gene-sets and GO terms obtained from the first step using the 'Post-Analysis' feature of EnrichmentMap and extracted the TF-target gene-sets associated with 'ROS biosynthesis' and 'leukocytes infiltration process'. These TFs were considered as CIRI-associated TFs. In the final step, the transcriptional expression patterns of these CIRI-related TFs and their functions involved in CIRI pathogenesis were identified. CIRI, cerebral ischemia-reperfusion injury; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROS, reactive oxygen species; TF, transcription factor.

after reperfusion in tMCAO mice, respectively (Table II). As CIRI is generally considered to peak at ~24 h after reperfusion (2,27), we further identified the GO terms specifically regulated 24 h after reperfusion by Venn analysis of the enriched GO terms between the I3 and I24 groups. To improve understanding of the biological significance of these I24-specific GO terms, 631 GO terms were grouped into several major clusters with semantic annotations (Fig. 2), most of which were previously reported to be associated with the pathogenesis of CIRI, including 'lymphocytes mediated immunity' (55 terms), 'lymphocytes proliferation' (55 terms), 'nucleocytoplasmic transport' (24 terms), 'JAK-STAT cascade' (19 terms), 'Toll-like receptor pathway' (16 terms), 'reactive oxygen species (ROS) biosynthetic process' (13 terms), 'cytokine secretion' (12 terms), 'endopeptidase activity' (12 terms) and 'coagulation' (11 terms). A total of 631 GO terms were specifically enriched 24 h following reperfusion; four terms were downregulated and 627 terms were upregulated (Fig. 3A).

Table II. Number of gene-sets differentially enriched in tMCAO at 3 h (S3I3) and 24 h (S24I24) after reperfusion.

Gene-sets	S3I3 dataset			S24I24 dataset		
	Upregulated	Downregulated	Total	Upregulated	Downregulated	Total
Transcription factor-targets	34	1	35	65	0	65
Gene ontology-terms	423	0	423	949	4	953

tMCAO, transient middle cerebral artery occlusion.

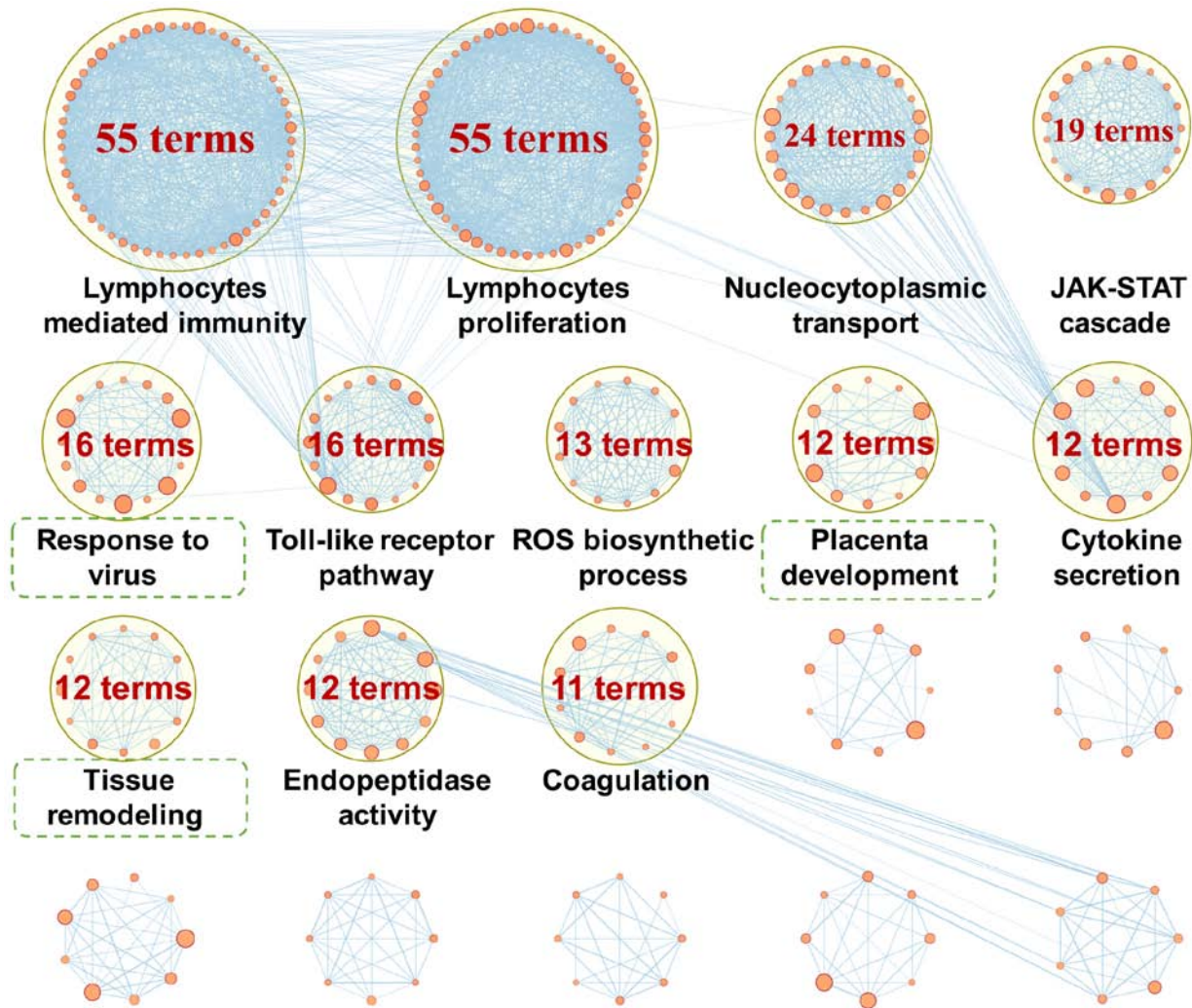


Figure 2. Clusters of functionally-associated I24-specific GO terms. The enrichment results of I24-specific biological pathways and processes were mapped to a network of GO terms (nodes), where the node size represents the number of genes within each GO term and the thickness of blue lines (edges) represents the degree of GO term similarity. The network was automatically clustered and circled by AutoAnnotate software; each cluster was textually annotated and visualized in the EnrichmentMap software. This clustered network map was manually curated removing general and uninformative sub-networks, resulting in a simplified network. The labels marked with dashed lines indicate the clusters that are functionally unassociated with the present study. GO, Gene Ontology; STAT, signal transduction and activator of transcription.

Identification of key transcription factors associated with CIRI. Oxidative stress and inflammation are the two most important mechanisms in the pathogenesis of CIRI, in which ROS overproduction and leukocyte infiltration are key initiation processes (1). Therefore, TFs involving both ROS generation and leukocytes infiltration processes are likely to participate in the pathogenesis of CIRI.

Following GSEA using mm_TF gene-sets database, we identified 35 and 65 TF-target gene-sets that were significantly enriched at 3 and 24 h after reperfusion in tMCAO mice, with 40 TF-target gene-sets specifically enriched at 24 h after reperfusion (I24-specific TF-target gene-sets, Table II and Fig. 3A). Then, the gene set similarity of I24-specific TF-target gene-sets to I24-specific GO terms

Table III. General description of candidate cerebral ischemia-reperfusion injury-related TF-target gene-sets and their target leading edge genes.

TF gene-sets	Symbol	Name	Description	Leading edge genes
ZHAO_SP3	<i>sp3</i>	SP3	Trans-acting transcription factor 3	Tgm1/Coll1a2/Ccl2/Mmp9/Ucp2/Cdkn1a/Rac2/Tal1/Fbln1/Pcyl1a/ Abcc1/Aebp1
ZHAO_CEBPB	<i>cebpb</i>	C/EBP beta	C/EBPβ	Saa3/Il6/Abcb1b/Cdkn1a/C3/Nupr1/Coll1a1
WINGENDER_RELTA	<i>rela</i>	P65	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	Il6/Myd88/Tlr13/Tlr2/Ccnd1/Lcn2/Serpine1/Zfp36
WINGENDER_CEBPA	<i>cebpa</i>	C/EBP alpha	C/EBPα	Il6/Fabp4/Hmox1/Lcn2/Serpine1/Cebpa/Abcc3/
TFACTS_TP53	<i>trp53</i>	P53	Transformation related protein 53	Bcl3/Upp1/Igfbp3/Casp1/Spp1/Xpc/Eomes/Ccnd1/Brca1/Birc5/Ezh2/Cdkn1a/Myc/Mki67/C1s1/Trp53/Abcc3/Rela/Phlda3/Notch1/Bak1/Abcc1/Rrm1/Afp/Prkab1
TFACTS_TFAP2A	<i>tfap2a</i>	AP-2 alpha	Transcription factor AP-2 α	Il6/Timp1/Igfbp3/Nos3/Mmp9/Hk2/Th/Hmox1/Pecam1/Gfap/Cdkn1a/Myc/Plaur/Hook2/Cebpa/Fbln1/Plat/Mcam/Adm/Calb2/Tnpo1/Coll1a1/Taf7/Cyp11a1
TFACTS_STAT5B	<i>stat5b</i>	STAT5B	Signal transducer and activator of transcription 5B	Socs3/Myd88/Tlr2/Fcgr1/A2m/Cd84/Ccnd1/Klk8/Igf1/Cish
TFACTS_STAT5A	<i>stat5a</i>	STAT5A	Signal transducer and activator of transcription 5A	Socs3/Myd88/Tlr2/Fcgr1/A2m/Cd84/Ccnd1/Klk8/Igf1/Cish
TFACTS_SPI1	<i>spi1</i>	PU.1	Spleen focus forming virus proviral integration oncogene	Ncf4/Vav1/Fcgr1/Cd163/Itgb2/Nos3/Ptpcr/Prtn3/Tlr4/Msr1/Cd68/Cd72/Ctsk/Tal1/Pdgfrb/Il1b/Chil1/Lsp1/Icam1/Fli1
TFACTS_GLI2	<i>gli2</i>	GLI2	GLI-Kruppel family member GLI2	Il6/Tnc/Tgfb1/Il1r2/Igfbp3/Rapgef1/Tead1/Ccnd1/Ccl17/Irf9/Fen1/Bmp7/Cdkn1a/Myc/Hpgd/Npy2r/Efna1/Ifngr1/Itga6/Lum/Cks1b/Cflar/Col5a2/Edn1/Lamb3/Mad211
TFACTS_FOS	<i>fos</i>	FOS	FBJ osteosarcoma oncogene	Mmp3/Il6/Tgm1/Itga5/Timp1/Th/Msr1/Plaur/Nqo1/Trp53
TFACTS_EGR1	<i>egr1</i>	EGR1	Early growth response 1	Il6/Cd44/Coll1a2/Spint1/Apoa1/Cebpb/Th/Hmox1/Nudt6/Tfpi2/Pecam1/Twist1/Serpine1/Trp53/Elk1/Cebpa/Rela/Cd9/Icam1
TFACTS_JUND	<i>jund</i>	JUND	Jun D proto-oncogene	Il6/Fos11/Timp1/Spp1/Th/Cdkn1a/Plaur/Nqo1/Plat

C/EBP, CCAAT/enhancer binding protein; TF, transcription, factor.

were determined using the ‘Post-Analysis’ feature of EnrichmentMap; 19 and 16 TF-target gene-sets were significantly associated with ‘ROS biosynthetic process’ and ‘leukocytes infiltration’, respectively (Fig. 3B). Of these TF-target gene-sets, 15 were finally determined as candidate CIRI-related TF-target gene-sets that were associated with ‘ROS biosynthesis’ and ‘leukocyte infiltration’. In addition, we further employed these 15 CIRI-related

TF-target gene-sets in the I3I24 dataset for GSEA to identify CIRI-related TFs that were significantly induced after 24 h of reperfusion than after 3 h. From the GSEA results, two TFs, upstream transcription factor 1 (*Usf1*) and *Cebpd* were excluded as they were not significantly enriched in the I24 group under the cutoff adjusted $P \leq 0.05$ (Fig. 3C). An overview of these 13 candidate CIRI-associated TF-target gene-sets is presented in Table III.

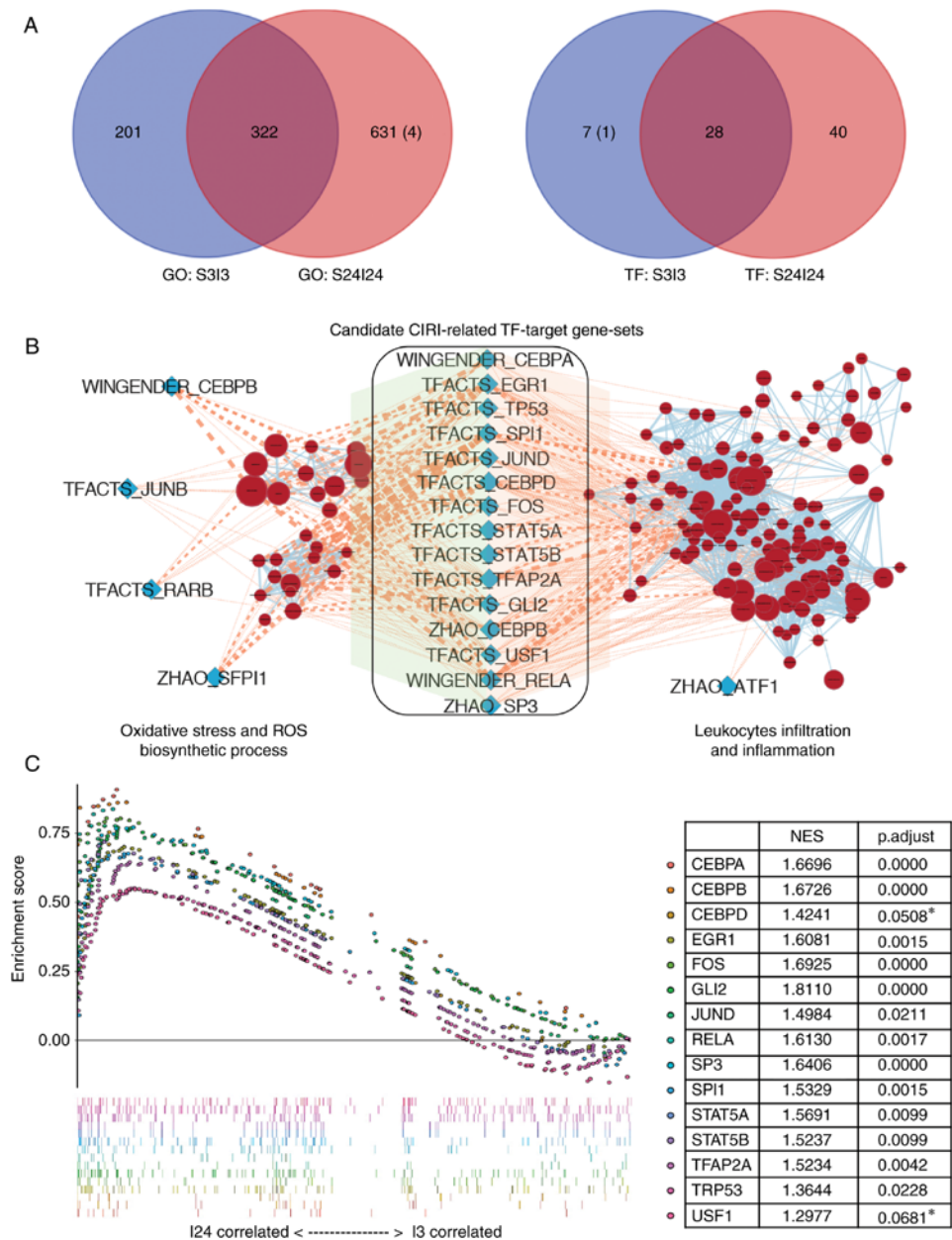


Figure 3. Identification of key transcription factors associated with CIRI. (A) Venn diagrams of the numbers of GO terms (left panel) or TF-target gene-sets (right panel) significantly enriched in the ischemic brains of middle cerebral artery occlusion mice after 3 h (S3I3) or 24 h (S24I24) of reperfusion. The number of terms enriched in the sham control group was shown in parentheses. (B) Enrichment map for CIRI-associated GO terms and overlap with TF-target gene-sets. The map presented the specifically enriched GO terms of ‘ROS biosynthesis’ and ‘leukocytes infiltration’ process (brick red circles) in I24 group vs. sham controls. The blue diamonds represented the TF-target gene-sets that were specifically enriched in the I24 group. Pink edges indicated overlap between the TF-target gene-sets and enriched GO terms; thickness represented significance. Only edges with nominal $P < 0.05$ based on a Mann-Whitney U test were visualized. (C) Identification of CIRI-related TF-target gene-sets by gene set enrichment analysis between I3 and I24 groups. A total of two TFs, *Usf1*, and *Cebpd*, with adjusted $P > 0.05$, were marked with asterisks and excluded for the further study. CEBP, CCAAT enhancer binding protein; CIRI, cerebral ischemia-reperfusion injury; GO, gene ontology; ROS, reactive oxygen species; STAT, signal transduction and activator of transcription; TF, transcription factor; TRP53, transformation related protein 53; *Usf1*, upstream transcription factor 1.

Gene expression analysis of CIRI-related TFs and their target genes. Although all the CIRI-related TF-target gene-sets were specifically induced in the injured brain after 24 h of reperfusion, gene expression analysis demonstrated that these CIRI-related TFs and their target genes exhibited variable transcriptional expression patterns in S3I3 or S24I24 dataset. As presented in Fig. 4A, one group of TFs including *Stat5b*, transformation related protein 53 (*Trp53*), TF AP-2 α (*Tfap2a*), spleen focus forming virus proviral integration oncogene (*Sp1*), and *Sp3* exhibited no significant difference in expression in the S3I3 and

S24I24 datasets, while another group of TFs with *Fos* and *Egr1* were significantly upregulated in both datasets. GLI family zinc finger 2 (*Gli2*) and *Jund* exhibited opposing expression patterns, in which *Gli2* was downregulated in the S24I24 dataset while *Jund* was upregulated in the S3I3 dataset. The last group of TFs with *Stat5a*, *Rela*, *Cebpb* and *Cebpa* were overexpressed specifically in the S24I24 dataset but not in S3I3.

In addition, we performed leading-edge analysis with the 13 CIRI-related TF-target gene-sets following GSEA between the I3 and I24 groups to identify the target genes regulated by

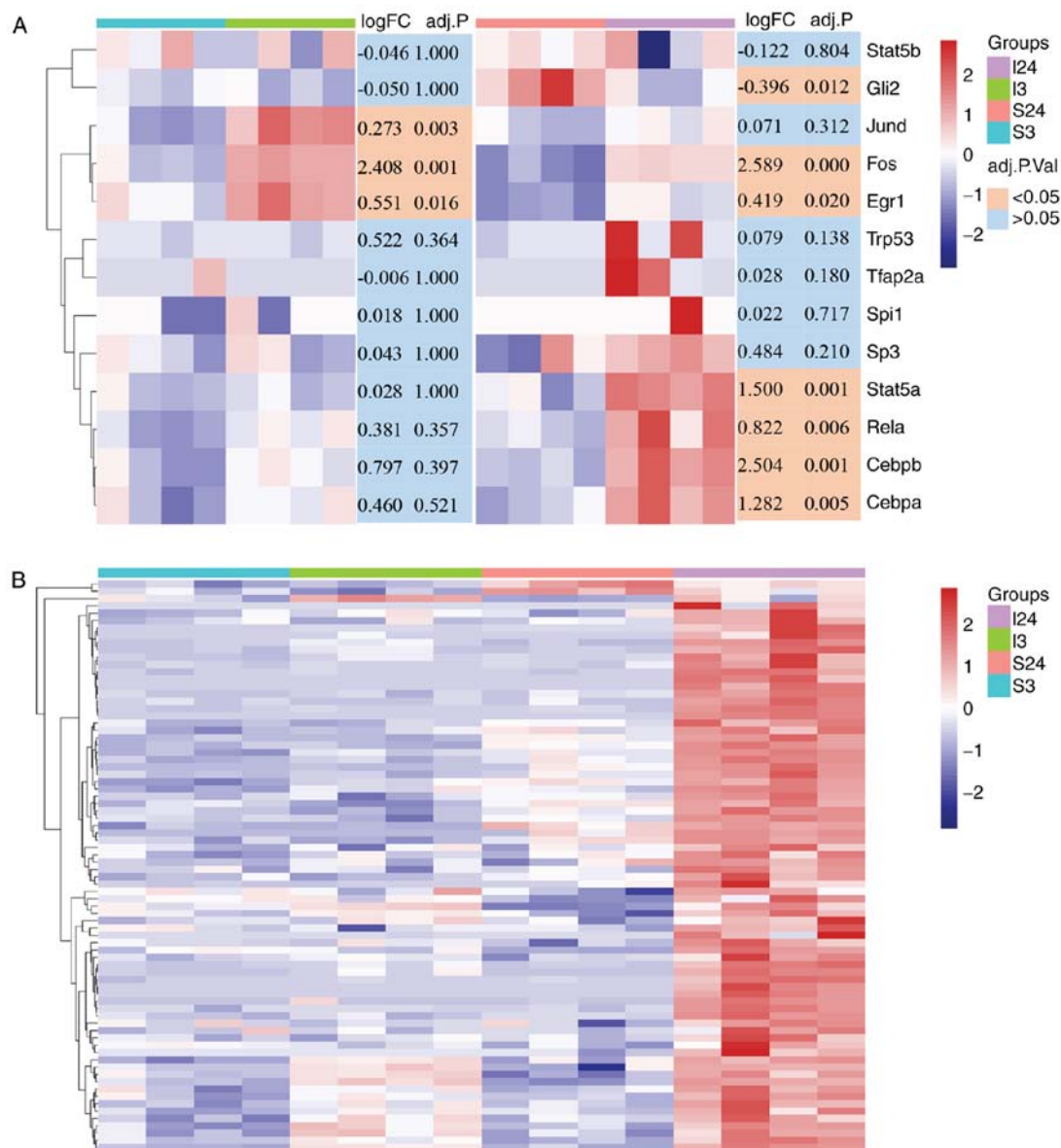


Figure 4. Heatmap analysis of CIRI-related TFs and their target genes. (A) The transcriptional expression patterns of CIRI-related TF genes in the ischemic brain after 3 and 24 h of reperfusion compared with the relevant sham controls. The color blocks in the lower right of heatmap represent the significance of the differential expression of CIRI-related TF genes, orange, adjusted $P < 0.05$; light blue, adjusted $P > 0.05$. (B) Heat map of CIRI-related TFs target genes ($n=78$) significantly upregulated (adjusted $P < 0.05$) in the I24 group as compared with sham control subjects (S24). These genes were identified by leading-edge analysis after gene set enrichment analysis between I3 and I24 groups, and the log2 transformed data were used for heatmap analysis. The y-axis data are individual genes, the color bar in the heatmap right represents the gene expression level (blue, downregulation and red, upregulation). The color blocks in the upper right of the heatmap indicate the experimental groups (purple, I24 group; green, I3 group; pink, S24 group and cyan, S3 group). CEBP, CCAAT enhancer binding protein; CIRI, cerebral ischemia-reperfusion injury; Egr1, early growth response-1; Gli2, GLI family zinc finger 2; Spi1, spleen focus forming virus proviral integration oncogene; Stat, signal transduction and activator of transcription; TF, transcription factor.

these CIRI-related TFs. A total of 123 target genes regulated by the 13 CIRI-associated TFs were identified in the I3I24 dataset, of which 78 genes were significantly overexpressed in the I24 group and were presented as a heatmap (Fig. 4B). The statistical results of expression of the 78 target genes in the S3I3 and S24I24 datasets were listed in Table S1.

Enrichment analysis for target genes of CIRI-associated transcription factors. The 78 overexpressed genes in the I24 group were used for enrichment analysis to determine significant KEGG pathways in which CIRI-related TFs were involved. The results demonstrated that these target genes of CIRI-related TFs were mainly involved in the 'PI3K-Akt signaling pathway'

(adjusted $P=3.54 \times 10^{-5}$), 'tumor necrosis factor (TNF) signaling pathway' (adjusted $P=4.01 \times 10^{-7}$), 'hypoxia-inducible factor-1 (HIF-1) signaling pathway' (adjusted $P=3.81 \times 10^{-7}$), 'focal adhesion' (adjusted $P=1.55 \times 10^{-4}$) and 'JAK-STAT signaling pathway' (adjusted $P=5.74 \times 10^{-5}$) (Fig. 5A). In addition, the KEGG pathways involving each CIRI-related TF were further analyzed by calculating the gene-sets similarity of CIRI-related TFs with the KEGG pathways enriched in the S24I24 dataset using the 'Post-Analysis' feature of EnrichmentMap (Fig. 5B and C, $P \leq 0.05$). Of these CIRI-related TFs, 5 TFs (*Cebpa*, *Gli2*, *Sp3*, *Tfap2a* and *Spi1*) were reported to be associated with CIRI, which were mainly involved in the pathways related to inflammation and responses to reperfusion, including the

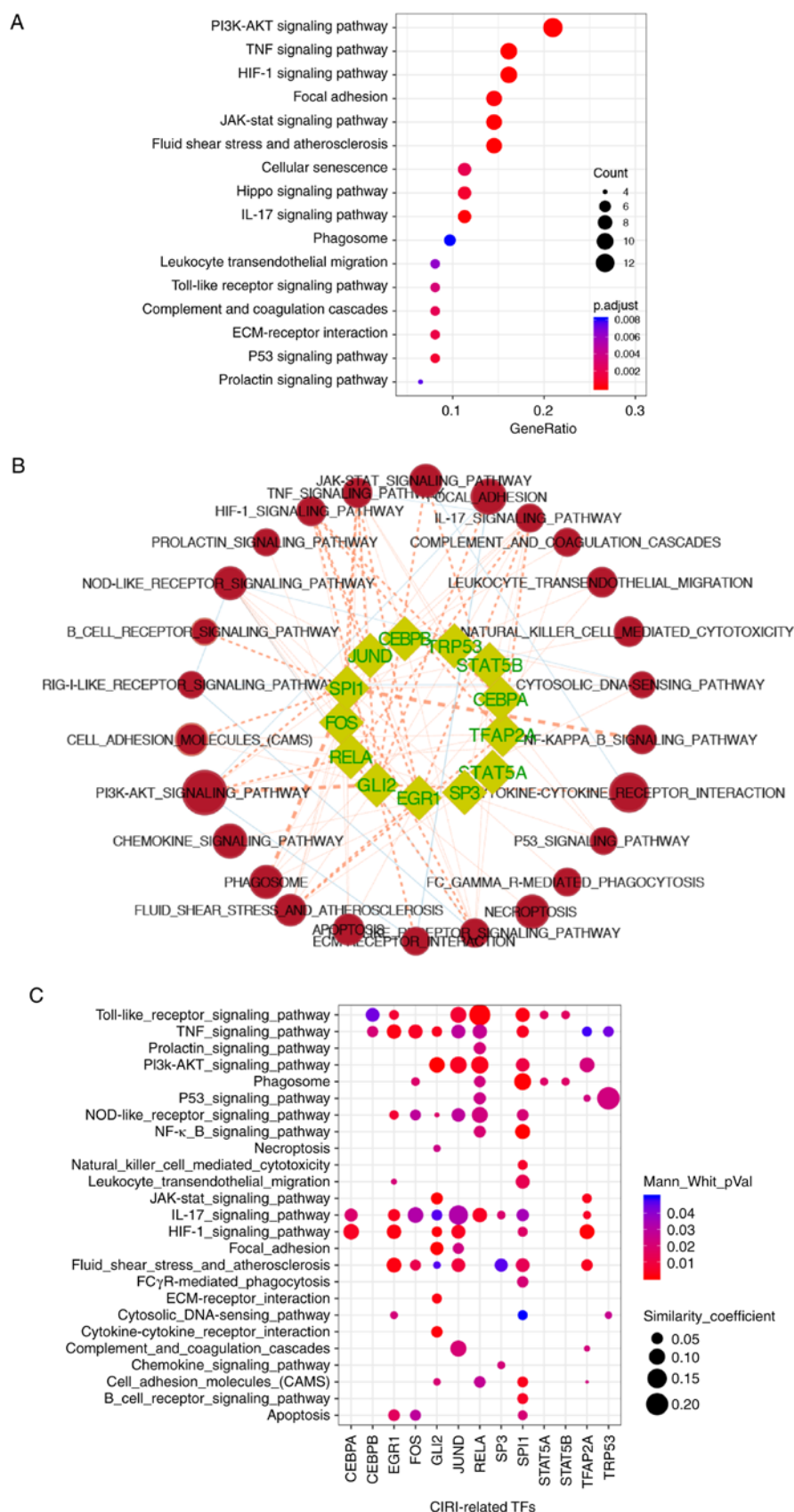


Figure 5. Functional enrichment analysis of CIRI-associated TFs and their target genes. (A) Dot plot showing enrichment of KEGG pathway for the list of CIRI-related TFs target genes (adjusted $P < 0.05$). The dot sizes represent the numbers of genes, the color of dots corresponds to the adjusted P -value. (B) Enrichment map for KEGG pathways and overlap with TF-target gene-sets. The map indicated the specifically enriched KEGG pathways (brick red filled circles) in I24 group vs. sham controls. The yellow diamonds represented the TF-target gene-sets that were specifically enriched in the I24 group. Pink edges indicated overlap between the TF-target gene-sets and enriched KEGG pathways; thickness represented significance. Only edges with nominal $P < 0.05$ based on a Mann-Whitney U test were visualized. (C) Dot plot of KEGG pathway enrichment for each CIRI-related TF. The dot sizes represent the coefficient similarity of the KEGG pathway and CIRI-related TF; the color of dots corresponds to the Mann-Whitney U test nominal P -value. CIRI, cerebral ischemia-reperfusion injury; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; PI3K, phosphatidylinositol 3-kinase; TF, transcription factor.

'TNF signaling pathway' (*Gli2*, *Spi1* and *Tfap2a*, $P=0.0035$, 0.0035 and 0.048 , respectively), 'interleukin (IL)-17 signaling pathway' (*Cebpa*, *Gli2*, *Sp3*, *Spi1* and *Tfap2a*, $P=0.019$, 0.047 , 0.019 , 0.035 and 0.005 , respectively), and fluid shear stress and atherosclerosis (*Gli2*, *Sp3*, *Spi1* and *Tfap2a*, $P=0.047$, 0.046 , 0.013 and 0.003 , respectively). In particular, *Spi1* was observed to be significantly involved in the majority of the enriched KEGG pathways of S24I24, including 'NF- κ B signaling pathway' ($P<0.001$), 'phagosome' ($P<0.001$), 'Toll-like receptor signaling pathway' ($P=0.002$), 'TNF signaling pathway' ($P=0.004$), 'PI3K-Akt signaling pathway' ($P=0.010$), 'HIF-1 signaling pathway' ($P=0.017$), 'apoptosis' ($P=0.021$), 'cell adhesion molecules' ($P=0.003$), 'NOD-like receptor signaling pathway' ($P=0.021$), 'cytosolic DNA-sensing pathway' ($P=0.050$), 'natural killer cell mediated cytotoxicity' ($P=0.005$), 'IL-17 signaling pathway' ($P=0.035$), 'B cell receptor signaling pathway' ($P=0.004$), 'FC γ R-mediated phagocytosis' ($P=0.021$), 'leukocyte trans-endothelial migration' ($P=0.013$), and 'fluid shear stress and atherosclerosis' ($P=0.013$).

Discussion

In the present study, the gene expression profiles of ischemic stroke were systematically analyzed at different time points (3 and 24 h) after reperfusion using the GSEA bioinformatics method to reveal the possible transcriptional regulatory mechanisms associated with CIRI at various functional levels. We identified 13 key CIRI-related TFs, of which 5 CIRI-related TFs (*Cebpa*, *Gli2*, *Sp3*, *Tfap2a* and *Spi1*) were reported, and the biological pathways involved following ischemic stroke were determined. To the best of our knowledge, this is the first bioinformatics study to characterize CIRI-related TFs in the context of the pathogenesis of CIRI.

Oh *et al* (28) investigated genetic alterations in the peripheral blood after acute ischemic stroke, suggesting the detrimental effects of peripheral white blood cells on the ischemic brain after reperfusion. In the present study, the GO terms specifically enriched at 24 h post-reperfusion in an MCAO rat model were analyzed to determine the CIRI-related TFs associated with ROS overproduction and leukocyte infiltration. It has been reported that differentially expressed genes at 24 h after cerebral ischemia/reperfusion in MCAO rats are associated with inflammation, apoptosis, stress and immune responses, and are involved in the pathogenesis of CIRI (29,30). Our results from GSEA are consistent with previous findings, indicating that I24-specific GO terms were mainly enriched in immune cells activity and inflammation, and their related pathways, including the Jak-stat cascade and the Toll-like receptor pathway. In addition, GSEA also revealed several functional clusters of CIRI-related GO terms that have not previously been reported in high throughput studies (30), such as 'nucleocytoplasmic transport,' 'endopeptidase activity' and 'coagulation'. Importantly, the enrichment of nucleocytoplasmic transport-associated genes could reveal the enhancement of transcriptional activity in the ischemic brain 24 h after reperfusion, further indicating the pivotal roles of novel transcripts induced by reperfusion in the pathogenesis of CIRI.

Transcription factors can be induced in ischemic stroke, and are involved in neuronal injury or neuroprotection at different stages after reperfusion by regulating post-ischemic

inflammation (6). In the present study, it was determined that 8 CIRI-related TFs, including *Cebpb*, *Egr1*, *Rela*, *Jund*, *Stat5a/b*, *Fos* and *Trp53* were functionally activated at 24 h after reperfusion, as other studies have previously reported (6,31,32). Of these TFs, the activation of *Jund* (31), *Stat5a/b* (32), *Fos* and *Trp53* is known to prevent ischemic neuronal damage, whereas the induction of *Cebpb*, *Egr1* and *Rela* promotes inflammation and neuronal death following cerebral ischemia (6). KEGG pathway analysis revealed that these CIRI-related TFs were not only involved in pro-inflammatory pathways to promote cell death but in neuroprotective pathways to promote neuron survival. These results demonstrated that TFs were important endogenous modulators, which may be actively involved in the balance of brain damage and repair during the pathogenesis of CIRI, by coordinating numerous downstream genes that govern inflammation.

Furthermore, the present study reported that three CIRI-related TFs (*Jund*, *Fos* and *Egr1*), belonging to the immediate early gene family, were significantly upregulated as early as 3 h after reperfusion. These TFs could be rapidly induced at the onset of hypoxic conditions to trigger (*Egr1*) or suppress (*Jund* and *Fos*) the first wave of inflammatory responses in the ischemic brain or other organs (11,33). An additional group of CIRI-related TFs, including *Stat5a*, *Rela*, and *Cebpa/b*, can be induced by *Fos* and *Egr1*, and were significantly overexpressed 24 h after reperfusion. These TFs may act in a positive regulatory loop and promote of CIRI at 24 h after reperfusion (34).

Other CIRI-related TFs, including *Cebpa*, *Gli2*, *Sp3*, *Tfap2a* and *Spi1* were first identified to be associated with CIRI in this study. The TFs *Cebpa* and *Spi1* are two myeloid-specific proteins involved in the differentiation of monocyte lineages (35). A previous study revealed that the decrease in CEBP α/β DNA-binding activity was inevitably associated with CIRI in a long-term (30 days) global cerebral ischemia model (36). In addition, the expression of *Cebpa* was induced in microglia after hypoxic-ischemic brain injury (37). In addition, *Cebpa* can combine with *Spi1* to enhance monocyte-specific promoter activity and affect NF- κ B activity in response to TNF- α signaling (38). It is well reported that increased inflammation and inflammatory proteins, such as TNF- α , serve critical roles in the pathogenesis of CIRI via NF- κ B signaling pathway (34). Therefore, *Spi1* and *Cebpa* may serve a synergistic pro-inflammatory role in the pathogenesis of CIRI. *Sp3* is a transcriptional repressor that, in complex with RE1-silencing transcription factor, inhibits the gene expression of Na⁺-Ca²⁺ exchanger 1 in the tMCAO rat model (39). Our functional enrichment analysis demonstrated that *Sp3* was activated in the I24 group and was functionally associated with the activation of the IL-17 signaling pathway. Previous studies have reported that inhibiting the IL-17 signaling pathway reduced neutrophil infiltration and protected against ischemic stroke (40). *Sp3* activation may promote neutrophil invasion via the IL-17 signaling pathway, which then promotes the pathogenesis of CIRI-induced cerebral edema.

Gli2 is generally considered as a potent oncogene by activating the Sonic hedgehog (Shh) signaling pathway in embryonal cancer cells (41,42). Previously, the Shh signaling pathway was shown to serve an important role in neuroprotection and neurogenesis via a mechanism of anti-oxidative stress

in a rodent stroke model after cerebral ischemia (43). In the present study, the transcriptional activity of *Gli2* was significantly activated and closely associated with the PI3K-Akt signaling pathway, suggesting the neuroprotective effect of *Gli2* in response to CIRI. *Tfap2a* has been considered to be a tumor-related gene that regulates tumor growth and survival via the HIF-1 α signaling pathway (44). It was previously demonstrated that *Tfap2a* served an important role in the repair of myocardial ischemic injury via an Akt-dependent signaling pathway (45). The present study reported that *Tfap2a* activation was involved in the HIF-1 and PI3K-Akt signaling pathways, suggesting other biological functions of *Tfap2a* in the pathogenesis of CIRI in addition to tumorigenesis. Collectively, these findings indicated that these CIRI-related TFs served complex and critical roles in the pathogenesis of CIRI at the level of various pathways following ischemia/reperfusion. This supports the hypothesis that TFs could be effective targets for alleviating CIRI.

The lack of further experimental verification of these CIRI-TFs is the largest limitation of the present study. In particular, the newly identified CIRI-associated TFs, including *Gli2*, *Spi1* and *Tfap2a* require further investigation to determine their protein expression profiles and exact roles in the pathogenesis of CIRI.

In summary, the functional gene-sets enriched in ischemic stroke at different time points (3 and 24 h) after reperfusion based on microarray expression data were analyzed in the present study. The results indicated 13 CIRI-related TFs, including *Cebpb*, *Cebpa*, *Egr1*, *Fos*, *Rela*, *Jund*, *Stat5a/b*, *Trp53*, *Gli2*, *Sp3*, *Tfap2a* and *Spi1*, which may serve significant regulatory roles in the pathogenesis of CIRI. Although some of these TFs were reported in previous studies, five TFs including *Cebpa*, *Gli2*, *Sp3*, *Tfap2a* and *Spi1* have not been reported in CIRI to the best of our knowledge. These findings may provide insight into the potential molecular mechanism underlying the pathogenesis of CIRI and aid the identification of novel targets in the treatment of CIRI; however, further investigation is required.

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Availability of data and materials

The microarray data used during the current study can be downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32529>). The mouse gene-sets of transcription factor target genes (mm_TF), Gene Ontology (mm_GO) and metabolic pathways (mm_metabolic) used in the present study can be downloaded from GSKB database (<http://ge-lab.org/#/data>). The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YYZ, JL and WJJ made substantial contributions to the design of the present study. YEL, KW and AFL conducted data collection and preprocessing. Data analysis and interpretation was performed by YYZ, KW, WW, YEL, JZ, CL, YQZ and APZ. YYZ and JL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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