Cold-inducible RNA-binding protein as a novel target to alleviate blood–brain barrier damage induced by cardiopulmonary bypass

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ABSTRACT

Background: Hypothermia provides neuroprotection and alleviates cerebral injury after cardiopulmonary bypass (CPB). The mechanism of cold-inducible RNA-binding protein (CIRP), which has been reported to be facilitated by hypothermia and act as a critical regulatory protein in the brain, remains unclear in CPB. Hence, the role of CIRP on hypothermia CPB-induced brain injury was investigated in a rat model.

Methods: *Cirp*^{-/-} rats were generated using the transcription activator-like effector nucleases-based genome editing technique. The animals were randomly allocated to 3 groups (n = 5, each group): sham group, CPB group, and CPB in *Cirp*^{-/-} group (*Cirp*^{-/-} group). Three biological replicates received RNA sequencing in the CPB and *Cirp*^{-/-} groups. The relative protein expression of the hippocampus was detected. The integrity of the blood–brain barrier (BBB) was measured using transmission electron microscopy and immunoglobulin G immunostaining. Glial fibrillary acidic protein in serum was detected. The brain was fixed for histopathological assessment.

Results: More differentially expressed genes of BBB leakage were clustered functionally by gene ontology and *Kyoto Encyclopedia of Genes and Genomes* pathway analyses. Transforming growth factor- β 1, matrix metalloproteinase-9, tumor necrosis factor- α , and malondialdehyde in the hippocampus were higher in the *Cirp*^{-/-} group, whereas the interleukin-4 level was opposite. Furthermore, more serious BBB disruption in the *Cirp*^{-/-} group was shown using transmission electron microscopy and immunoglobulin G extravasation. Moreover, *Cirp*^{-/-} showed enhanced tight junction protein degradation and histopathologic injury in the hippocampus (pathological score, surviving hippocampal neurons, and terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick-end labeling-positive neurons). Therefore, CIRP significantly alleviated neurologic injury.

Conclusions: CIRP exerted important neuroprotective effects by alleviating BBB breakdown, which might be associated with transforming growth factor- β 1-matrix metalloproteinase-9 signals in hypothermia CPB. (J Thorac Cardiovasc Surg 2019;157:986-96)

Increased brain injury after CIRP ablation on a rat cardiopulmonary bypass model.

Central Message

The expression of CIRP increased in the brain during hypothermia CPB, and CIRP exerted an important neuroprotection effect by alleviating BBB breakdown in hypothermia CPB.

Perspective

CIRP could be facilitated when temperature decreased to 32°C during CPB, and had a neuroprotective effect on brain injury by alleviating BBB leakage, which might be associated with TGF- β 1–MMP-9 signals. It would be a novel therapeutic target to prevent CPB-related brain injury.

See Commentary on page 997.

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Abbreviations and Acronyms

BBB	= blood–brain barrier
cAMP	= adenosine 3',5'-cyclic monophosphate
CIRP	= cold-inducible RNA-binding protein
CPB	= cardiopulmonary bypass
DEGs	= differentially expressed genes
GO	= gene ontology
IgG	= immunoglobulin G
IL	= interleukin
KEGG	= <i>Kyoto Encyclopedia of Genes and Genomes</i>
MDA	= malondialdehyde
MMP-9	= matrix metalloproteinase-9
TGF	= transforming growth factor
TNF	= tumor necrosis factor
ZO	= zona occludens

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Cardiopulmonary bypass (CPB)–related brain injury is one of the most disturbing outcomes in patients who undergo open heart surgeries, which increases the risk of mortality and postoperative complications.¹ Because of its high incidence, postoperative cognitive deficits including learning disabilities, memory deficits, hyperactivity, and behavioral disorders bring heavy psychological and economic burden to patients.² Hence, neuroprotection during cardiac surgery is especially important. The neuroprotective effect of hypothermia is mediated by reducing metabolism, relieving cerebral edema, and maintaining the blood–brain barrier (BBB).^{3,4} However, the neuroprotective regulatory mechanism of hypothermia remains unclear.

Cold-inducible RNA-binding protein (CIRP), which was first identified from mouse testis cells, can be facilitated by hypothermia in the brain, heart, and liver.⁵ It is constitutively, but weakly, expressed in various tissues, and is significantly enhanced under hypothermia.⁵ As a critical regulatory protein, CIRP responds to stress such as hypothermia, hypoxia, and ultraviolet light, and transfers from the nucleus to the cytoplasm to activate cell protective reactions.^{6,7} Studies on therapeutic hypothermia of traumatic brain injury and cardiac arrest have suggested

VIDEO 1. Surgical procedure in the rat model of hypothermia cardiopulmonary bypass. Video available at: [https://www.jtcvs.org/article/S0022-5223\(18\)32495-4/fulltext](https://www.jtcvs.org/article/S0022-5223(18)32495-4/fulltext).

that CIRP plays a leading role in the antiapoptotic effect of hypothermia.^{8,9} Moreover, when CIRP is endogenously induced under hypothermia or artificially overexpressed, hydrogen peroxide-induced neuronal apoptosis is dramatically inhibited, implying the potential neuroprotective role of CIRP.^{10,11}

On the basis of this information, it was postulated that CIRP might participate in hypothermia-mediated neuroprotection during CPB. The aim of this study was to show the protective effects of CIRP on BBB leakage induced by hypothermia CPB in a rat model.

METHODS

The experimental protocol was approved by the Care of Experimental Animals Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College. All of the experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Experimental Animals and Model of CPB

To study the pathophysiology of CIRP protein in the brain during CPB, genetically engineered rat lines were generated via transcription activator-like effector nucleases-based genome editing to target exon 3 of *Cirp* and ablate its expression in intact rats, as previously reported.¹² Sequencing analysis was also performed to detect base pair deletions and frameshift mutations of *Cirp* as previously shown.¹² The following experiments were performed on wild type and *Cirp*^{−/−} adult male rats weighing 300 to 400 g. The animals were randomly allocated to 3 groups (n = 5, each group): sham group, CPB group, and CPB in *Cirp*^{−/−} group (*Cirp*^{−/−} group).

CPB was performed (Video 1), as previously reported, with minor modifications (Figure 1, A).^{13,14} Briefly, rats were anesthetized with 1.5% sevoflurane after orotracheal intubation with a 16-gauge cannula (Insyte BD Medical, Sandy, Utah), and mechanically ventilated at 60 breaths per minute^{−1} with 8 mL/kg tidal volume (Harvard Apparatus, Holliston, Mass). A 24-gauge catheter was cannulated in the left femoral artery for arterial blood pressure monitoring. CPB was established via the tail artery (cannulated using a 20-gauge catheter) and right external jugular vein–right atrium (cannulated using a homemade multiorificed catheter) after median sternotomy. A 3.5-mm angioplasty balloon catheter, which acted as an endoaortic cross-clamp, was retrogradely inserted into the ascending aorta via the right common carotid artery. In the sham group, rats were anesthetized, cannulated, and heparinized with median sternotomy, but did

FIGURE 1. Model of cardiopulmonary bypass and the cerebral expression of cold-inducible RNA-binding protein (CIRP) after cardiopulmonary bypass. A, Schematic overview of the rat cardiopulmonary bypass (CPB) apparatus. B, A schematic diagram of the transcription activator-like effector nucleases (TALEN)-based genome editing strategy applied to generate the *Cirp*^{-/-} rats. C, Representative chromatogram showing a microdeletion and representative sequences of mutated alleles identified from clonal amplicons. Black dashes indicate the deleted bases. D and E, The Western blot analysis of CIRP in the hippocampus of wild type and *Cirp*^{-/-} rats. *****P* < .0001, between the sham and *Cirp*^{-/-} group. F and G, A representative Western blot image showing the CIRP expression between the sham and CPB groups, and the densitometric quantification of CIRP expression. ****P* < .001, between the sham and CPB group. The horizontal lines show the 5-number summary, which includes the sample minimum, the lower quartile, the median, the upper quartile, and the sample maximum in the box and whiskers graphs.

not undergo CPB. The entire circuit was primed with 12 mL 6% hydroxyethyl starch (Hextend; Hospira Inc, Lake Forest, Ill), 1 mL of heparin (250 IU/kg), 5% sodium bicarbonate, and 1 mL 20% mannitol. Blood was pumped from a venous reservoir through a custom-designed small-volume oxygenator (Kewei, Beijing, China) using a twin-roller pump (Stöckert, Munich, German). Flow rate was maintained at more than 120 mL/kg/min during CPB. After cooling to a rectal temperature of 32°C within 15 minutes, the endoaortic clamp was quickly inflated, while 0.9 mL St Thomas' solution was concomitantly administered through the central lumen of the angioplasty catheter. Cardioplegic arrest was confirmed using electrocardiography, and the mechanical ventilation was terminated. After 30 minutes of cardioplegic arrest, the balloon was deflated and removed, and mechanical ventilation was restarted. After a 30-minute rewarming, CPB was discontinued, and the animals were maintained, anesthetized with 1.0% sevoflurane, and ventilated for another 60 minutes, after which rats were sacrificed using decapitation. Arterial blood samples for blood gas analyses were collected just before CPB, before aortic cross-clamping, 30 minutes after aortic cross-clamping, and immediately after the end of CPB.

RNA Sequencing Analyses

Three biological replicates of each treatment received RNA sequencing. RNA extraction, library preparation for transcriptome sequencing, clustering, sequencing, quality control, transcriptome assembly, gene expression quantification, and bioinformatics analysis were performed, as previously reported.¹⁵ Briefly, sequencing was performed using an Illumina HiSeq platform (San Diego, Calif), and the raw data in fastq format were processed using in-house Perl scripts. The DESeq R package (version 1.10.1; AT&T Bell Laboratories, New Zealand) was used to identify differentially expressed genes (DEGs; *P* < .05), which were subsequently converted to official gene symbols and grouped according to a common biological property, according to gene ontology (GO) and *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway analyses.

Enzyme-Linked Immunosorbent Assay

Sixty minutes after CPB termination, blood samples were collected and centrifuged at 3000 rpm for 20 minutes at 4°C and the serum was obtained. Glial fibrillary acidic protein concentrations in serum and transforming

FIGURE 2. RNA sequencing analysis of differential gene expression in the hippocampus of wild type and *Cirp*^{-/-} rats after cardiopulmonary bypass. A, Volcano plot of differentially expressed genes (DEGs) between samples. The X-axis represents the fold change of DEGs in the different experimental groups. The -log₁₀ (adjusted *P* value; *padj*) in the Y-axis shows that DEGs become more significant as the value increases. The dots represent the genes, the green dots indicate nondifferentially expressed genes, the red dots represent the upregulated differentially expressed genes, and the blue dots represent the down-regulated differentially expressed genes (Table E2). B, C, and D, The identified genes were functionally clustered by gene ontology (GO) analysis. The GO analysis was conducted at level 2 under the 3 main GO categories including biological process (BP), molecular function (MF), and cellular component (CC). E, The mostly enriched *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway between the cardiopulmonary bypass and *Cirp*^{-/-} groups. The Y-axis represents the different KEGG categories, whereas the X-axis represents the rich factor. The size of the dot indicates the number of DEGs involved in the pathway. The color bars on the right represent the *P* value of the KEGG pathway. Each group includes 3 biological duplicates. TRP, Transient receptor potential; cAMP, adenosine 3',5'-cyclic monophosphate.

growth factor (TGF)-β1 in hippocampus tissue homogenates were measured using enzyme-linked immunosorbent assay kits from BioSource (Camarillo, Calif), in accordance with the manufacturer's instructions.

Western Blot Analysis

Equal amounts of protein (40 μg) were separated in 4% to 12% Bis-Tris gels in a morpholine ethyl sulfonate-sodiumdodecyl sulphate

FIGURE 3. Cold-inducible RNA-binding protein might be involved in the transforming growth factor (*TGF*)- β 1–matrix metalloproteinase (*MMP*)-9 signaling pathway during cardiopulmonary bypass. **A**, The expression levels of *TGF*- β 1 in the hippocampus. **B** and **C**, The Western blot images of *MMP*-9 and the graphic presentation of *MMP*-9 protein abundance was shown. **D–H**, The expression levels of tumor necrosis factor (*TNF*)- α , interleukin (*IL*)-4, *IL*-5, *IL*-10, and *IL*-13 in the hippocampus. **** $P < .0001$, between sham and cardiopulmonary bypass (*CPB*) group; *** $P < .001$, between sham and *CPB* group; ** $P < .01$, between sham and *CPB* group; * $P < .05$, between sham and *CPB* group; ### $P < .001$, between *CPB* and *Cirp*^{-/-} group; ## $P < .01$, between *CPB* and *Cirp*^{-/-} group; # $P < .05$, between *CPB* and *Cirp*^{-/-} group. The horizontal lines show the 5-number summary, which includes the sample minimum, the lower quartile, the median, the upper quartile, and the sample maximum in the box and whiskers graphs. *ns*, No statistical difference.

running buffer, and were electroblotted onto the nitrocellulose membrane (all obtained from Thermo Fisher Scientific, Waltham, Mass). The nitrocellulose filter membranes were incubated with anti-CIRP (1:1000; ProteinTech, Chicago, Ill), anti-occludin (1:50,000; Abcam, Cambridge, Mass), anti-zona occludens (*ZO*)-1 (1:1000; ProteinTech), anti-matrix metalloproteinase-9 (*MMP*-9) (1:500; Aviva Systems Biology, San Diego, Calif), and anti- β -actin (1:3000; Sigma, St Louis, Mo) at 4°C overnight. Then, the membranes were probed with appropriate secondary antibodies conjugated with horseradish peroxidase (ZSGB-Bio, Beijing, China). The specific bands of target proteins were visualized by chemiluminescence, and analyzed semiquantitatively with Quantity One system (version 4.6.2; Bio-Rad, Hercules, Calif).

Inflammatory Cytokine Measurement

Various inflammatory cytokines in hippocampus tissue homogenates were measured using Proinflammatory Panel 2 (rat) kits (K15059D-1) obtained from Meso Scale Discovery (Gaithersburg, Md), according to the manufacturer's protocol. These inflammatory cytokines included tumor necrosis factor- α (*TNF*- α), interleukin (*IL*)-4, *IL*-5, *IL*-10, and *IL*-13.

Transmission Electron Microscopy

Part of the right hippocampus was fixed with 2.5% glutaraldehyde and 1% osmium tetroxide solution. Then, the specimens were dehydrated in ethanol, infiltrated with propylene oxide epoxy, and embedded in epoxy. The ultrathin sections were collected on copper grids and were stained with uranyl acetate and lead citrate. Ultrastructural sections were reviewed and photographed using a transmission electron microscope (TEM-1400plus; JEOL, Tokyo, Japan).

Immunohistochemistry

BBB disruption was assessed using immunoglobulin G (IgG) immunostaining, as previously reported.¹⁶ Briefly, the deparaffinized sections were incubated with biotinylated goat anti-rat IgG (1:200; Vector Laboratories, Burlingame, Calif) overnight at 4°C, followed by incubation with an avidin-biotin-horseradish peroxidase complex reagent (1:200; Vector Laboratories) at room temperature for 2 hours. The staining was developed using the 3,3'-diaminobenzidine method. The quantification was performed using ImageJ software (Version 1.51, National Institutes of Health, USA) as previously shown.¹⁶

Hematoxylin and Eosin Staining, and Terminal Deoxynucleotidyl Transferase-Mediated 2'-Deoxyuridine, 5'-Triphosphate Nick-End Labeling Assay

Hematoxylin and eosin staining was performed as the standard protocol. Neurologic damage was evaluated according to the following pathological scores: 0 = no damage; 1 = 0% to 12.5% damage; 2 = 12.5% to 25% damage; 3 = 25% to 50% damage; 4 = >50% damage.¹⁷ Histologic examination was evaluated by an experienced pathologist with a blind protocol. For Nissl staining, the brain slices were mounted onto slides and stained with 0.2% Crystal Violet (Sigma-Aldrich, Saint Louis, Mo). To determine apoptosis-like cell death, terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick-end labeling staining (Promega, Madison, Wis) was performed, according to manufacturer's instructions.

FIGURE 4. Cold-inducible RNA-binding protein maintained the integrity and permeability of the blood–brain barrier during cardiopulmonary bypass. A, Electron microscope examination of the microvasculature in the rat hippocampus. The ultrastructure of the microvasculature showed no alterations in the sham group. Compared with the cardiopulmonary bypass (CPB) group, *Cirp*^{-/-} induced more obvious interstitial edema around the vessels and more serious destruction of the endothelial compartment. Scale bar = 2 μ m. Triangles indicate microvascular endothelium, and asterisks the capillary lumen. B and C, The representative immunostaining images of immunoglobulin (Ig)G extravasation (arrows, original magnification 40 $\times$) and graphic presentation of the percentage area with IgG-positive staining in the hippocampus was shown. D and E, The Western blot analysis of occludin in rat hippocampus. F and G, The Western blot analysis of zona occludens (ZO)-1 in rat hippocampus. **** P < .0001, between sham and CPB group; *** P < .001, between sham and CPB group; ** P < .01, between sham and CPB group; #### P < .0001, between CPB and *Cirp*^{-/-} group; ## P < .01, between CPB and *Cirp*^{-/-} group. The horizontal lines show the 5-number summary, which includes the sample minimum, the lower quartile, the median, the upper quartile, and the sample maximum in the box and whiskers graphs.

Malondialdehyde Measurement

Malondialdehyde (MDA) is a marker of oxidative stress and free oxygen radical-mediated damage. The MDA level of the hippocampus was measured by using an MDA assay kit (ab118970, Abcam), according to manufacturer's instructions.

Statistical Analysis

Numerical data were expressed as mean $\pm$ standard deviation, and were compared using 1-way analysis of variance followed by Student–Newman–Keuls test. Student t test was used for 2-group analyses. Most of the data sets passed the normality test. The Kruskal–Wallis test was used for nonparametric values, and the Mann–Whitney U test was used as a post-test to identify the specific differences among the groups. Statistical significance was defined as P < .05. The data were collected and statistically analyzed using SPSS 16.0 software (SPSS Inc, Chicago, Ill), and all graphs were performed using GraphPad Prism7 (GraphPad Software, La Jolla, Calif).

RESULTS

Induction of CIRP in Rat Brain After CPB

With the use of the transcription activator-like effector nucleases-based genome editing technique, *Cirp*^{-/-} rats were successfully established (Figure 1, B). Sequencing analysis of the targeted *Cirp* locus revealed a mutation comprising a 7-base pair deletion (Figure 1, C). Western blot analysis verified the gene-targeting efficiency of CIRP, showing a significant reduction in CIRP protein expression (0.00 ± 0.00 vs 0.51 ± 0.04 ; P < .0001; Figure 1, D and E).

All rats experienced the CPB procedures and full cardioplegic arrest over 30 minutes, and were weaned from CPB without inotropic agents. The physiological data during the operation are summarized in Table E1. The preoperative data in the 3 groups were comparable.

The expression of CIRP protein in the brain of rats between the sham and CPB groups at 1 hour after surgery are shown in Figure 1, F. The semiquantitative analysis of Western blot showed that the expression of CIRP protein significantly increased in the CPB group compared with the sham group (0.06 ± 0.01 vs 0.03 ± 0.01 ; P = .0008; Figure 1, G).

Differential Gene Expression in the Hippocampus of *Cirp*^{-/-} Rats During CPB

To identify molecules responsible for the increase of CIRP during CPB, RNA sequencing analyses were conducted, and the hippocampal gene expression profiles in CPB and *Cirp*^{-/-} group were examined. A total of 148 DEGs were identified (P < .05; Figure 2, A and Table E2), and these were functionally clustered by GO (Figure 2, B–D) and KEGG pathway analyses (Figure 2, E). In the category of biological process of GO functional enrichment analysis (Figure 2, B), concerns were raised that several DEGs were involved in “regulation of blood vessel diameter,” “regulation of blood vessel size,” and “negative regulation of hydrolase activity,” suggesting that the role of CIRP might be related to angiogenic

FIGURE 5. Cold-inducible RNA-binding protein alleviated neuronal injury and oxidative stress during cardiopulmonary bypass. A, Ischemic neurons identified using hematoxylin and eosin staining in representative brain sections (arrows, original magnification 40 $\times$) obtained from rats in each group. No structural alterations were shown in the sham group. *Cirp*^{-/-} promoted more ischemic neurons compared with the cardiopulmonary bypass (CPB) group. B, Graphical presentation of the pathological scores. C, In the sham group, the structure of most neurons in the hippocampus was clear, and some uniformly stained Nissl bodies appeared in the cytoplasm. *Cirp*^{-/-} obviously promoted cell loss induced by CPB, compared with the CPB group. D, The number of intact neurons in the hippocampus. E, The terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick-end labeling (TUNEL) assay performed for the sham group, CPB group, and *Cirp*^{-/-} group (arrows, original magnification 20 $\times$). F, The summary of TUNEL-positive cells at 1 hour after CPB. G, The plasma levels of glial fibrillary acidic protein (GFAP; ng/mL). H, Malondialdehyde (MDA) contents in the hippocampus. * $P < .05$, between sham and CPB group; *** $P < .001$, between sham and CPB group; ## $P < .01$, between CPB and *Cirp*^{-/-} group; #### $P < .001$, between CPB and *Cirp*^{-/-} group; ##### $P < .0001$, between CPB and *Cirp*^{-/-} group. The horizontal lines show the 5-number summary, which includes the sample minimum, the lower quartile, the median, the upper quartile, and the sample maximum in the box and whiskers graphs. HE, Hematoxylin and eosin.

molecules and matrix metalloproteinases during CPB.¹⁸ As for DEGs enriched in the category of molecular function and cellular components, the results are shown in Figure 2, C and D. The KEGG pathway analysis revealed that more DEGs were involved in “adenosine 3',5'-cyclic monophosphate (cAMP) signaling pathway,” “gap junction,” and “inflammatory mediator regulation of transient receptor potential channels” (Figure 2, E), which belonged to BBB disruption processing.¹⁹⁻²¹ These results suggest that CIRP ablation in the hippocampus during CPB was correlated to BBB leakage.

CIRP Might Be Associated With the TGF- β 1-MMP-9 Signaling Pathway to Maintain the Integrity of the BBB

According to the prediction of GO and KEGG pathway analyses, the expression of TGF- β 1 and MMP-9 were examined, which was the common part of the enriched biological process and cAMP-protein kinase A signals.²² The expression of TGF- β 1 and active MMP-9 of hippocampus significantly increased in the *Cirp*^{-/-} group, compared

with the CPB group (273 ± 81 vs 169 ± 65 TGF- β 1, and 0.62 ± 0.12 vs 0.33 ± 0.06 active MMP-9; $P = .018$, and $< .001$, respectively; Figure 3, A–C). A similar pattern of changes occurred to TNF- α in the hippocampus (3.61 ± 0.96 vs 2.12 ± 0.35 ; $P = .003$; Figure 3, D). In contrast, the production of anti-inflammatory cytokine IL-4 significantly decreased in the *Cirp*^{-/-} group, compared with the CPB group (2.49 ± 0.43 vs 3.69 ± 0.73 ; $P = .002$; Figure 3, E). There were no significant differences in the levels of cytokines IL-5, IL-10, and IL-13 (Figure 3, F–H) between these 2 groups (37 ± 7 vs 35 ± 3 IL-5, and 21 ± 6 vs 25 ± 9 IL-10, and 3.26 ± 1.09 vs 3.34 ± 0.94 IL-13; $P = .540$, .405, and .884, respectively).

Furthermore, the integrity of the BBB was evaluated using transmission electron microscopy, immunohistochemistry, and Western blot analysis to show whether CIRP had a protective effect on BBB leakage. The edema of vascular foot processes and narrowed vascular lumen, which resulted from BBB damage, were observed in wild type

FIGURE 6. The neuroprotective effect on brain injury of cold-inducible RNA-binding protein (CIRP) during cardiopulmonary bypass. The putative role of CIRP responses to cardiopulmonary bypass (CPB)-induced brain injury. CIRP could be facilitated when the temperature decreased to 32°C during CPB and might be involved in the transforming growth factor (TGF)- β 1–matrix metalloproteinase (MMP)-9 signaling pathway to alleviate blood–brain barrier (BBB) leakage, which therefore inhibited the release of inflammatory mediator and oxidative stress from the blood, and alleviated cerebral injury during CPB. The arrows indicate the tentative effects of CIRP. ECM, Extracellular matrix; TNF- α , tumor necrosis factor- α ; MDA, malondialdehyde.

and *Cirp*^{-/-} rats after CPB, whereas in the *Cirp*^{-/-} group, the interstitial edema was obvious around the vessels, and the destruction of the endothelial compartment was more serious and discontinuous (Figure 4, A). Using immunohistochemistry, it was found that *Cirp*^{-/-} increased the IgG extravasation in the hippocampus (6.36 ± 0.68 vs 1.92 ± 0.54 ; $P < .0001$; Figure 4, B and C). Western blot analysis results also confirmed that the tight junction protein levels of occludin and ZO-1 decreased after CPB, compared with the sham group, and *Cirp* ablation could effectively accelerate these decreasing trends (0.03 ± 0.01 vs 0.08 ± 0.04 occludin, and 0.04 ± 0.01 vs 0.09 ± 0.03 ZO-1; $P = .007$, and $.0058$, respectively; Figure 4, D–G). These results were consistent with the KEGG pathway analysis, and suggested the possible role of CIRP in maintaining the integrity of the BBB during CPB.

CIRP Alleviated Neuronal Injury and Oxidative Stress During CPB

Ischemic neuronal change was identified in the CA1 section of the hippocampus. In the sham group, neurons were morphologically intact with a clear shape and abundant cytoplasm. In the *Cirp*^{-/-} and CPB groups, ischemic neurons exhibited shrunken cell bodies, triangulated pyknotic nuclei, and eosinophilic cytoplasm. The pathological score of the *Cirp*^{-/-} group was higher than that of the CPB group (3.0 [interquartile range, 2.0 – 3.0] vs 1.0 [interquartile range, 0.5 – 1.0], $P < .0001$; Figure 5, A and B). Nissl

staining revealed remarkable neuron loss in *Cirp*^{-/-} rats (Figure 5, C). The deficiency of CIRP robustly increased neuron loss (802 ± 216 vs 3369 ± 564 ; $P < .0001$; Figure 5, D). The number of terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick-end labeling-positive cells was significantly increased in the *Cirp*^{-/-} group, compared with the CPB group (15.98 ± 3.05 vs 0.70 ± 0.14 ; $P = .0012$; Figure 5, E and F). In addition, glial fibrillary acidic protein levels, a cerebral injury marker in plasma, were significantly higher in *Cirp*^{-/-} rats, compared with wild type rats (49 ± 12 vs 24 ± 7 ; $P = .0011$; Figure 5, G), indicating that CIRP deficiency was associated with more serious brain cellular injury during CPB. Moreover, MDA levels were significantly elevated in the *Cirp*^{-/-} group, compared with the CPB group (223 ± 43 vs 101 ± 30 ; $P = .0001$; Figure 5, H).

DISCUSSION

In the present study, the role of CIRP responses to CPB-induced brain injury was shown, to our knowledge, for the first time. The results revealed that *Cirp*^{-/-} was associated with aggravated cerebral injury in rats after 75-minute CPB, as evidenced by significantly increased hippocampal neuronal loss and local brain oxidative stress damage. The present results also suggest that *Cirp* ablation exacerbated the BBB leakage and aggravated the inflammatory responses of the brain, as predicted by RNA sequencing

FIGURE 7. Putative mechanisms for how cold-inducible RNA-binding protein (CIRP) prevents brain injury induced by cardiopulmonary bypass (CPB). The arrows indicate the tentative effects of CIRP. *TGF-β1*, Transforming growth factor-β1; *TβRII*, transforming growth factor β receptor II; *TβRI*, transforming growth factor β receptor I; *AC*, adenylyl cyclase; *cAMP*, adenosine 3',5'-cyclic monophosphate; *PKA*, protein kinase A; *MMP-9*, matrix metalloproteinase-9; *ECM*, extracellular matrix; *BBB*, blood-brain barrier; *TNF-α*, tumor necrosis factor-α; *IL-4*, interleukin-4; *MDA*, malondialdehyde.

analyses, which might be associated with the TGF-β1–MMP-9 pathway. The neuroprotective effect of CIRP is summarized in Figure 6.

An increasing number of studies have reported on the incidence of CPB-induced brain injury, in which the molecular mechanism remains unclear.² Because of its cytoprotective effect, hypothermia has been widely applied in cardiac surgery.^{23,24} Previous studies on the molecular biological mechanisms of hypothermia during CPB mainly focused on its role on metabolism reduction. However, the precise mechanism remains undetermined. When the body temperature declines, various proteins are induced to accommodate to the temperature change. CIRP is the first cold-shock protein expressed in mammalian cells.⁵ It has a regulatory effect on cell growth and apoptosis under cold induction. CIRP mRNA could be found in great quantities at low temperatures, but it could hardly be detected at normal temperatures, indicating that the stability of CIRP mRNA is regulated by temperature.²⁵ Furthermore, hypothermia after cerebral ischemia/reperfusion injury can

promote the production of CIRP.²⁶ The molecular characteristics of CIRP encouraged the investigators to verify the neuroprotective effect of CIRP against CPB-related brain injury.

In the present study, it was found that CIRP was significantly elevated in the hippocampus after hypothermic CPB. This result was consistent with previous studies, which suggested that hypothermia, hypoxia, and inflammation might be involved in the upregulation of CIRP, serving as the response of neurons to ischemia.²⁶ To further explore the regulatory mechanism of CIRP, gene expression differences were compared between wild type and *Cirp*^{−/−} rats after CPB. According to the KEGG analysis, the enriched signals of “cAMP signaling pathway,” “gap junction,” and “inflammatory mediator regulation of transient receptor potential channels” were noteworthy, because all of these participated in BBB disruption,^{19–21} which was the focus of concern in this study.

Although the mechanisms of cerebral injury after CPB are not fully understood, injury to BBB leakage has been

regarded as a crucial part in the pathophysiological process.²⁷ BBB disruption occurred in 67% of patients who undergo open heart surgery,²⁸ and Jonas and colleagues reported that an increase in permeability of the BBB might be a sign and a mechanism of brain damage in the piglet model of CPB.²⁹ Prolonged CPB time stimulates a neutrophil-mediated decrease in β -catenin expression in cerebral microvascular endothelial cells,³⁰ suggesting that CPB-induced BBB leakage could injure the brain by exposing it to harmful substances otherwise confined to the vasculature.³¹ Therefore, the prevention of BBB disruption might alleviate cerebral injury after CPB. In the present study, the investigators attempted to propose a new target for hypothermia CPB associated with the maintenance of BBB integrity.

According to RNA sequencing analyses, the TGF- β 1 pathway, as part of the cAMP-protein kinase A signals, might be involved in the regulation between CIRP and the BBB during CPB. The TGF- β 1 pathway was related to brain injury.³² As an angiogenic and profibrogenic factor, TGF- β 1 induced the expression and deposition of extracellular matrix components, and eventually resulted in communicating hydrocephalus by increasing endothelial permeability and decreasing ion and fluid transport.³³ Furthermore, as an upstream signal-regulated molecule, TGF- β 1 induced MMP-9 expression in cultured human meningeal cells.³⁴ MMP-9 was a major gelatinase and collagenase, and was known to participate in the breakdown of the BBB under pathological conditions, such as brain ischemia and hydrocephalus.³⁵ MMP-9 degraded fibronectin and collagen-extracellular matrix components generated through the TGF- β 1 signaling pathway³⁶ and enhanced BBB permeability via brain endothelial cell disruption.³⁷ By verifying the predictive molecule from RNA sequencing analyses, the expression of TGF- β 1 and active MMP-9 of hippocampus increased in the *Cirp*^{-/-} group during CPB. These results, together with the high TNF- α and low IL-4 levels in the hippocampus in the *Cirp*^{-/-} group, suggested that CIRP might be associated with the TGF- β 1-MMP-9 signaling pathway, which alleviated the disruption of the BBB, as confirmed by the decrease in tight junction protein levels and increase in permeability to IgG when *Cirp* was knockout during CPB. As a result, the BBB leakage promoted the release of the inflammatory mediator TNF- α from the blood and inhibited the expression of anti-inflammatory cytokine IL-4 in the brain. Thus, CPB-induced BBB disruption might be closely associated with the reduced expression of CIRP. The proposed mechanism as to how CIRP might attenuate the BBB leakage induced by CPB is summarized in Figure 7.

Neuronal damage continued to exist although CIRP was induced under hypothermia CPB. The explanation might be that the increased expression of CIRP after CPB could attenuate, but not reverse, the injury under physiopathologic

conditions. Thus, exogenous CIRP supplement might become a novel therapeutic target in the prevention of brain injury caused by CPB. However, besides CPB, the pathophysiology of brain injury during open heart surgery is multifactorial. Initiated by major surgery and regulated by neuroendocrine activation, intraoperative formation of gaseous emboli and aggregated platelets, systemic inflammatory response, hypoperfusion, hypotension, surgical trauma, and blood loss all enhance the risk of cerebral injury.³⁸ Therefore, the expression and function of CIRP cannot be explained simply by a single mechanism during CPB. Nevertheless, the present study revealed a new endogenous mechanism for maintaining BBB integrity under hypothermia CPB.

There were several limitations that merit comment in the present study. First, it was difficult for rats to survive after median thoracotomy with great invasion in the present study. To explore the effect of CIRP on postoperative behavior predicted by GO functional enrichment analysis, a functional experiment needs to be conducted with improving the model in the follow-up study. Second, further investigation is needed to identify the specific messenger molecules of CIRP, to modulate the TGF- β 1-MMP-9 signaling pathway. Last, potential differences in the nervous systems of humans and rodents, and clinical settings, such as the major surgery that was not present in our model, were not examined and should be further investigated.

CONCLUSIONS

These present findings showed that the expression of CIRP increased in the brain after cooling to 32°C during CPB, and that CIRP exerted an important neuroprotective effect by alleviating BBB breakdown, which might be associated with TGF- β 1-MMP-9 signals in a rat model of hypothermia CPB.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: cold-inducible RNA-binding protein, hypothermia, cardiopulmonary bypass, blood-brain barrier

TABLE E1. Physiologic variables and blood gas analyses during cardiopulmonary bypass

Group	Time point	Weight, g	MAP, mm Hg	HR, bpm	Hct	Hb, g/L	pHa	Lac, mmol/L	SaO ₂ , %	PaO ₂ , mm Hg	PaCO ₂ , mm Hg
Sham	T0	330.21 ± 13.53	122.31 ± 12.31	375.02 ± 8.02	0.395 ± 0.02	132.2 ± 5.7	7.37 ± 0.04	0.91 ± 0.15	99.90 ± 0.00	491.04 ± 37.73	43.83 ± 5.09
	T1		126.11 ± 3.57	420.84 ± 25.45	0.360 ± 0.04	120.0 ± 14.7	7.48 ± 0.01	0.97 ± 0.25	99.81 ± 0.16	394.30 ± 47.34	37.40 ± 1.35
	T2		120.69 ± 15.05	439.26 ± 69.64	0.357 ± 0.04	119.0 ± 14.7	7.52 ± 0.09	1.60 ± 0.20	99.89 ± 0.12	362.90 ± 42.52	38.13 ± 0.78
	T3		118.95 ± 7.56	414.06 ± 49.07	0.337 ± 0.02	112.3 ± 5.5	7.56 ± 0.15	1.67 ± 0.47	99.77 ± 0.20	339.27 ± 22.52	40.13 ± 3.65
CPB	T0	329.00 ± 14.53	124.31 ± 14.33	398.45 ± 28.60	0.390 ± 0.03	130.0 ± 8.9	7.32 ± 0.06	0.93 ± 0.25	99.77 ± 0.23	399.30 ± 123.64	45.16 ± 1.95
	T1		85.43 ± 17.07*	346.38 ± 31.77*	0.189 ± 0.03*	63.3 ± 8.7*	7.51 ± 0.04	1.79 ± 0.80	97.90 ± 0.37	304.30 ± 47.34	37.27 ± 20.50
	T2		79.98 ± 14.31*	308.52 ± 40.08*	0.203 ± 0.02*	67.6 ± 7.2*	7.58 ± 0.01	2.44 ± 1.46	99.90 ± 0.20	248.80 ± 53.52	37.05 ± 12.26
	T3		104.61 ± 21.12	369.15 ± 37.27	0.203 ± 0.02*	67.8 ± 5.5*	7.57 ± 0.04	2.70 ± 1.10*	99.80 ± 0.17	239.37 ± 22.52*	34.62 ± 12.41
<i>Cirp</i> ^{-/-}	T0	333.31 ± 13.09	125.16 ± 16.68	385.02 ± 22.04	0.403 ± 0.01	134.3 ± 4.0	7.36 ± 0.01	0.73 ± 0.47	99.90 ± 0.12	391.04 ± 47.74	47.26 ± 3.45
	T1		87.73 ± 14.27*	335.84 ± 25.33*	0.197 ± 0.02*	66.3 ± 6.7*	7.54 ± 0.15	1.69 ± 0.92	98.07 ± 1.17	298.62 ± 123.91	33.02 ± 17.53
	T2		82.71 ± 12.22*	301.26 ± 79.64*	0.204 ± 0.03*	68.3 ± 8.8*	7.57 ± 0.11	2.64 ± 1.46	99.07 ± 0.58	234.37 ± 76.60	32.05 ± 9.13
	T3		113.95 ± 5.56	344.06 ± 59.07	0.204 ± 0.02*	68.6 ± 8.6*	7.59 ± 0.69	3.07 ± 1.60*	98.96 ± 1.21	254.17 ± 52.53*	38.03 ± 8.21

MAP, Mean arterial pressure; HR, heart rate; bpm, beats per minute; Hct, hematocrit; Hb, hemoglobin; pHa, arterial pH; Lac, lactate; SaO₂, arterial oxygen saturation; PaO₂, partial pressure of arterial oxygen; PaCO₂, partial pressure of arterial carbon dioxide; T0, before CPB; T1, before aortic cross-clamping; T2, 30 minutes after aortic cross-clamping; T3, immediately after the end of CPB; CPB, cardiopulmonary bypass. *P < .05 versus sham.

TABLE E2. Differentially expressed genes between the CPB and *Cirp*^{-/-} group

ID	Gene name	Log ₂ fold change	P value	padj
ENSRNOG00000049743	NEWGENE_620381	5.756687	2.60E-25	5.91E-21
ENSRNOG00000008428	Drd2	5.236001	2.42E-24	2.75E-20
ENSRNOG000000011207	Gh1	-10.9314	1.31E-22	9.93E-19
novel.3609	-	4.622191	1.34E-20	7.62E-17
ENSRNOG000000028356	LOC103694872	4.36004	6.93E-20	3.15E-16
ENSRNOG000000049580	Gpr6	4.575496	1.27E-19	4.83E-16
ENSRNOG00000003800	Rgs9	4.50444	1.63E-19	5.22E-16
ENSRNOG00000006589	LOC103694877	-4.45155	1.83E-19	5.22E-16
novel.12386	-	6.613628	3.54E-19	8.96E-16
novel.17638	-	-4.44188	2.17E-18	4.94E-15
ENSRNOG00000008943	Penk	4.063058	2.83E-18	5.86E-15
ENSRNOG000000052099	LOC103694869	5.457769	2.44E-16	4.63E-13
ENSRNOG000000007374	Tac1	3.840393	2.69E-16	4.71E-13
novel.14959	-	3.945332	4.33E-16	7.04E-13
novel.7447	-	4.094205	4.70E-16	7.13E-13
ENSRNOG000000026679	Scn4b	3.671938	9.05E-16	1.29E-12
ENSRNOG000000023688	Drd1	4.144552	2.83E-15	3.79E-12
novel.6887	-	-4.73698	3.30E-15	4.17E-12
ENSRNOG000000012556	Isl1	6.639481	7.65E-15	8.74E-12
ENSRNOG000000046250	LOC100911027	-4.26365	7.68E-15	8.74E-12
novel.1668	-	-6.74895	8.99E-15	9.74E-12
ENSRNOG000000028404	Ppp1r1b	3.461061	1.85E-14	1.92E-11
ENSRNOG000000049944	LOC100911440	-3.55439	2.70E-14	2.67E-11
ENSRNOG000000025037	Ankk1	9.538262	2.89E-14	2.74E-11
ENSRNOG000000049298	LOC100909595	-4.27853	5.71E-14	5.20E-11
ENSRNOG000000054508	Foxp2	3.745592	1.92E-13	1.68E-10
novel.6691	-	3.518378	4.03E-13	3.39E-10
novel.3607	-	4.514926	4.50E-13	3.66E-10
ENSRNOG000000025012	Chat	5.12503	7.89E-13	6.19E-10
ENSRNOG000000018780	Sh3rf2	5.112869	9.25E-13	7.02E-10
ENSRNOG000000002462	LOC108348055	3.811961	1.75E-12	1.29E-09
ENSRNOG000000018736	Pnpla2	-4.24752	2.36E-12	1.68E-09
novel.9466	-	4.759742	2.56E-12	1.77E-09
novel.10387	-	3.548767	4.04E-12	2.71E-09
ENSRNOG000000050855	LOC100911994	3.806039	8.10E-12	5.27E-09
novel.9329	-	-3.5714	8.34E-12	5.27E-09
ENSRNOG000000047618	LOC100910196	4.597373	1.73E-11	1.06E-08
ENSRNOG000000046120	RGD1561252	-3.75616	2.92E-11	1.75E-08
ENSRNOG000000011542	Apopt1	3.520596	3.62E-11	2.11E-08
novel.12385	-	8.893641	4.50E-11	2.56E-08
ENSRNOG000000011824	Trh	3.337768	6.41E-11	3.56E-08
ENSRNOG000000003209	Pcp4l1	2.89843	8.38E-11	4.54E-08
ENSRNOG000000045949	LOC100911581	-3.40651	8.76E-11	4.63E-08
ENSRNOG000000048130	LOC100911725	-5.21115	1.62E-10	8.40E-08

(Continued)

TABLE E2. Continued

ID	Gene name	Log ₂ fold change	P value	padj
ENSRNOG00000028041	Tnnt1	3.218826	2.63E-10	1.33E-07
novel.7455	–	3.156254	2.86E-10	1.41E-07
novel.6266	–	8.72119	2.96E-10	1.43E-07
ENSRNOG00000010053	Calcr	5.754447	3.13E-10	1.48E-07
novel.17647	–	5.732464	4.00E-10	1.86E-07
ENSRNOG00000061943	Rn60_7_1214.2	–4.47812	4.08E-10	1.86E-07
ENSRNOG00000050827	Pdcd5	–2.9381	5.20E-10	2.32E-07
ENSRNOG00000022946	Slc22a3	4.115772	5.50E-10	2.39E-07
ENSRNOG00000007112	Pcsk1n	–3.60924	5.57E-10	2.39E-07
ENSRNOG00000055229	LOC108348118	–4.4434	5.95E-10	2.51E-07
ENSRNOG00000014761	Rasd2	2.693744	7.41E-10	3.06E-07
ENSRNOG00000049495	Krt71	3.040058	8.14E-10	3.31E-07
ENSRNOG00000016000	LOC100911417	2.741865	1.49E-09	5.96E-07
ENSRNOG00000037188	Mug1	5.568427	1.84E-09	7.23E-07
novel.3942	–	–4.33395	1.91E-09	7.35E-07
novel.17965	–	3.149788	2.02E-09	7.65E-07
ENSRNOG00000010478	LOC500712	5.543393	2.40E-09	8.96E-07
ENSRNOG00000001701	Cbr3	2.796487	2.55E-09	9.35E-07
ENSRNOG00000008356	Myo5c	3.283644	3.15E-09	1.14E-06
novel.9369	–	4.077866	6.45E-09	2.29E-06
ENSRNOG00000012038	Htr1d	4.696278	8.28E-09	2.90E-06
ENSRNOG00000055934	Dmkn	3.234468	1.44E-08	4.95E-06
novel.10141	–	4.000001	1.50E-08	5.11E-06
ENSRNOG00000009530	Uchl3	3.260809	1.69E-08	5.65E-06
novel.70	–	–4.10877	1.87E-08	6.15E-06
novel.107	–	–8.31898	2.10E-08	6.81E-06
ENSRNOG00000049491	RT1-DMb	–4.06376	2.89E-08	9.25E-06
ENSRNOG00000050602	Kif28p	8.226635	2.93E-08	9.25E-06
ENSRNOG00000019473	NEWGENE_1582994	–3.19466	5.48E-08	1.71E-05
novel.18207	–	2.551516	5.71E-08	1.75E-05
novel.11466	–	–5.31473	7.22E-08	2.19E-05
novel.913	–	2.480194	7.98E-08	2.39E-05
ENSRNOG00000002151	LOC103693564	–8.15904	8.20E-08	2.42E-05
novel.17330	–	3.784693	1.12E-07	3.27E-05
ENSRNOG00000026371	Krt17	4.43387	1.15E-07	3.31E-05
ENSRNOG00000009143	Lrrc57	–3.56108	1.74E-07	4.96E-05
ENSRNOG00000011310	Pde10a	2.657965	1.84E-07	5.16E-05
ENSRNOG00000010822	Dlx6	2.688737	2.30E-07	6.39E-05
novel.17289	–	2.263178	2.39E-07	6.55E-05
ENSRNOG00000009735	Fibcd1	–4.4691	2.67E-07	7.24E-05
ENSRNOG00000040201	Atp6ap11	2.583491	3.08E-07	8.26E-05
novel.18225	–	–3.19674	3.30E-07	8.74E-05
ENSRNOG00000045760	Ebna1bp2	2.926169	3.41E-07	8.92E-05
novel.11413	–	2.326483	3.52E-07	9.09E-05

(Continued)

TABLE E2. Continued

ID	Gene name	Log ₂ fold change	P value	padj
novel.10511	–	4.960079	4.78E-07	0.000122185
novel.1048	–	7.896671	5.06E-07	0.000127933
novel.15609	–	–2.50346	6.82E-07	0.000170436
novel.16165	–	2.638777	7.44E-07	0.000183948
ENSRNOG00000018250	Tnni3	2.951473	7.87E-07	0.000192516
novel.17838	–	4.882351	9.27E-07	0.000224341
ENSRNOG00000062141	Slc18a3	2.517415	1.09E-06	0.000258229
ENSRNOG00000058862	AABR07051308.1	7.800873	1.09E-06	0.000258229
ENSRNOG000000021555	Mis18a	–2.83549	1.38E-06	0.000324878
ENSRNOG00000002229	Adcy5	2.063366	1.47E-06	0.000340775
novel.14186	–	–7.77357	1.61E-06	0.000370791
ENSRNOG00000003171	Mpz	3.756283	1.66E-06	0.000378378
novel.259	–	2.02752	2.06E-06	0.00046441
ENSRNOG00000012302	Gucy1a3	2.041653	2.10E-06	0.000468072
ENSRNOG00000027894	Iqgap3	2.296892	2.20E-06	0.000486914
ENSRNOG00000060949	Anxa8	7.698259	2.40E-06	0.000525986
ENSRNOG00000025889	Gnas	3.272291	2.52E-06	0.000541813
ENSRNOG00000045772	LOC100911545	3.272291	2.52E-06	0.000541813
ENSRNOG00000046192	Nsun4	–3.80989	2.92E-06	0.000621263
ENSRNOG00000007647	Oprk1	2.427701	3.37E-06	0.000710423
ENSRNOG00000038370	Ahsg	7.644078	3.61E-06	0.000752787
ENSRNOG00000001369	Oas1a	–4.17303	4.01E-06	0.000823146
ENSRNOG00000003119	Gc	4.000788	4.01E-06	0.000823146
ENSRNOG00000057060	LOC108348049	–2.91718	4.84E-06	0.000984419
ENSRNOG00000024848	Fga	7.587783	5.45E-06	0.001087
ENSRNOG00000003566	ApoH	7.587783	5.45E-06	0.001087
novel.12981	–	2.307147	5.80E-06	0.00114807
ENSRNOG00000002802	Cxcl1	–2.49077	6.32E-06	0.001239602
ENSRNOG00000019985	Asic4	1.946177	6.48E-06	0.001259617
novel.14040	–	3.590771	6.96E-06	0.001341588
ENSRNOG000000033613	RGD1565459	4.620359	7.53E-06	0.001440588
ENSRNOG00000015267	Aldh18a1	2.995367	7.68E-06	0.001456248
ENSRNOG00000017374	Prl	–7.53376	8.28E-06	0.001557509
novel.9533	–	3.518856	9.35E-06	0.001743561
ENSRNOG00000019719	Kcna5	2.584711	1.04E-05	0.00191662
novel.11870	–	–2.20459	1.20E-05	0.002194958
novel.4379	–	–7.46702	1.27E-05	0.002257673
ENSRNOG00000046980	LOC108348151	7.468141	1.27E-05	0.002257673
novel.71	–	1.88223	1.27E-05	0.002257673
novel.18705	–	2.446084	1.27E-05	0.002257673
ENSRNOG00000009371	Abl1	2.299046	1.32E-05	0.002303517
ENSRNOG00000050660	Grcc10	–2.30709	1.32E-05	0.002303517
ENSRNOG00000024330	Ngp	–2.57653	1.33E-05	0.002317368
ENSRNOG00000021225	Oxt	2.851458	1.41E-05	0.002431351

(Continued)

TABLE E2. Continued

ID	Gene name	Log ₂ fold change	P value	padj
ENSRNOG00000060572	Tlk1	2.576805	1.48E-05	0.002529312
ENSRNOG00000010597	Slc5a7	1.967894	1.65E-05	0.002798929
novel.11984	–	2.749849	1.66E-05	0.002802539
ENSRNOG00000033026	Dclk3	1.866587	1.74E-05	0.002910833
ENSRNOG00000018257	Hpx	2.899361	1.78E-05	0.002961488
ENSRNOG00000010440	Gnal	1.829045	1.88E-05	0.003108202
ENSRNOG00000055705	LOC103694905	–7.39704	1.96E-05	0.003205799
novel.6068	–	2.336162	2.01E-05	0.003270511
ENSRNOG00000018613	Crip3	3.792127	2.06E-05	0.003318338
novel.6089	–	–2.00019	2.08E-05	0.003327142
novel.77	–	2.036851	2.30E-05	0.0036328
ENSRNOG00000008644	Nkx2-1	2.480539	2.30E-05	0.0036328
novel.8248	–	1.776533	2.93E-05	0.00460042
ENSRNOG00000012315	Agmat	2.845384	3.04E-05	0.004683547
novel.15258	–	–7.32349	3.05E-05	0.004683547
novel.15492	–	7.337672	3.05E-05	0.004683547

padj, Adjusted *P* value.