Abstract—Ischemic preconditioning (IPC) is a phenomenon in which a short-term sublethal ischemic exposure induces tolerance to a subsequent lethal ischemic insult; however, the detailed mechanism underlying IPC-induced neuroprotection remains obscure. Here, we applied middle cerebral artery occlusion, a preconditioning ischemic insult mouse model, to investigate the molecular mechanism underlying cerebral IPC. RNA sequencing and whole-genome bisulfite sequencing were performed to explore the gene expression profile and DNA methylation changes after cerebral IPC treatment. In this study, we identified 636 differentially expressed genes enriched for several pathways that were partially overlapping or interconnected in terms of similar gene function. The involvement of several genes in IPC-induced neuroprotection was first reported. Genes induced by IPC, including *Arid5a*, *Nptx2* and *Stc2*, demonstrated a neuroprotective effect against oxygen–glucose deprivation induced neurotoxicity *in vitro*. Thus, our findings provide new insights into IPC signaling pathways and offer a novel therapeutic strategy towards stroke. © 2019 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: neuroprotection, ischemic preconditioning, middle cerebral artery occlusion, oxygen–glucose deprivation, DNA methylation.

INTRODUCTION

The non-idiopathic stroke is characterized by temporary or permanent neuronal impairment caused by acute dysfunction of cerebral blood circulation to central lobes or hemorrhage-induced intracerebral bleeding (Huang and McNamara, 2004). During an ischemic stroke, a local shortage of blood supply to the brain leads to a dramatic decrease of oxygen and glucose, a breakdown of ATP production, and ultimately dysfunction of brain energy metabolism (Sims and Muyderman, 2010). Recent epidemiological reviews of stroke incidence in China illustrate that both morbidity (5.54–8.34 per 1000) (Wu et al., 2013) and mortality (109.13–121.57 per 100,000) (Sun et al., 2013) increase within two decades, presenting a lethal public health challenge. Presently, effective treatment or drug options are lacking in clinical practice. Limited knowledge of the molecular mechanisms underlying the pathological process of ischemia–reperfusion injury (IRI) is a major challenge restricting the development of treatments and drugs. Among several models that are widely used to explore the mechanisms and treatments of stroke, cerebral ischemic preconditioning (IPC) is counterintuitive but intriguing and promising. Cerebral IPC can be defined as a phenomenon in which both a short-term and non-lethal ischemic episodes increase the preventative tolerance of neurons in response to subsequent long-term and lethal ischemic damage, thus acting as a neuroprotective shielding to brain cellular stress (Yang et al., 2017). Kitagawa and colleagues first reported that two minutes of cerebral ischemia in advance ameliorated the neuronal death caused by a subsequent 5 min treatment (Kitagawa et al., 1990). The concept of cerebral IPC has been applied in both clinical trials and animal models. For example, a randomized
pilot study during a phase II trial of patients with acute ischemic stroke symptoms showed improved neurological outcomes when treated with Remote Ischemic Conditioning After Stroke Trial (RECAST). This clinical trial showed that Remote Ischemic Conditioning (RIC) induced neuroprotection to mitigate symptoms such as intermittent limb ischemia in stroke patients (England et al., 2017). Besides, various mouse models have allowed for a controlled and standardized reproduction of cerebral ischemia; these advances have led to the application of various mouse systems to simulate IPC, such as remote limb ischemic preconditioning models, global ischemia models, and focal ischemia models (Schaller and Graf, 2002). Here, we will explore the mechanisms that underlie middle cerebral arterial occlusion (MCAO), a focal ischemia model that is implemented in this study. In this model system, a surgical filament is used to obstruct the middle cerebral artery, which leads to the blockage of blood, and thus infarction of cerebrum (Chiang et al., 2011). If the occlusion is removed during a transit interval (such as 15 min, 30 min or 1 h) and blood flow is allowed to return the cerebrum, then this procedure can be viewed as comparable to an IPC treatment (Stenzel-Poore et al., 2003).

As a phenomenon showing potential clinical importance, cerebral IPC attracts intensive research programs with identification of several novel genes of interest and their function in both pathophysiology and neuroprotection. Previous clinical trial suggested that Heat Shock Protein 27 (HSP27) mediated neuroprotective effects (England et al., 2017). In mouse models, RIC induced neuroprotection was promoted through the adenosine monophosphate-activated protein kinase (AMPK) mediated immune regulation and autophagy (Guo et al., 2018; Vaibhav et al., 2018). Additionally, cerebral IPC also induces the upregulation of Notch1, which suggests the activation of related Notch signaling pathways during neuroprotection (Chen et al., 2018). Specifically, it was further described that nitric oxidation-induced activation of the Ras/extracellular regulated kinase cascade played an important role in IPC (Gonzalez-Zulueta et al., 2000). Besides, IPC induced neuroprotection can be abolished by protein synthesis inhibitors, suggesting that new protein synthesis is required for protection (Zhang et al., 2009).

Epigenetic mechanisms are also involved in IPC, such as DNA methylation, histone modification, non-coding RNA, and micro-RNA; the role of DNA methylation as an independent factor in IPC is still controversial (Thompson et al., 2013; Lopez et al., 2016). Regardless, it has been observed in stroke patients, RIC was able to modify expression patterns of several genes through regional DNA methylation, such as CEACAM8 and CRISP3 (Nikkola et al., 2018). In terms of mouse models, the 30 min MCAO was shown to reduce DNA methylation, and the scopes of infarcts induced by MCAO treatment were smaller in DNA methyltransferase knockout mice than in control mice (Endres et al., 2000). These results suggest a significant linkage does exist between neuroprotection and DNA hypo-methylation. However, it was uniquely noted that decreased levels of DNA methyltransferase 1 (Dnmt1), rather than the absence of Dnmt1, showed neuroprotective effects (Endres et al., 2001). Generally, various ischemic conditions have been shown to produce diverse and dynamic DNA methylation profiles (Meller et al., 2015). Overall, the relation between DNA methylation and cerebral IPC needs further exploration to uncover and translate the molecular mechanisms underlining such an association.

In this study, we used an ischemic preconditioning mouse model through MCAO and performed RNA-seq and whole-genome bisulfite sequencing to characterize the gene expression profile and changes in whole-genome methylation after cerebral IPC treatment. Arid5a, Stc2, and Nptx2 were identified as IPC-inducible genes and provided neuroprotective functions during ischemic injury.

**EXPERIMENTAL PROCEDURES**

**Transient Focal Ischemia**

Cerebral focal ischemia was induced by occlusion of the middle cerebral artery. To avoid possible sex-specific sensitivity to ischemic tolerance, only C57 male mice which were purchased from the Laboratory Animal Center of the Military Medical Sciences Academy were subjected to MCAO as described previously (Andrab et al., 2011; Zhang et al., 2016, 2018). In brief, mice were anesthetized with 1.5–2% isoflurane and maintained at 37 °C throughout the surgical procedure using a heating pad and rectal probe (Harvard Apparatus, USA). A monofilament coated with silicone was inserted via a small nick in the right external carotid artery and slowly pushed through the right internal carotid artery until it reached the base of the middle cerebral artery to block the blood flow into the middle cerebral artery brain territory.

For MCAO ischemic precondition, 15 min of occlusion was performed in the mice as described previously (Stenzel-Poore et al., 2003). Blood flow to the brain during the entire occlusion phase and reperfusion was monitored by Laser Doppler flowmetry with a probe placed directly on the exposed skull over the lateral parietal cortex. After 15 min of occlusion, the filament was removed, and reperfusion was verified. Mice with average blood flow records that dropped below 80% of the baseline value were included in the experiments. Besides, any mice died during the surgery were excluded for analysis. Based on our previous studies (Zhang et al., 2016, 2018) and other researchers’ findings (Andrab et al., 2011) to characterize the early changes in the DNA methylation and RNA transcription, animal brains were removed at 6 h after IPC, and the ipsilateral cortex which was subject to IPC treatment and the corresponding contralateral undamaged cortex which served as the control of the same brain were collected and frozen at ~80 °C for the following experiments. All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committees at the Peking Union Medical College.

**Whole Transcriptome Library Preparation and RNA-Sequencing Analysis**

Total RNA was isolated from ipsilateral and contralateral cortices from three mouse brains and subjected to quality control according to the manufacturer’s instructions. Two
micrograms of RNA per sample were used as input material for library construction. Ribosomal RNA (rRNA) was removed by an Epicenter Ribo-zero rRNA Removal Kit (Epicenter, USA) according to the manufacturer's instructions. Subsequently, strand-specific libraries were generated with the dUTP method using the resulting RNA by an NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations. RNA-seq was performed on the Illumina HiSeq 2000 platform, and 100-bp paired-end reads were generated according to Illumina's protocol. Prior to RNA-seq data analysis, the adapter sequences were removed from the raw sequencing data, and the individual libraries were converted to the FASTQ format. Sequence reads were aligned to the mouse genome (mm10) with TopHat2 (v2.0.9), and the resulting alignment files were reconstructed with Cufflinks (v2.1.1) and Scripture (beta2). The RefSeq database (build 37.3) was chosen as the annotation reference for mRNA analyses. The read counts of each transcript were normalized to the length of the individual transcript and to the total mapped fragment counts in each sample and were expressed as fragments per kilobase of exon per million fragments of mRNA mapped (fragments per kilobase million; FPKM) in each sample. Differential mRNA expression between the IP-treated and control groups was analyzed, and an adjusted P-value < .05 (Student's t test with Benjamini-Hochberg false discovery rate (FDR) adjustment) was used as the cutoff for significantly differentially expressed genes (DEGs). DEGs were analyzed by enrichment analyses to detect overrepresented functional terms present in the genomic background. A gene ontology (GO) analysis was performed using the GO-seq R package (Young et al., 2010), in which gene length bias was corrected, and a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using KOBAS software (Mao et al., 2005).

WGBS Library Preparation, Sequencing and Analysis

Genomic DNA samples were isolated from ipsilateral and contralateral cortices of mouse brains using a DNeasy Blood & Tissue Kit according to the manufacturer's instructions. A total of 500 ng of DNA spiked with 5 ng of lambda DNA (Promega, Madison, WI) per sample was fragmented to 250–350 bp by sonication with a Covaris M220, followed by end repair and methylated adaptor ligation. These DNA fragments were treated with bisulfite using an EZ DNA Methylation-Gold™ Kit. The obtained single-strand DNA fragments were amplified by PCR using KAPA HiFi HotStart Uracil + ReadyMix (2x) followed by purification by AMPure XP magnetic beads. The library concentration was quantified by a Qubit 2.0 Fluorometer, and the insert size was assessed on an Agilent Bioanalyzer 2100 system. After clustering the index-coded samples, which was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS, the library was sequenced on an Illumina HiSeq 2500 platform, and 150 bp paired-end reads were generated according to Illumina's protocol. Prior to data analysis, adapter oligonucleotide sequences were removed from the raw sequencing data, and the individual libraries were converted to the FASTQ format. Additionally, reads with a percentage of Ns (unknown bases) that was larger than 10% and low quality (PHRED score ≤ 5, percentage of low-quality bases ≥ 50%) were also filtered out. Sequencing reads were analyzed using the BSmooth software package (Hansen et al., 2012). The sodium bisulfite non-conversion rate was calculated as the percentage of cytosines sequenced at cytosine reference positions in the lambda genome. To identify differentially methylated regions (DMRs), the BSeq package in BSmooth was employed. Only CpGs appearing at least twice in at least two samples per group were included in the analysis. DMRs were identified using a smooth window containing either 70 CpGs or a width of 1 kb, whichever was larger. Next, regions satisfying the following criteria were deemed putative DMRs: 1) a t-statistic cut-off of (−2.5, 2.5); 2) must contain at least 3 CpG sites; and 3) must have a methylation difference of at least 10%. All data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE122107.

Bisulfite Sequencing

Bisulfite conversion of genomic DNA was performed according to the instructions of EZ DNA Methylation-Direct™ Kit (Zymo Research, USA) followed by nested PCR for the amplification of the DMRs (Bakulski et al., 2015). The primer sequences were listed in Table 3. The PCR products were detected on 2% agarose gel and then sequenced on the PyroMark Q24 platform.

Gene Expression Validation by Western Blotting

Total protein extracts were prepared from the ipsilateral and contralateral cortices of eight mice and size-separated through SDS/PAGE as described previously (Zhang et al., 2011a, b). After electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane. Membranes were blocked in TBST buffer (20 mM Tris base, 150 mM NaCl, 0.05% Tween 20, pH 7.4) containing 5% nonfat milk at room temperature for 2 h prior to incubation with primary antibody at 4 °C overnight. TBST-washed membranes were incubated with goat anti-rabbit (Santa Cruz, sc-2004, Lot # A1416) or goat anti-mouse (Santa Cruz, sc-2005, Lot # J2215) secondary antibodies in TBST with 5% nonfat milk and then washed with TBST, and membrane-bound antibody was visualized in a chemiluminescence assay. Beta-tubulin was used for normalization. Anti-Arid5a polyclonal antibody (Lot #ab81207) was purchased from Abcam, anti-Nptx2 polyclonal antibody (Lot #10889–1-AP) and anti-Stc2 monoclonal antibody (Lot #60063–1-Ig) were purchased from Protimech, and anti-beta-tubulin antibody (Lot # 030 M4788) was purchased from Sigma Applied Science.

Construction of the Overexpression Vectors and Lentivirus Generation

Arid5a, Nptx2 and Stc2 open reading frames (ORF) were purchased from OriGene and subsequently cloned into a
cFUGW-GFP lentiviral vector using the multiple clone sites. Lentiviral constructs were generated by transient transfection of 293 T cells by using the FuGENE HD Transfection Reagent by Roche Applied Science as described previously (Zhang et al., 2011a, b). In brief, cells were co-transfected with the vector plasmid (cFUGW-GFP, cFUGW-Arid5a, cFUGW-Npx2 and cFUGW-Stc2), the trans-complementation plasmids (pLP1 and pLP2), and the plasmid encoding the vesicular stomatitis virus envelope glycoprotein (VSVG). Sodium butyrate was added to the media 4–6 h after transfection. The medium was replaced 12 h after transfection and collected at 36 h and 60 h later. Supernatants were centrifuged at 800 × g for 10 min, treated with DNase and passed through a filter with 0.45-mm pores. Viral particles were then concentrated by ultracentrifugation (90 min, 25,000 r/min, rotor SW28) and resuspended in 0.1 M PBS.

Neuronal Cultures and Ischemic Modeling
Primary cortical neuron cultures were prepared from gestational day 14.5 fetal mice as previously described (Dai et al., 2010). In mature cultures, neurons represent approximately 80% of the total number of cells. Neurons were transduced with the overexpressing lentiviral constructs at 7 days in vitro (DIV). Three days later, an in vitro equivalent of ischemic modeling was used, whereby the neurons were subjected to oxygen–glucose deprivation (OGD) as previously described (Dai et al., 2010; Zhang et al., 2018). Briefly, OGD was performed by complete replacement of the media with deoxygenated, glucose-free Earle's balanced salt solution. Cultures were kept in an anaerobic chamber (Biospherix Ltd., USA) for 120 min at 37 °C. OGD was terminated by replacement of the Earle’s balanced salt solution with oxygenated growth media. Cells were washed twice with control salt solution (CSS) at a pH of 7.4 containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 25 mM Tris-Cl, 15 mM glucose. Neurotoxicity was assessed by computer-assisted cell counting after staining with 1 μg/ml Hoechst 33342 (Molecular Probes) and 5 μM propidium iodide (PI) (Sigma, St. Louis, MO). The numbers of total and dead cells were counted with Axiovision 4.7 software (Zeiss, Thornwood, NY), and the percentage of neuronal death was quantified and expressed as the mean plus standard error of the mean (SEM) from three independent experiments performed in triplicate. Data were analyzed by Student's t test for independent means.

RESULTS
Transcriptome Profile of IPC-treated Cerebral Cortices
To investigate the mechanisms underlying the neuroprotective function of ischemic preconditioning, mice weighing 20–23 g were subject to 15 min MCAO (Fig. 1A). Laser Doppler ultrasonography and perfusion imagery were applied to monitor brain blood flow. The baseline blood flow was considered to be 100% for all mice; any mice with average blood flow records that dropped below 80% value were included in the further molecular analysis experiments (Fig. 1B). After common carotid artery occlusion (CCAO), the first blood flow decline was noted and occlusion of the middle cerebral artery led to the second reduction in blood flow (Fig. 1B). Over the 15 min period of occlusion, cortical perfusion monitored by Laser Doppler ultrasonography was reduced 84.12 ± 0.32% of baseline. The reduction was stable throughout the occlusion period and recovered to pre-ischemic levels upon removal of the filament (Fig. 1B). Mice were divided into three groups: for RNA-seq, for proteins examination, and for the DNA methylation analysis (Fig. 1A).

To explore the transcriptional profile of IPC-treated cerebral cortices, total RNA was extracted from the ipsilateral and contralateral cerebral cortices from the same mouse brain. The contralateral undamaged cerebral cortices were used as controls. RNA-sequencing results demonstrated a significantly different gene expression pattern. The IPC-Induced Global DNA Methylation Profile and DMRs
To further explore how the DEGs were regulated during IPC, we also performed WGBS to characterize the profile
whole-genome DNA methylation after IPC treatment. A total of 1,668,004,204 clean reads were obtained. The mapping rates were 95.06% and 95.82% in the IPC-treated and control cerebral cortices, respectively, and the bisulfite conversion rates were over 99.5% in both groups. We also calculated the methylation level of each cytosine. A total of 43,735,674 CpG sites were mapped, and the coverage rates were 81.89% and 80.79% in the IPC-treated and control cortices, respectively. The percentages of methylated cytosines (mCs) in the IPC-treated and control cortices were 72.96% and 72.90%, respectively. All of these results suggest that the WGBS libraries were well prepared and sequenced in both groups.

Next, we performed the DMR analysis and identified 10,799 DMRs and 6865 DMR-related genes in the genomic regions (including the 5’UTR region, 3’UTR region, promoter, upstream, downstream, intron and exon) in the IPC-treated samples compared to those in the controls (Fig. 3A). We found that more DMRs were distributed in the intronic regions, although the promoter regions were also noted to be important (Fig. 3B).

The Link Between DNA Methylation and Gene Expression

To ascertain the potential relationship exists between DNA methylation and individual gene expression induced by IPC, we linked and cross-analyzed the gene list from WGBS and actively compared this data to identified DEGs obtained using RNA-seq. The results showed that the RNA expression levels of 355 genes were significantly linked to the DNA methylation changes in 531 DMRs (Fig. 4A). We then classified DMRs into four groups according to the hypo- or hypermethylation and down- or upregulation of RNA expression (Fig. 4B). We found that 151 upregulated genes showed hypomethylation in their DMRs and that only 15 downregulated genes displayed hypermethylation in their DMRs. We also found negative correlations between RNA expression and DMRs located in exons, in introns, overlapping the 3’UTR, overlapping the 5’UTR, overlapping an exon downstream, and overlapping an exon upstream (Fig. 4C). These results suggest extensive demethylation in the regulatory regions of many neuroprotective genes, initiating gene expression and protein synthesis after IPC treatment.

Overexpression of IPC-induced Genes Elicits Neuroprotection

In consideration of the results from both RNA-seq and DNA methylation, we selected 3 unique mRNA genes that were upregulated, namely Arid5a, Nptx2 and Stc2, and two of which were shown to be hypo-methylated in their DMRs. The overall mean methylation level at the Arid5a DMR was significantly reduced 15.08% in IPC-treatment. For the Nptx2
Fig. 2. Gene ontology (GO) and pathway analysis of DEGs. (A) Heat map of DEGs in the IPC treatment vs. Control groups. (B) GO term analysis for differentially regulated genes between the IPC and Control groups. The vertical axis represents the GO category (biological process, cellular component and molecular function), and the horizontal axis represents the -log10 (P-value) of the significant GO terms. (C) Scatter plot of the KEGG enrichment analysis for differentially regulated genes between the IPC and Control groups. The size of each circle stands for the number of significantly DEGs enriched in the corresponding pathway. The enrichment factor was calculated using the number of enriched genes divided by the number of all background genes in the corresponding pathway. The q value is depicted according to the color scale, and q values <0.05 are to be considered to indicate statistical significance.
DMR, the mean methylation level was reduced 13.31\% except for the DMR in inside exon (Table 2). Individual CpG analysis was determined by bisulfite pyro-sequence indicating that IPC induced hypo-methylation of Arid5a DMR and Nptx2 DMR at several sites (Fig. 5A and B). Western blots verified that the expression levels of Arid5a, Nptx2 and Stc2 were significantly increased after IPC in the mouse MCAO model (Fig. 5C and D).

To ascertain whether these upregulated genes have potential neuroprotective actions, we prepared lentiviruses expressing Arid5a, Nptx2 and Stc2 individually and used the vectors to transduce primary cortical neurons followed by exposure to OGD. Western blots showed that lentiviral transduction led to significant overexpression of Arid5a, Nptx2 and Stc2 proteins in HEK293T cells (Fig. 5E). For the primary neuronal cultures, we found that overexpression of Arid5a provided approximately 60\% protection against OGD-induced cell death, whereas overexpression of Nptx2 and Stc2 showed approximately 50\% and 35\% protection, respectively (Fig. 5F and G). Taken together, these results indicate that IPC-induced gene upregulation provides neuroprotection against subsequent lethal ischemic injury.

**DISCUSSION**

IPC is a powerful endogenous phenomenon in which a brief sublethal ischemic insult induces robust protection against subsequent lethal ischemia (Yang et al., 2017). Although the exact mechanisms of IPC-induced neuroprotection remain unclear, this powerful mechanism holds substantial promise to identify novel neuroprotective agents that could be used to treat various neurological disorders including stroke. In this study, we characterized the profiles of gene expression and whole-genome methylation by RNA-seq and WGBS in an IPC-treated mouse model of MCAO and identified several novel genes related to cerebral IPC. Therefore, our findings provide novel insights for the investigating IPC signaling pathways and the mechanisms of ischemia-induced neuronal cell death and survival. We also identified Arid5a, Stc2, and Nptx2 as IPC-inducible genes that were upregulated and demonstrated neuroprotective functions during ischemic injury.

It is believed that new protein synthesis is required for IPC-induced protection (Barone et al., 1998). Currie and colleagues reported that the expression levels of Hsp70 and Hsp27 were significantly increased in the MCAO mouse model (Currie et al., 2000; Dhodda et al., 2004). Here, we found that Hspa1b and Hspa1a ranked as the top 2 genes in the MCAO model (Table 2). Individual CpG analysis was determined by bisulfite pyro-sequence indicating that IPC induced hypo-methylation of Arid5a DMR and Nptx2 DMR at several sites (Fig. 5A and B). Western blots verified that the expression levels of Arid5a, Nptx2 and Stc2 were significantly increased after IPC in the mouse MCAO model (Fig. 5C and D).

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**Table 1.** Identified genes upregulated during IPC treatment.

| Gene | Full gene name | Con | IPC | Log2 ratio | FDR  |
|------|----------------|-----|-----|------------|------|
| Hspa1b | heat shock 70 kDa protein 1b | 168 | 11,915 | 5.81 | 2.14E-100 |
| Hspa1a | heat shock 70 kDa protein 1a | 118 | 7309 | 5.62 | 4.75E-03 |
| Arid5a | AT rich interactive domain 5a | 245 | 1132 | 1.86 | 1.34E-07 |
| A2m | alpha-2-macroglobulin | 124 | 616 | 1.96 | 5.50E-06 |
| Nptx2 | neuronal pentraxin 2 | 1055 | 4172 | 1.63 | 9.37E-04 |
| Hsp70 | heat shock 105 kDa/110 kDa protein 1 | 4617 | 33,237 | 2.5 | 2.91E-04 |
| Dnajb1 | DnaJ homolog, subfamily B, member 1 | 862 | 4774 | 2.13 | 3.35E-04 |
| Atf3 | activating transcription factor 3 | 11 | 359 | 4.71 | 7.79E-04 |
| Btg3 | BTG family, member 3 | 369 | 1197 | 1.34 | 3.84E-03 |
| Arc | activity-regulated cytoskeleton | 1637 | 14,575 | 2.81 | 1.32E-03 |
| Piezo1 | piezo-type mechanosensitive ion channel 1 | 191 | 815 | 1.75 | 1.38E-03 |
| Hmox1 | heme oxygenase 1 | 105 | 1057 | 2.98 | 1.09E-04 |
| Peg10 | paternally expressed 10 | 598 | 1794 | 1.22 | 6.97E-03 |
| Hsp60 | heat shock 10 kDa protein 1 | 463 | 2427 | 2.03 | 7.51E-04 |
| Mmp12 | matrix metalloproteinase 12 | 1 | 53 | 5.74 | 2.45E-06 |
| Stc2 | stanniocalcin 2 | 64 | 507 | 2.63 | 5.96E-06 |
| Serpine1 | serpin peptidase inhibitor, clade E, member 1 | 23 | 75 | 4.09 | 3.04E-05 |
| Lrc32 | leucine rich repeat containing 32 | 88 | 558 | 2.32 | 8.05E-06 |
| Ccl4 | chemokine (C-C motif) ligand 4 | 3 | 75 | 4.09 | 3.04E-05 |
| Numb | numb homolog | 1005 | 2709 | 1.08 | 6.10E-04 |
| Rnd3 | Rho family GTPase 3 | 285 | 918 | 1.33 | 5.29E-04 |
| Stc5a3 | solute carrier family 5, member 3 | 1825 | 5823 | 1.31 | 7.67E-03 |
| Chac1 | ChaC, cation transport regulator homolog 1 | 118 | 821 | 2.44 | 7.67E-03 |
| Phlda1 | pleckstrin homology-like domain, A, member 1 | 271 | 771 | 1.15 | 9.38E-03 |
| Jun | jun proto-oncogene | 1115 | 4732 | 1.75 | 3.68E-02 |
| Samd4 | sterile alpha motif domain containing 4 | 687 | 2170 | 1.31 | 1.25E-01 |
| Hspd1 | heat shock 60 kDa protein 1 | 1975 | 7256 | 1.52 | 8.59E-01 |
| Cckbr | cholecystokinin B receptor | 830 | 2179 | 1.04 | 9.19E-01 |
| Dnajb1 | DnaJ homolog, subfamily A, member 1 | 3558 | 10,585 | 1.22 | 3.41E-05 |
| Hspb1 | heat shock 27 kDa protein 1 | 120 | 1016 | 2.73 | 7.22E-05 |
Fig. 3. Analysis of the profile of whole-genome DNA methylation in the IPC-treated cerebral cortex. (A) Circos plot of the DMR distribution across the whole genome in the IPC-treated cerebral cortex. The outer circle represents the average methylation levels of the IPC-treated group. The middle circle indicates the average methylation levels of the control group. The inner circle indicates the different methylation levels. DMRs were more frequent on chromosomes 1, 4, 5 and 15. (B) The distribution of DMRs in various gene structures. (C) The lengths of DMRs.
vivo can reduce brain damage following the ischemic insult in mice by buildup of a "neuroprotective shield" after by the formation of a calcium/calmodulin complex which initiates a response at the genomic level (Zhang et al., 2011a, b). The expression of this neuroprotective gene was also found to increase during as a response to IPC-induced protection. Heme oxygenase 1 (Hmox1) acts as a phase 2 electrophilic inducer downstream of the Keap1-Nrf2 pathway, this gene encodes for an enzyme which catabolizes free heme groups, up-regulates IL-10 IL-1RA, thus preventing against oxidative stress through anti-inflammatory mechanisms (Piantadosi et al., 2011). Specifically, resistance to oxidative stress and neuronal cell death in the cerebellar granule neurons of Hmox1 transgenic mice were protected from stressful pathological events (Chen et al., 2000). Activity-regulated cytoskeleton-associated protein (Arc) (also known as Arg3.1) is one of neuronal activity regulated molecules; Arc mRNA can rapidly accumulate in dendritic spines in response to neuronal activity, this change in function is able to target and activate synapses (Lyford et al., 1995; Farris et al., 2014; Na et al., 2016). Additionally, Arc plays a significant role in the regulation surface amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor trafficking, which provides necessary contributions to neuroprotection and homeostatic maintenance of neuronal plasticity (Peebles et al., 2010). The expression of the previously described genes and their products are consistent with our RNA-seq results. The consistency between our results and previously described studies in the literature demonstrate the accuracy and reliability of employing IPC models for this paper.

In this study, we also confirmed that three IPC-induced genes (Stc2, Arid5a and Nptx2) have neuroprotective functions against the in vitro ischemia OGD induced cytotoxicity. Stc2 is a glycoprotein hormone that has been identified as a biomarker of tumor onset, and this gene is highly expressed in several cancer types and facilitates tumor invasion and metastasis (Kita et al., 2011). Furthermore, Stc2 is a HIF-1 target protein that is involved in the regulation of cell proliferation under hypoxia (Law and Wong, 2010). Stc2 is also termed as a necessary survival component in relation to unfolded protein response (UPR) (Ito et al., 2004) and as a

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**Table 2.** DMR-related genes that were most likely related with the upregulated genes.

| Gene  | DMR                        | MCAO methy-level | Control methy-level | Direction | Genomic description |
|-------|----------------------------|------------------|---------------------|-----------|---------------------|
| Arid5a | Chr1(36309076–36,309,909) | 0.547960057      | 0.697549311         | hypo      | inside intron       |
| Arid5a | Chr1 (36324051–36,324,359) | 0.546945062      | 0.687466176         | hypo      | downstream          |
| Arid5a | Chr1 (36303012–36,303,337) | 0.110986999      | 0.265011815         | hypo      | upstream            |
| Arid5a | Chr1 (36324694–36,324,935) | 0.579443291      | 0.73846478          | hypo      | downstream          |
| Nptx2 | Chr5 (144556271–144,556,337) | 0.74075543      | 0.820342792         | hyper     | inside exon         |
| Nptx2 | Chr5 (144636366–144,637,103) | 0.665392099     | 0.771857442         | hypo      | downstream          |
| Nptx2 | Chr5 (144457483–144,457,925) | 0.472346672      | 0.620632668         | hypo      | upstream            |
| Nptx2 | Chr5 (144594841–144,595,080) | 0.665984874      | 0.810551314         | hypo      | downstream          |
negative modulator of Ca^{2+} cytoplasmic entry maintenance and Ca^{2+} homeostasis (Zeiger et al., 2011). Stc2 showed neuroprotective effects in response to hippocampal degeneration by means of mitigating the levels of nitric oxide, TNF-α, and IL-1β (Byun et al., 2010); these results are in agreement with our findings.
our study indicates that *Arid5a* expression was induced in the process of neuronal cell death or degeneration (Tanaka et al., 1998). *Arid5a* is a member of the Arid protein family, which have diverse functions that are directly involved in the development of both tissue-specific gene expression and regulation of cell growth. *Arid5a* is a protein that can regulate both IL-6 and TNF-α in response to LPS by stabilizing the local expression of IL-6 and T-bet mRNA, respectively (Masuda et al., 2013; Zaman et al., 2016). In our present study, we found that IPC can induce *Arid5a* upregulation after MCAO, and recombinant lentivirus-mediated overexpression of *Arid5a* in primary neurons protected against OGD-mediated neurotoxicity. Those results have the potential to define a novel molecular function for *Arid5a* as a novel modulator of neuronal cell death. *Nptx2* (also known as *Narp*) is a secreted immediate early gene regulated by synaptic activity in brain, and it plays a crucial role in the formation of excitatory synapses, especially GluA4-containing AMPA receptors (O’Brien et al., 1999; Gu et al., 2013). A recent study showed that postmortem human AD cortex and CSF displayed pro-

ductions providing novel insights into the mechanisms underlying IPC-induced neuroprotection, which are imperative for the identification of neuroprotective agents that could be used to treat various neurological disorders including stroke.

**Author Contributions**

MC designed and performed experiments and wrote the manuscript. ZL set up the MCAO mouse model and neuronal culture. YZ and YL assisted with analysis of the RNA-seq and WGBS data. YZ, YH and HC assisted with designing the scheme of this study. YL, JJ, WH and JZ designed the scheme of this study or revised the manuscript.

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**Conflict of Interest Statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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