Research Article

Genome-wide interrogation of transfer RNA-derived small RNAs in a mouse model of traumatic brain injury

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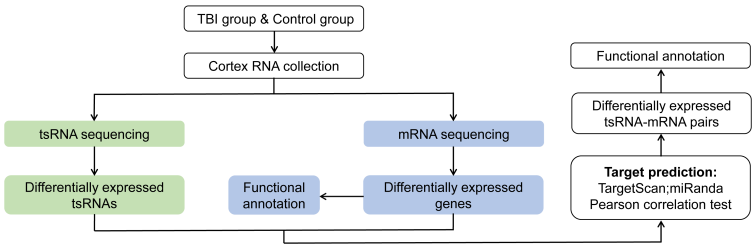
Graphical Abstract *Transfer RNA-derived small RNAs (tsRNAs) might be implicated in secondary brain injury*



Abstract

Transfer RNA (tRNA)-derived small RNAs (tsRNAs) are a recently established family of regulatory small non-coding RNAs that modulate diverse biological processes. Growing evidence indicates that tsRNAs are involved in neurological disorders and play a role in the pathogenesis of neurodegenerative disease. However, whether tsRNAs are involved in traumatic brain injury-induced secondary injury remains poorly understood. In this study, a mouse controlled cortical impact model of traumatic brain injury was established, and integrated tsRNA and messenger RNA (mRNA) transcriptome sequencing were used. The results revealed that 103 tsRNAs were differentially expressed in the mouse model of traumatic brain injury at 72 hours, of which 56 tsRNAs were upregulated and 47 tsRNAs were downregulated. Based on microRNA-like seed matching and Pearson correlation analysis, 57 differentially expressed tsRNA-mRNA interaction pairs were identified, including 29 tsRNAs and 26 mRNAs. Moreover, Gene Ontology annotation of target genes revealed that the significantly enriched terms were primarily associated with inflammation and synaptic function. Collectively, our findings suggest that tsRNAs may be associated with traumatic brain injury-induced secondary brain injury, and are thus a potential therapeutic target for traumatic brain injury. The study was approved by the Beijing Neurosurgical Institute Animal Care and Use Committee (approval No. 20190411) on April 11, 2019.

Key Words: gene set enrichment analysis; inflammation; integrated analysis; neurodegenerative disease; next-generation sequencing; secondary injury; synaptic function; transfer RNA-derived small RNAs; transfer RNAs; traumatic brain injury

Chinese Library Classification No. R446; R742; Q344+.13

Introduction

Traumatic brain injury (TBI) pathogenesis is a complex process that results from primary and secondary insults (Galgano et al., 2017). Primary injury is caused by mechanical force and occurs at the moment of injury; it is followed by delayed and protracted secondary injury (Bae et al., 2018). Secondary injury occurs as a consequence of diverse pathological mechanisms, including excitotoxicity (Fujikawa, 2015; Tehse and Taghibiglou, 2019), oxidative stress (Cornelius et al., 2013; Greco et al., 2016; Chen et al., 2017), cerebral metabolic dysfunction (Glenn et al., 2003; Soustiel et al., 2005), cerebrovascular pathology (Len and Neary, 2011; Ramos-Cejudo et al., 2018; Sun et al., 2021), chronic inflammatory events (Kumar et al., 2015; Corrigan et al., 2016; Clark et al., 2019), and mitochondrial dysfunction (Hiebert et al., 2015; Pandya et al., 2019). Because of the heterogeneous nature of its complicated pathogenesis, no effective therapy is available to improve clinical outcomes for patients with TBI. A better understanding of the precise molecular mediators underlying TBI pathogenesis, especially in secondary injury-associated processes, is critical for developing effective therapeutic approaches for TBI patients.

Transfer RNA (tRNA)-derived small RNAs (tsRNAs) are a recently established family of regulatory small non-coding RNAs that modulate diverse biological processes, including sperm maturation (Peng et al., 2012; Sharma et al., 2016), the onset and progression of multiple types of cancers (Balatti et al., 2017), stress responses (Fu et al., 2009; Thompson and Parker, 2009; Yamasaki et al., 2009; Kumar et al., 2016), transposon control (Martinez et al., 2017; Schorn et al., 2017;

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Zhang et al., 2017), intergenerational epigenetic inheritance (Chen et al., 2016; Shi et al., 2019; Zhang et al., 2019b), and neuronal function (Karaiskos and Grigoriev, 2016). tsRNAs are derived from tRNAs, which are highly conserved and essential components of translation machinery. Pre-tRNAs are transcribed by RNA polymerase III and generate mature tRNA after endonucleolytic cleavage by RNases P and Z and the subsequent addition of a CCA tail (Phizicky and Hopper, 2010). Mature tRNAs generally resemble a cloverleaf secondary structure, composed of the dihydrouridine loop, anticodon loop, variable loop, TψC loop, and amino acid acceptor stem (Schimmel, 2018). Based on the mapped region of mature tRNA or pre-tRNA, tsRNA are classified into four major categories: 5′ or 3′ tRNA halves, tRNA-derived RNA fragment (tRF)-5, tRF-3, and tRF-1 (Kumar et al., 2016). With the rapid advances of next-generation sequencing coupled with its plummeting price, the numbers and types of identified tsRNA are increasing. However, the functional roles of tsRNA in physiological and pathological states are far less understood.

The endonucleolytic cleavage of tRNA is a conserved response to genetic and environmental stress, including inflammation, oxidative stress, and metabolic perturbation (Thompson and Parker, 2009; Anderson and Ivanov, 2014; Li et al., 2018; Hogg et al., 2019). Increasing evidence indicates that tsRNA abnormalities are involved in neurological dysfunction and may contribute to the development of neurodegenerative diseases (Schaffer et al., 2014; Karaiskos and Grigoriev, 2016; Hogg et al., 2019; Qin et al., 2020). These findings prompted us to speculate that tsRNA might be implicated in TBI-associated pathophysiological changes. To address this question, a combination of genome-wide tsRNA and messenger RNA (mRNA) sequencing was adopted to investigate the underlying role of tsRNA in TBI.

Materials and Methods

Animals

Adult male C57BL/6 mice (*n* = 30, 8–10 weeks, 25–30 g) were obtained from Beijing Vital River Experimental Animals Technology Co., Ltd. (Beijing, China; license No. SCXK-(Jing) 2016-0006). All animals were housed individually in temperature- (22 ± 2°C) and humidity- (50–60%) controlled animal quarters with food and water *ad libitum*, and were maintained on a 12-hour light/dark cycle. All animal procedures were approved by the Beijing Neurosurgical Institute Animal Care and Use Committee (approval No. 20190411) on April 11, 2019. All experiments were designed and reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Controlled cortical impact models

Mice were randomly divided into two groups (*n* = 15 per group): control and TBI. As in our previously published work (Zhang et al., 2020), mice were anesthetized with 2% isoflurane (RWD Life Science Co., Shenzhen, China) inhalation and positioned in a stereotaxic frame (RWD Life Science Co.). The temperature of each mouse was maintained at (37.0 ± 0.5)°C during surgery using a thermal plate. A midline scalp incision was made to expose the skull. Next, a 4.0-mm craniotomy was made over the right parietal bone using an electric drill (RWD Life Science Co.), without damaging the dura mater. Mice were then subjected to controlled cortical impact with a 3-mm diameter flat tip using an electromagnetic controlled cortical impact device (Pinpoint PCI3000 Precision Cortical Impactor, Hatteras Instruments, Cary, NC, USA) at a velocity of 3 m/s, with a 20 ms dwell time and a depth of 1.5 mm. The controlled cortical impact model was considered successful when cerebral cortical contusion was noticeable (Song et al., 2019). Following the impact, the burr hole was sealed with bone wax and the scalp incision was sutured closed. Animals in the control group underwent the same process, but without the cortical impact.

RNA extraction

Mice were deeply anesthetized and transcardially perfused with 100 mL ice-cold 0.9% saline at 72 hours post-injury. After removal of the brain, cerebral perilesional cortex tissue around 1 mm from the margin of the contusion site was acquired on a chilled stainless-steel plate overlying crushed ice. Subsequently, the RNA was extracted from the cortices using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Pooled samples can reduce variability and compensate for losses in the number of replicates (Takele Assefa et al., 2020). Therefore, following RNA isolation, the cortex lysates from three mice were pooled into one sample, and one group contained three biological replicates. The RNA purity and integrity were evaluated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and an RNA 6000 Nano LabChip Kit (Agilent Technologies). Finally, the resulting RNA from each sample was split into two halves and used for mRNA sequencing (mRNA-seq) or tsRNA sequencing.

mRNA library construction and sequencing

Poly(A) RNA was purified from total RNA with RNA integrity number > 7.0 using poly-T oligo-attached magnetic beads (New England Biolabs, Ipswich, MA, USA) with two rounds of purification. Subsequently, RNA was fragmented into small pieces and reverse-transcribed to construct a cDNA library using the manufacturer's protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego, CA, USA). The average insert size for the paired-end libraries was 300 bp (± 50 bp) and the quality of the sequencing library was determined using an Agilent 2100 Bioanalyzer with an Agilent DNA 1000 chip kit (Agilent). Next, paired-end sequencing was performed using an Illumina HiSeq 4000 (Illumina).

tsRNA library construction and sequencing

tsRNA undergo a large number of post-transcriptional modifications, which can block reverse transcriptase-mediated extension during cDNA synthesis (Cozen et al., 2015). Therefore, Escherichia coli alpha-ketoglutarate-dependent dioxygenase (AlkB) was used to demethylate N1-methyladenosine, N3-methylcytidine, and N1-methylguanosine prior to reverse transcription. Additionally, the following treatments were performed to remove terminal modifications that interfere with adaptor ligation to the RNA ends using the rtStar™ tRF&tiRNA Pretreatment Kit (Arraystar Inc., Rockville, MD, USA): 3′-aminoacyl (charged) deacylation to 3′-OH, 3′-cP (2′,3′-cyclic phosphate) removal to 3′-OH, and 5′-OH (hydroxyl group) phosphorylation to 5′-P (Qin et al., 2019). The libraries with 134–160 bp polymerase chain reaction-amplified fragments (corresponding to an RNA size range of 14–40 nt) were sequenced for 50 cycles using an Illumina NextSeq 500 as per the manufacturer's instructions, thus generating 50 bp single-end read datasets.

Data processing and analysis

For mRNA-seq, clean reads were obtained from raw reads by removing reads that contained adaptors, primers, or Q nucleotide quality scores lower than 20. The clean reads were then aligned to the mouse genome using the HISAT (2.0) package (http://www.ccb.jhu.edu/software/hisat/index.shtml). Next, StringTie (version 1.3.0, http://ccb.jhu.edu/software/stringtie/) was used to assemble transcripts with mapped reads and perform gene- and transcript-level quantifications as fragments per kilobase of transcript per million RNA-sequencing mapped reads.

For tsRNA sequencing, cytoplasmic tRNA sequences were downloaded from GtRNAdb (http://gtrnadb.ucsc.edu/) and mitochondrial tRNA sequences were predicted using tRNAscan-SE (http://trna.ucsc.edu/tRNAscan-SE/). The

mature tRNA library was generated through the removal of intronic sequences and the addition of 3′-terminal CCA tails. In addition, to generate the precursor tRNA library ,40 bp flanking genomic sequences were added to each side of the tRNA sequences. The reads were first subjected to adaptor removal, and reads with a length shorter than 14 nt or longer than 40 nt were then discarded with Cutadapt (version 1.17, https://cutadapt.readthedocs.io/en/stable/). Subsequently, trimmed reads were aligned to the mature tRNA sequences, which accepted only single mismatches with Bowtie software (version 1.2.2, http://bowtie-bio.sourceforge.net/index.shtml). Reads that were not mapped to the mature tRNA sequences were then aligned to the precursor tRNA sequences. The abundance of tsRNA was calculated by the read counts and normalized as the counts per million of total aligned reads. Known tRNA fragments were downloaded from tRFdb (http://genome.bioch.virginia.edu/trfdb/).

Differential expression analysis

Differential expression analysis was performed on raw counts to compare gene expression between the TBI and control groups using the edgeR package (https://bioconductor.org/packages/release/bioc/html/edgeR.html) and the DESeq2 package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html), which is based on a negative binomial regression model. To correct for multiple testing, the false discovery rate (FDR) was calculated using the Benjamini–Hochberg procedure. For mRNA-seq, differentially expressed genes were defined as those having $|\log_2(\text{fold change})| > 1$ and FDR < 0.05. For tsRNA sequencing, differentially expressed tsRNAs were defined as those having a fold change > 1.5 and *P*-value < 0.05.

tsRNA target prediction and correlation analysis

Increasing evidence indicates that tsRNA can regulate gene expression through microRNA (miRNA)-like seed matching mechanisms (Kumar et al., 2014). Therefore, two commonly used miRNA target prediction algorithms, TargetScan and miRanda, were used for the tsRNA target analysis. In addition to a context score percentile > 50 in TargetScan and max energy < –10 in miRanda, putative targets needed to be simultaneously predicted by both algorithms. Furthermore, to reduce false positives produced by miRNA prediction algorithms, the Pearson correlation test was used to analyze pair-wise correlations between tsRNAs and putative target genes using the expression data of tsRNA and mRNA. If $r < -0.5$ and $P < 0.05$, there was a significant negative correlation between tsRNA and gene expression. On the basis of regulatory relationships between tsRNAs and predicted targets genes, a network of tsRNAs and their target genes was constructed using Cytoscape software (version 3.7.2, https://cytoscape.org/).

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis

To depict the biological functions of differentially expressed genes in mRNA-seq and tsRNA target genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (https://www.genome.jp/kegg/) and Gene Ontology (GO) analysis (http://geneontology.org/), respectively, were used. For the GO and KEGG analyses, a list of genes were imported to the web-based tool Metascape (https://metascape.org/gp/index.html#/main/step1), which is a biologist-oriented resource for the analysis of systems-level databases (Zhou et al., 2019). The FDR was calculated using the Benjamini–Hochberg procedure to account for multiple testing. Enriched terms with FDR < 0.05 were considered to be significantly enriched terms.

Protein-protein interaction network and key gene analysis

The protein–protein interaction network was obtained from the online STRING database (Search Tool for Recurring Instances of Neighbouring Genes; https://string-db.org/), which integrates both known and predicted protein–protein interactions (Szklarczyk et al., 2019). The potential interaction network was then imported into Cytoscape, which is a public source software for visualizing and analyzing molecular interaction networks. The application Cytohubba in Cytoscape was used to identify key genes based on the node degree method.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) is a robust threshold-free computational method that determines whether a priori defined sets of genes are differentially expressed in different phenotypes (Subramanian et al., 2005). First, mouse genes were assigned to the corresponding human orthologs using the GSEA "Collapse dataset to gene symbols" feature with respective symbol remapping chip (mouse_Gene_Symbol_Remapping_MSigDB.v7.0.chip). GSEA software (version 4.0.3, https://www.gsea-msigdb.org/gsea/index.jsp) was used to determine canonical pathway enrichment (C2 KEGG subset of canonical pathways). The parameter of permutation was 1000. The normalized enrichment score reflected the enrichment degree in the gene expression data. The FDR was the estimated probability that the enriched gene set with a given normalized enrichment score represented a false-positive finding. Gene sets with FDR < 0.25 were defined as significantly enriched.

Results

Genome-wide profiling of tsRNAs in the mouse cortex after TBI

To dissect the transcriptomic landscape of tsRNAs in the peri-injured cortex of a mouse model of TBI, six cDNA libraries were generated and sequenced. On average, 11.0 million raw reads per cerebral cortex sample were obtained. After the removal of adaptors and read length filtering, approximately 8.3 million clean reads were acquired per sample, of which an average of 18.8% and 1.04% were mapped to mature tRNA and pre-tRNA, respectively.

The sequencing results revealed that the most abundant tsRNAs were 22 nt in length, which accounted for 33.57% and 26.76% of the total clean reads in the control and TBI groups, respectively, and predominantly consisted of tRF-3b and tRF-5b (**Figure 1A** and **B**). Notably, the percentage of tsRNAs with 32 nt in length was significantly increased from 15.21% to 26.68% post-TBI; these were mainly comprised of tRF-5c (**Figure 1A** and **B**).

tRNA isodecoders share the same anticodon but differ in their body sequences (Geslain and Pan, 2010, 2011; Rudinger-Thirion et al., 2011). Mounting evidence indicates that tRNA isodecoders undergo post-transcriptional regulation beyond translation (Geslain and Pan, 2011). In the present study, 254 and 324 isodecoders were detected in the control and TBI groups, respectively. In the control group, His-GTG had the most subtypes, with 14 isodecoders. In the TBI group, Glu-TTC had the most subtypes, with 19 isodecoders after injury (**Figure 1C** and **D**).

The sequencing results demonstrated that 342 tsRNAs were acquired in all libraries, of which 302 tsRNAs were specifically detected in our work compared with the known tRNA fragments from the tRFdb (**Figure 2A**). Furthermore, there were 68 and nine tsRNAs specifically expressed in the TBI and control groups, respectively, while 213 tsRNAs appeared in both groups (**Figure 2B**). Notably, the number of 5′ ends of tRNA-derived fragments, particularly tRF-5c, was markedly increased after TBI (**Figure 2C**).

Identification of differentially expressed tsRNAs in a mouse model of TBI

To investigate the potential role of tsRNAs in secondary injury post-TBI, differentially expressed tsRNAs were determined. The expression correlation of tsRNAs was first performed in the control and TBI groups, which indicated a strong correlation of tsRNA expression between the two groups (r = 0.91, $P < 2.2 \times 10^{-16}$; **Figure 2D**). Subsequently, 103 tsRNAs were observed to be differentially expressed in the mouse model of TBI at 72 hours, of which 56 tsRNAs were upregulated and 47 tsRNAs were downregulated (**Figure 2E**). Furthermore, differentially expressed tsRNAs were able to distinguish fairly well between the two groups, which implies a potential biological significance of tsRNAs in secondary injury after TBI (**Figure 2F**).

Interrogation of protein-coding gene alterations and biological functions

To explore the biological processes that occur in response to TBI injury, differentially expressed protein-coding genes were determined. Consequently, 1922 genes were upregulated relative to the control group upon TBI injury, and 294 genes were downregulated (**Figure 3A**). To characterize the biological functions of the upregulated genes, GO and KEGG analyses were conducted using the web-based tool Metascape. GO annotation results revealed that molecular functions of upregulated genes were mainly focused on interactions between biomolecules, such as cytokine binding, pattern recognition receptor activity, and calcium ion binding (**Figure 3B**). Moreover, the KEGG enrichment analysis revealed that significantly enriched pathways were largely implicated in inflammatory processes, including cytokine–cytokine receptor interaction, the nucleotide-binding oligomerization domain-like receptor signaling pathway, and the nuclear factor-κB pathway (**Figure 3B**). To determine which of the 1922 upregulated genes had critical roles in secondary injury post-TBI, a protein–protein interaction network was constructed using STRING and the CytoHubba application of Cytoscape. The hub genes (e.g., Tnfa, Tlr2, Tlr4, Il-1b, and Stat3) were primarily involved in inflammatory processes, which was consistent with the functional analysis by KEGG and GO (**Figure 3C**).

Given the bias caused by the arbitrary cutoff to identify significant genes, GSEA was adopted to identify the enriched biological processes. This analysis method uses information about all genes in the experiment to investigate the underlying biological mechanisms. After conversion to the corresponding human orthologs, an enrichment analysis was performed against the C2 KEGG subset of canonical pathways gene sets. This revealed that the enriched pathways by GSEA were also predominantly associated with inflammation (**Figure 4**), which further corroborated the functional identification of the KEGG and GO analyses.

Correlation analysis of tsRNAs and their target genes

In light of the miRNA-like regulatory roles of tsRNAs in gene expression, two commonly used miRNA target prediction algorithms, TargetScan and miRanda, were used for the interaction analysis of tsRNAs and genes, both of which were differentially expressed after TBI. The correlation analysis of tsRNAs and their target genes identified 57 tsRNA–mRNA interaction pairs, which included 29 tsRNAs and 26 mRNAs (**Figure 5A** and **B**). Out of 57 pairs, 47 displayed a pattern of tsRNA upregulation while their corresponding targets were downregulated. GO analysis was then performed to obtain global insights into the physiological roles of the target genes. The GO annotation results demonstrated that the significantly enriched terms were primarily involved in inflammation and synaptic communication (**Figure 5C**). To further depict the interaction of tsRNAs and their target genes, the network between tsRNAs and their target genes was constructed using Cytoscape. This revealed that multiple tsRNAs can target the same gene, while a single tsRNA can also regulate multiple genes (**Figure 6**).

Discussion

Although the mechanisms underlying TBI-associated secondary injury have been intensively investigated, there are no effective treatments available to date, which implies that alternative mechanisms are not yet fully understood. Given the pleiotropic role of tRNA-derived fragments in the central nervous system under physiological and pathophysiological conditions, the present work aimed to investigate whether tsRNAs are involved in secondary injury after TBI. To test this hypothesis, integrated next-generation sequencing analysis of tsRNA and mRNA was performed. The findings indicated that TBI exerted a large influence on tsRNA expression in the peri-injured cortex. Moreover, the mRNA targets of dysregulated tsRNA, identified by miRNA-like seed matching and expression correlation analysis, were primarily implicated in inflammation and synaptic functions.

tsRNAs can fine-tune gene expression at both the transcriptional and post-transcriptional levels in prokaryotes and eukaryotes, thereby playing key regulatory roles in various physiological and pathological events (Kim et al., 2017; Zhu et al., 2018). Recently, tsRNAs have gained more attention in neurological diseases (Qin et al., 2020). The interaction between cleavage, polyadenylation factor I subunit (CLP1), and the tRNA splicing endonuclease (TSEN) complex is necessary for tRNA splicing and maturation. Mutations in genes of the CLP1 and TSEN subunits result in neurological disorders, such as pontocerebellar hypoplasia (Abbott et al., 2014; Weitzer et al., 2015; Hayne et al., 2020). Hanada et al. (2013) reported that the absence of CLP1 activity in mice causes the accumulation of tyrosine pre-tRNA-derived small RNA fragments, which in turn sensitizes cells to oxidative stress-induced p53-dependent cell death, ultimately leading to a progressive loss of spinal motor neurons in the peripheral nerves, and thus muscle paralysis. Notably, in five unrelated families of the same ethnic group, a homozygous missense mutation (p.R140H) of CLP1 disturbed CLP1–TSEN complex integrity and caused the accumulation of linear tRNA introns. Abnormalities in tRNA-derived fragments led to severe sensory-motor deficits, cortical dysgenesis, and microcephaly in the affected individuals (Karaca et al., 2014). Concomitantly, in four independent consanguineous Turkish families, Schaffer et al. (2014) observed that the p.R140H mutation of CLP1 also results in cerebellar neurodegeneration because of accumulated pre-tRNA and reduced mature tRNA through p53-mediated neuronal loss. Together, these findings highlight that aberrant expression of tRNA-derived fragments is intimately associated with peripheral and central nervous system dysfunction.

In addition to genetic alterations, tsRNAs are often elevated during a variety of stress conditions, including neurological diseases (Thompson and Parker, 2009; Blanco et al., 2014; Hogg et al., 2019; Wang et al., 2019). Elkordy et al. (2018) demonstrated that oxidative stress, such as by the application of arsenite and hydrogen peroxide, induces tRNA cleavage and increases angiogenin-mediated generation of tRNA halves in a rat neuronal cell line (PC12). Moreover, the amount of tRNA halves is related to the degree of cell damage. Based on the observation that the generation of tRNA halves responds rapidly to oxygen–glucose deprivation, tRNA halves may be a novel biomarker for ischemia–reperfusion in PC12 cells (Elkordy et al., 2019). Intriguingly, aging can also greatly affect tsRNA expression, and 3′ tsRNAs display monotonic increases with age (Karaiskos and Grigoriev, 2016). Furthermore, Zhang et al. (2019a) identified eight differentially expressed tsRNAs in the brains of senescence-accelerated mouse-prone 8 mice, whose potential target genes were mainly implicated in

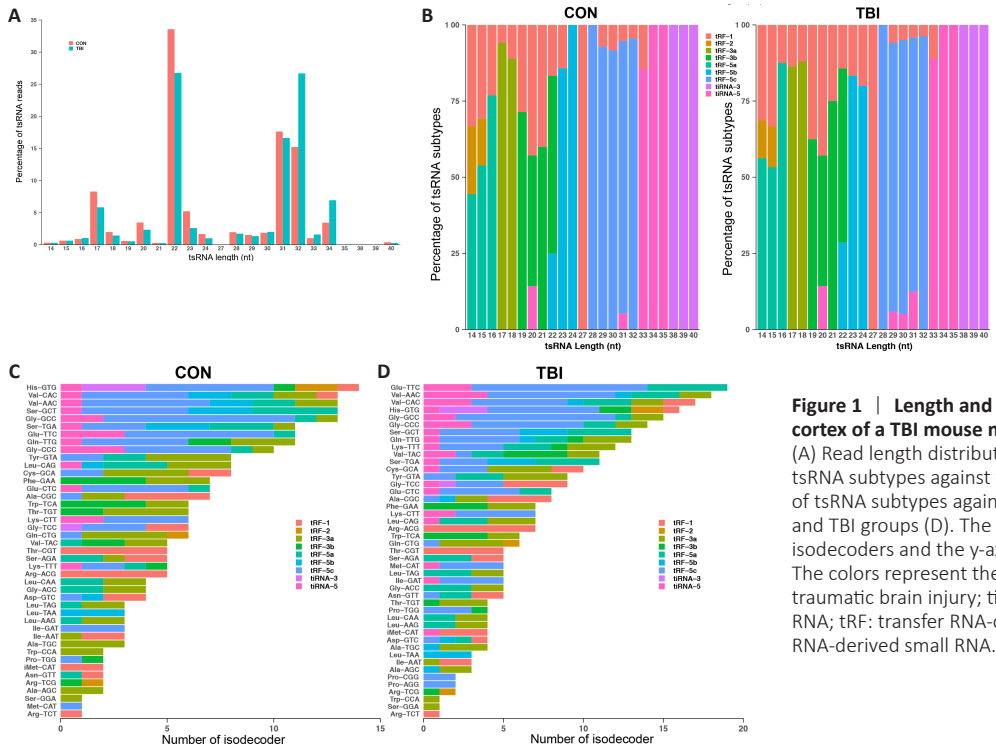

Figure 1 | Length and subtype distribution of tsRNAs in the cortex of a TBI mouse model.
(A) Read length distribution for tsRNAs. (B) The percentage of tsRNA subtypes against the tsRNA length. (C, D) The number of tsRNA subtypes against tRNA isodecoders in the control (C) and TBI groups (D). The x-axis indicates the number of tRNA isodecoders and the y-axis represents the tRNA isodecoders. The colors represent the subtypes of tsRNAs. CON: Control; TBI: traumatic brain injury; tiRNA: transfer RNA-derived stress-induced RNA; tRF: transfer RNA-derived RNA fragment; tsRNA: transfer RNA-derived small RNA.

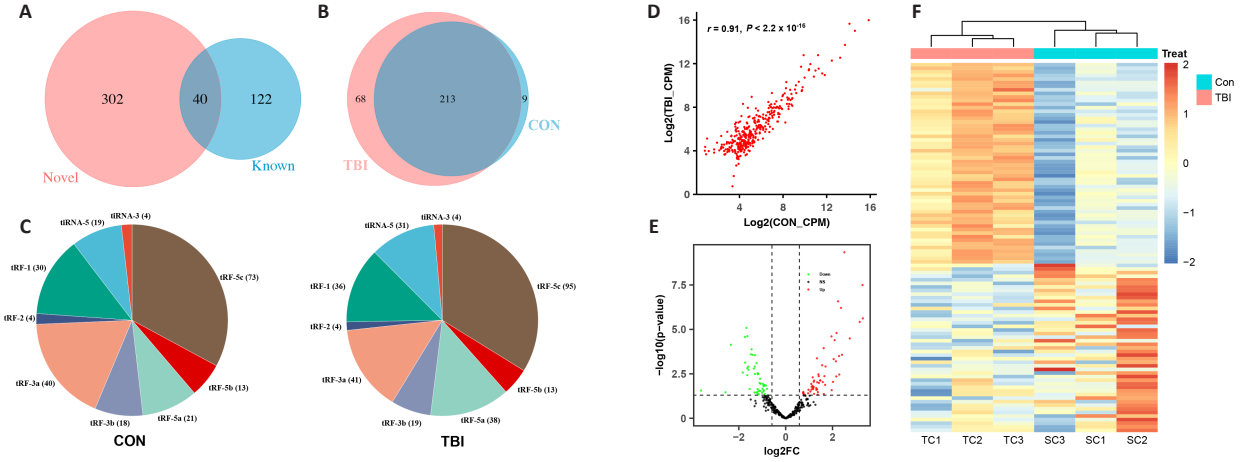

Figure 2 | Expression patterns of tsRNA and the identification of differentially expressed tsRNAs in the cortex of a TBI mouse model.
(A) Venn diagram comparing the tsRNAs detected in the cortex of the TBI mouse model and the tsRNAs from the tRFdb database. (B) Venn diagram showing the commonly and specifically expressed tsRNAs in the control and TBI groups. (C) Pie chart illustrating the number of each subtype of tsRNAs in the control and TBI groups. (D) Scatter plot indicating the correlation of tsRNA expression between the control and TBI groups, analyzed using Pearson's correlation coefficient. $r = 0.91$, $P < 2.2 \times 10^{-16}$. (E) Volcano plot displaying differentially expressed tsRNAs. Upregulated and downregulated differentially expressed tsRNAs are highlighted in red and green, respectively. (F) Heatmap showing the hierarchical clustering of differentially expressed tsRNAs between the control and TBI groups. Blue indicates low expression levels and red indicates high expression levels. CON: Control; CPM: counts per million of total aligned reads; FC: fold change; SC: control cortex; TBI: traumatic brain injury; TC: TBI cortex; tiRNA: transfer RNA-derived stress-induced RNA; tRF: transfer RNA-derived RNA fragment; tsRNA: transfer RNA-derived small RNA.

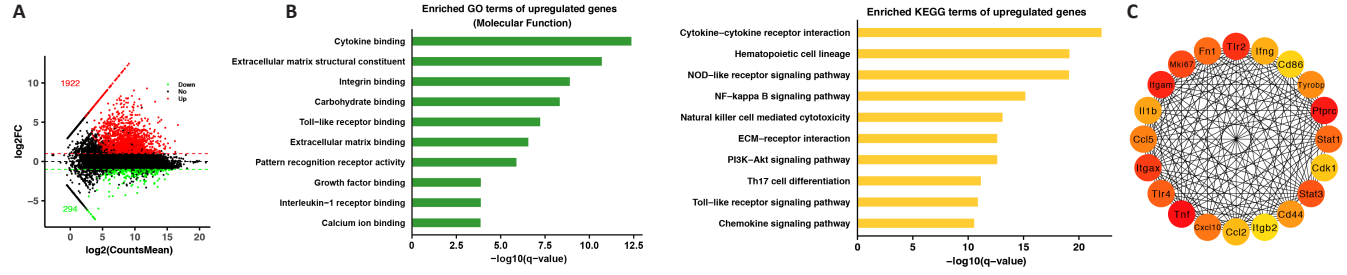

Figure 3 | Identification of differentially expressed genes (DEGs) in the cortex of a TBI mouse model and their functional annotation.
(A) Log-intensity ratios (M-values) versus log-intensity averages (A-values) plot showing the log2 fold changes (FC) of gene levels and the average counts. The red and green dots represent upregulated and downregulated DEGs, respectively. The values in red and green indicates the number of upregulated and downregulated DEGs, respectively. (B) GO molecular function term enrichment (left) and KEGG term enrichment (right) of upregulated DEGs. (C) Interaction network analysis of common DEGs. The color intensity indicates the degree of the node. The yellow and red colors represent low and high node degrees, respectively. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TBI: traumatic brain injury.

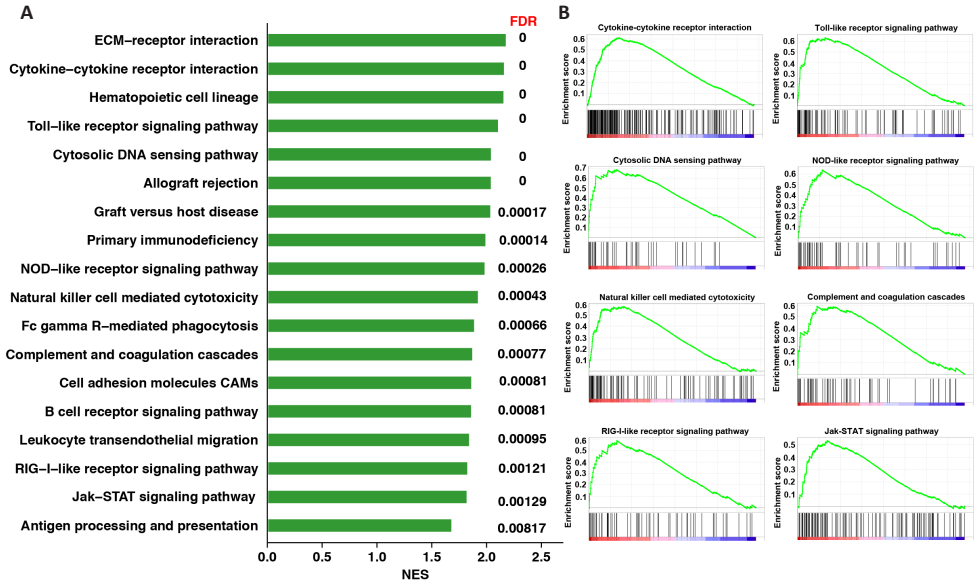

Figure 4 | Gene set enrichment analysis of differentially expressed genes (DEGs) in the cortex of a TBI mouse model based on the KEGG gene set.

(A) Bar plots indicating enriched KEGG pathways. (B) GSEA enrichment plots of representative gene sets that were significantly enriched. The enrichment score reflects the degree to which the genes in a gene set were overrepresented at the extremes (top or bottom) of a ranked gene list. FDR: False discovery rate; GSEA: gene set enrichment analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; NES: normalized enrichment score; TBI: traumatic brain injury.

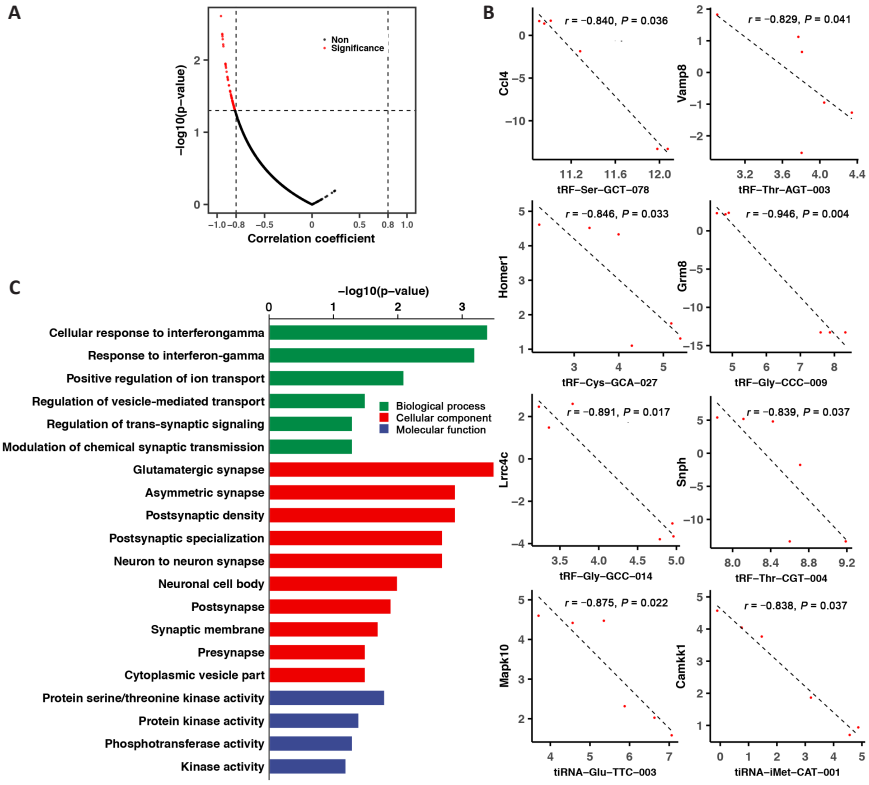

Figure 5 | Integrated analysis of tsRNAs and their target mRNAs.

(A) Volcano plot showing the correlation between tsRNAs and their targets, both of which were differentially expressed after TBI. The red dots represent significantly correlated pairs. (B) Representative significantly correlated tsRNA–mRNA pairs. (C) GO enrichment analysis of predicted mRNA targets for tsRNAs. GO: Gene Ontology; TBI: traumatic brain injury; tsRNA: transfer RNA-derived small RNA.

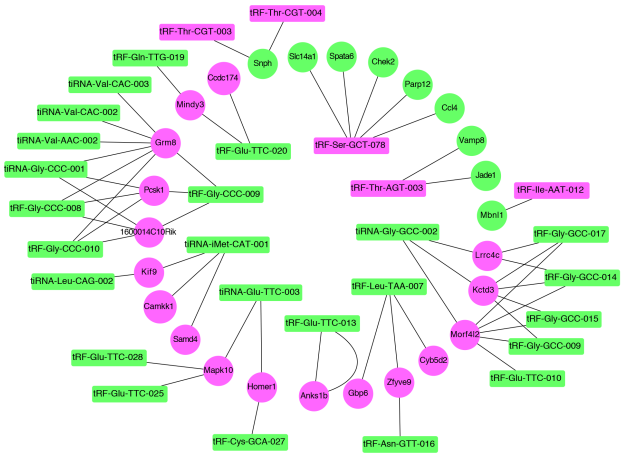

Figure 6 | Interaction network of significantly correlated tsRNA-mRNA pairs.

The green and rose/violet dots represent upregulation and downregulation, respectively. tsRNA: Transfer RNA-derived small RNA.

neuronal function, including synapse formation and synaptic vesicle cycle pathways. In the present work, we observed that 103 tsRNAs were differentially expressed in a mouse model of TBI at 72 hours. Functional interrogation of the potential target genes of dysregulated tsRNA demonstrated that synapse-related functions, such as the regulation of vesicle-mediated transport and trans-synaptic signaling, were significantly enriched. Recently, it has been suggested that tsRNAs might be associated with the pathological processes of traumatic spinal cord injury through mitogen-activated protein kinase and brain-derived neurotrophic factor (Qin et al., 2019).

Accumulating evidence suggests that tsRNAs are involved in the pathophysiology of the inflammatory response (Dhahbi, 2015; Zhu et al., 2020), such as in cancer (Shan et al., 2020), alcoholic fatty liver disease (Zhong et al., 2019), and neurological disorders in particular (Su et al., 2020; Winek et al., 2020). The balance between the inflammatory response and immune suppression largely determines the post-stroke prognosis, and involves the cholinergic blockade of immune reactions (Hoover, 2017; Martín et al., 2018). Winek et al. (2020) reported that tsRNAs undergo significant changes in the peripheral blood of ischemic stroke patients at 2 days post-stroke; 143 tsRNAs were differentially expressed, of which 87% were upregulated. Notably, tsRNAs predominantly originate from lymphocytes and monocytes, particularly $CD14^{+}$ monocytes. Further investigations have shown that tsRNAs might target cholinergic-associated monocytic transcription factors and regulate acetylcholine-mediated post-stroke immune suppression. Intriguingly, tsRNAs have been implicated in lifelong neuropathology in offspring (Su et al., 2020). Su et al. (2020) demonstrated that tsRNAs respond rapidly to maternal immune activation at the maternal–fetal interface, which can induce autism-related phenotypes in the offspring. Moreover, maternal immune activation triggers a decrease in 5′ tRNA halves and an increase in 3′ tsRNAs. In the mouse model of TBI in the current study, 54% of the differentially expressed tsRNAs were upregulated, similar to the pattern observed in the peripheral blood of ischemic stroke patients. In contrast to the changing trend of tsRNAs in the maternal immune activation autism model, 5′ tsRNAs were significantly upregulated post-TBI, which indicates that the biological function of disease-induced tsRNAs might be context specific. More importantly, TBI-associated tsRNAs were also involved in inflammation, such as tRF-Ser-GCT-078 and tRF-Thr-AGT-003, which target Ccl4 and Vamp8, respectively. Collectively, these data suggest that tsRNAs are potential contributors to regulating neuroinflammatory responses.

tsRNAs exert biological functions through multiple mechanisms, such as transcriptional silencing and repression and translational inhibition and enhancement (Xie et al., 2020; Yu et al., 2020). tsRNAs may interact with Argonaute proteins and function in a manner similar to miRNAs to regulate gene expression (Kumar et al., 2014). Maute et al. (2013) reported that tRNA-derived fragments, designated CU1276, repressed endogenous replication protein A1 in a 3′ untranslated region in a sequence-specific manner, thereby modulating proliferation and the DNA damage response. Interestingly, unlike conventional miRNA seed locations, Karaiskos and Grigoriev (2016) demonstrated that the potential seed regions of tsRNAs might exist at both ends of different tsRNAs. In particular, Jehn et al. (2020) reported that 5′ tRNA halves, which are highly expressed in the primate hippocampus, receive the most efficient gene silencing through the interactions between mid-regions of 5′ tRNA halves and coding DNA sequences or 3′ untranslated regions of target mRNAs. In addition to their direct binding to the 3′ untranslated region of mRNA transcripts, tsRNAs can interact with the RNA-binding protein Y-box binding protein-1 in a motif-specific manner and consequently suppress the stability of corresponding transcripts (Goodarzi et al., 2015). Furthermore, tsRNAs can fine-tune gene expression in terms of translational levels. Ivanov et al. (2011) revealed that angiogenin-induced tRNA fragments are able to displace eukaryotic initiation factor 4G/A from mRNA, and particularly uncapped mRNA, to inhibit translation in stressed cells. In contrast, Kim et al. (2017) demonstrated that LeuCAG-derived tsRNAs enhance the translation of two ribosomal protein mRNAs, RPS28 and RPS15, and ultimately regulate ribosome biogenesis. Together, these findings indicate that complex processes are associated with tsRNA-mediated biological functions. Based on potential miRNA-like actions, we identified 57 tsRNA–mRNA interaction pairs, of which 47 displayed the pattern of tsRNAs being upregulated while their corresponding targets were downregulated. Whether tsRNAs function in TBI through alternative mechanisms, such as translational regulation, remains unknown and requires further investigation.

To date, clinical trials for TBI have failed to show effective neuroprotection (Stein, 2015). Given the inherently heterogeneous nature of TBI and the multiplicity of its pathophysiological processes, the targeting of single events or molecules that are proposed to induce secondary injury is considered to be the most important factor underlying the failure of clinical trials (Loane and Faden, 2010). tsRNAs can act like miRNAs, and individual tsRNA can simultaneously fine-tune multiple genes, thereby influencing numerous biological processes. The manipulation of tsRNAs therefore provides a novel multipotential treatment for TBI. Our present and previous works have highlighted the intimate involvement of inflammatory mechanisms in the pathophysiological processes of TBI (Yang et al., 2021); thus, targeting inflammation-related tsRNAs might offer a potential strategy for the treatment of TBI. Moreover, the advancement of small RNA-based therapeutics, especially therapeutic small interfering RNA (siRNA), paves the way for the clinical translation of tsRNAs (Hu et al., 2020).

In summary, a greater understanding of the mechanisms underlying secondary injury will benefit the development of effective treatment modalities for TBI. The present study used integrated next-generation sequencing analysis of tsRNA and mRNA to provide the first evidence that tRNA-derived fragments might be involved in TBI-induced secondary injury. However, much remains to be elucidated regarding the detailed functions of tsRNA in secondary brain injury. For example, tRF-Ser-GCT-078 and tRF-Thr-AGT-003 have been implicated in inflammation, and the involvement of tRNA-derived stress-induced RNA (tiRNA)-Val-CAC-003 and tRF-Gly-Gcc-017 in synaptic function has been observed. However, more experimental evidence is needed to interrogate their functions in TBI using genetic approaches, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and siRNA. In addition, TBI-induced spatiotemporal changes of tsRNAs and their relationships, such as causality or synergism, require further investigation.

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