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Alterations of microRNAs expression profiles in small extracellular vesicle after traumatic brain injury in mice

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Abstract: Traumatic brain injury (TBI) is one of the leading causes of mortality and morbidity worldwide. Tools available for diagnosis and therapy are limited. Small extracellular vesicle (sEV) microRNAs (miRNAs) play an important role in TBI disease progression. This study aimed to investigate the alterations in sEV miRNAs expression in the mouse brain extracellular space after TBI. Twenty-four C57BL/6J mice were randomly divided into two groups (12/group). The TBI group was subjected to all surgical procedures and fluid percussion injury (FPI). The sham group only underwent surgery. Brain specimens were collected 3 h after TBI/sham. The brain sEV were isolated. Differentially expressed miRNAs were identified. A total of 50 miRNAs were observed to be differentially expressed (fold change ≥1.5 and P<0.05) after TBI, including 5 upregulated and 45 downregulated. The major enriched Gene Ontology terms were metabolic processes, cell, intracellular, organelle, cytoplasm, axon, binding, protein kinase activity, protein binding, and protein dimerization activity. The KEGG pathway analysis predicted that the pathways affected by the variation of miRNAs in sEVs after TBI included the Wnt signaling pathway and NF-κB signaling pathway. The changes in five miRNAs were confirmed by qRT-PCR. In conclusion, this study demonstrated the differential expression of a series of miRNAs in brain sEV after TBI, which might be correlated with post-TBI physiological and pathological processes. The findings might also provide novel targets for further investigating the molecular mechanisms underlying TBI and potential therapeutic interventions.
Key words: extracellular vesicle, gene ontology, Kyoto encyclopedia of genes and genomes, microRNA, traumatic brain injury

Introduction

Traumatic brain injury (TBI) is one of the leading causes of traumatic mortality and morbidity worldwide [1–3] and is responsible for 2.5 million emergency department visits each year in the United States [3]. The incidence of TBI is the highest in adults ≥75 years of age (2,232 per 100,000), followed by children <5 years of age (1,592 per 100,000) and adolescents of 15–24 years of age (1,081 per 100,000) [3]. Despite growing public concern about TBI, the tools available for diagnosis and therapy are limited. There are currently no

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pharmacotherapies that have been proven to improve post-TBI outcome [4–6].

Extracellular vesicles (EVs) are membranous nanoparticles that are found in all biological fluids investigated to date including amniotic fluid, blood, urine, saliva, breast milk, cerebrospinal fluid and ascetic fluid [7, 8]. EVs are heterogeneous in size, content, biogenesis, and membrane composition, which suggests variability in biological function. Terms used to classify EVs include exosomes, ectosomes (microvesicles or microparticles), apoptotic bodies and oncosomes [9, 10]. Exosomes are part of the broader population of EVs. In recent years, exosomes have sparked interest in the scientific community for their emerging role in cell-to-cell communication involved in physiological and pathological processes throughout the body [9]. Exosomes can be uptaken by distant cells and modify the functions and behaviors of these cells [11]. Exosomes appear to be involved in the pathogenesis of various diseases (cancers, degenerative diseases, and autoimmune diseases), but they can also have therapeutic implications [11]. Indeed, accumulating evidence indicates that exosomes are important intercellular mediators in post-TBI nerve repair and nerve injury [12–14]. Exosomes derived from mesenchymal stem cells (MSCs) can promote neurorestoration after TBI [15, 16]. Using a swine model of TBI, Williams *et al.* [17] showed that an early single-dose of exosomes attenuated neurologic injury, decreased brain lesion size, inhibited inflammation and apoptosis, and promoted neural plasticity over the first 7 days after TBI. Therefore, increasing attention has been paid to exosomes in medicine and healthcare.

MicroRNAs (miRNAs) are highly conserved, noncoding, single-stranded small-molecule RNAs composed of 19–28 nucleotides [18]. miRNAs inhibit translation either by binding to their target mRNA or by enhancing the degradation of their target mRNA [18]. miRNAs have been identified in exosomes, where they are protected from the extracellular conditions and modulate the recipient cells [11]. Given the transportability of exosomes, the role of miRNAs in exosomes is gaining increasing attention [11]. The miRNA profiles of exosomes may differ from those of the parent cells and play an important role in disease progression [19].

The central nervous system (CNS) contains the highest concentration and highest diversity of miRNAs, and 70% of the miRNAs are expressed in the brain, spinal cord, or peripheral nerves [20]. The expression of miRNAs evolves throughout neuro-development and varies among the brain regions [21]. Within neurons, miRNAs also display intracellular variation in localization [22]. miRNAs are involved in the pathophysiology of many diseases [11], but they are critical for neurodevelopment and brain function [23]. By regulating gene activity, miRNAs control cellular processes essential to neuronal injury and repair: differentiation, proliferation, apoptosis, and metabolism [24–26]. Our group previously reported that miR-21 could alleviate apoptosis of cortical neurons and secondary blood-brain damage after TBI in rats [27, 28]. Disruption in miRNA levels has been reported in numerous diseases of the CNS [29–31].

This study aimed to investigate the alterations in EV miRNAs expression in the mouse brain after TBI using miRNAseq to identify the important miRNAs involved in TBI and provide novel targets for future investigations. The diameter of extracellular vesicles obtained in this study is between 30–100 nm, so it is called small EVs (sEVs) in this article. With a combination of microRNA-seq data, bioinformatics analysis, and subsequent qRT-PCR, the potential roles of these differentially expressed miRNAs in sEVs from the brain extracellular space after TBI were explored.

Material and Methods

Animals

Experiments were performed in accordance with the principles and procedures of the Laboratory Animal Care and Use Committee of Tianjin Medical University (approval number IRB2022-DW-01). Adult male C57BL/6J mice (12–16 weeks old and 22–25 g) were housed for at least 7 days before surgery. Mice were allowed free access to water and food. Water and food were withheld overnight before surgery.

Experimental groups, surgical preparation, and TBI model

This study used samples and specimens that were obtained in a previous study by the authors' group [32]. Briefly, 24 mice were randomly divided into two groups (12 mice/group). The TBI group was subjected to all surgical procedures and fluid percussion injury (FPI). The sham group received an identical surgical procedure but did not receive an FPI. In this study, medium-sized mouse FPI was performed. After anesthesia with 10% chloral hydrate (0.3 ml/100 g), the skull was exposed, and a 3.0-mm diameter hole (2.0 mm posterior from the bregma and 2.0 mm lateral to the sagittal suture) was drilled. FPI was induced using a fast injection of saline (peak pressure was 1.9 ± 0.1 atm) through the skull window [32]. The specimens were obtained 3 h after FPI. The traumatically injured brain tissues was obtained in TBI group, and the corresponding brain tissues was obtained in sham group. Four random mouse brains in the

same group were mixed as one sample, so each group had three samples.

Isolation and detection of sEVs

The sEVs used in this study were those obtained for the previous study by our group [32]. The brain tissues were dissected in a cell culture medium (Hibernate-A, Invitrogen Inc., Carlsbad, CA, USA). The shredded brain tissues were resuspended in 3 ml of cell culture medium and treated with 20 U/ml papain (Worthington Biochemical Corp., Lakewood, NJ, USA) for 15 min at 37°C. The supernatant was discarded, and 6 ml of 4°C cell culture medium was added to stop the digestion. The samples were centrifuged for 30 min at 2,000 ×g at 4°C to discard cells and membranes. The supernatant was collected and filtered through a sterile 0.2-μm filter. Total exosome isolation reagents (Thermo Fisher Scientific, Waltham, MA, USA) were added to the supernatant at the proportion of 1 ml of supernatant to 500 μl of the isolation reagents, vortexed, and incubated overnight at 4°C. The supernatant was centrifuged at 10,000 ×g for 60 min at 4°C to pellet the sEVs. The sEVs were resuspended in 500 μl of TRIzol reagent (Invitrogen) for subsequent analyses.

sEVs were observed under a transmission electron microscope (TEM; HT7700, Hitachi, Tokyo, Japan). Western blot was used to detect typical exosomal markers. Equal amounts of protein were subjected to SDS-PAGE. The expression of typical exosomal markers was detected using the antibodies as follows: mouse anti-CD63 (1:400; Abcam, Cambridge, UK), mouse anti-astrocyte marker glial fibrillary acidic protein (GFAP) (1:1,000; Abcam), rabbit anti-TSG101 (1:1,000; Abcam), mouse anti-HSP70 (1:1,000; Abcam), goat anti-rabbit secondary antibody (1:5,000; Abcam), and goat anti-mouse secondary antibody (1:5,000; Abcam). Mouse anti-β-actin (1:1,000; Abcam) was used as an internal reference.

RNA extraction and quality control

Total RNA was isolated from the sEVs using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA quantification and quality were assessed by a NanoDrop ND-1000. For spectrophotometry, the optical density (OD) A260/A280 ratio had to be near 2.0 for pure RNA (ratios between 1.8 and 2.1 were acceptable). The OD A260/A230 ratio had to be more than 1.8 RNA integrity and DNA contamination were tested by denaturing agarose gel electrophoresis. DNA had to be completely absent, and RNA had to be not degraded. RNA integrity met the requirements when the RNA integrity number (RIN) was ≥7. The sequencing library was determined using an Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA was used for removing the rRNAs using Ribo-Zero rRNA Removal Kits (Illumina, Inc., San Diego, CA, USA). Libraries were controlled for quality and quantity using the BioAnalyzer 2100 system (Agilent Technologies).

RNA library construction and miRNA sequencing

The total RNA of each sample was used to prepare the miRNA sequencing library, which included the following steps: 1) 3'-adaptor ligation; 2) 5'-adaptor ligation; 3) cDNA synthesis; 4) PCR amplification; and 5) size selection of ~150 bp PCR amplicons (corresponding to ~22-nt miRNAs). The libraries were denatured as single-strand DNA molecules, captured on Illumina flow cells, amplified in situ as clusters, and finally sequenced for 50 cycles on Illumina HiSeq sequencer, following the manufacturer's instructions.

miRNA data analysis and bioinformatics

Data were generated after sequencing, image analysis, base calling, and quality filtering on the Illumina sequencer. First, Q30 was used to perform quality control. The adaptor sequences were trimmed, and the adaptor-trimmed-reads (≥15 nt) were left by the Cutadapt software (v1.9.3). The trimmed reads were aligned to the merged pre-miRNA databases (known pre-miRNA from miRBase plus the newly predicted pre-miRNAs) using the Novoalign software (v3.02.12) with at most one mismatch. The numbers of mature miRNA mapped tags were defined as the raw expression levels of that miRNA. The read counts were normalized by the TPM (tag counts per million aligned miRNAs) approach. Differentially expressed miRNAs between two samples were filtered through fold change. Differentially expressed miRNA between two groups were filtered by fold change and *P*-values. miRNA targets were performed using popular miRNA target prediction software. miRNA-targets networks were plotted using the Cytoscape software (v2.8.0). The GO and KEGG pathway analyses were performed based on the top 10 differentially expressed miRNA target genes.

Quantitative real-time polymerase chain reaction

To verify the accuracy of the miRNA-seq data, qRT-PCR was performed as a validation method. Each sample's target RNA and internal parameters were subjected to real-time PCR reactions on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Total RNA was reverse transcribed to synthesize cDNA using a Prime Script RT Reagent Kit (Perfect Real Time; TaKaRa, Osaka, Japan). The data were analyzed by the

$2^{-\Delta\Delta CT}$ method. The amplification cycle was 1) 10 min at 95°C; 2) 40 cycles of 10 s at 95°C and 60 s at 60°C; 3) 10 s at 95°C, 60 s at 60°C, and 15 s at 95°C. The temperature was slowly increased from 60°C to 99°C (Automatic instrument, ramp rate 0.05°C/s) to establish the melting curve of the PCR products. The primers of the five randomly selected miRNAs and internal parameters of hsa-miR-16-5p are shown in Table 1.

Statistical analysis

All statistical analyses were performed using SPSS 21.0 (IBM, Armonk, NY, USA). Data are presented as means ± SD and were analyzed using Student's *t*-test. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Detection of sEVs

EVs were characterized by TEM and Western blot, the results can be seen in our previous research [28]. Under TEM, these sEVs were irregular spheres ranging of 30–100 nm in diameter, with a clearly defined and relatively intact membrane. As Western blot analysis shows these sEVs expressed the typical exosomal markers such as CD63, TSG101, and HSP70. These sEVs also expressed the astrocytes marker GFAP, which confirmed they came from brain tissues.

RNA quality control

Supplementary Tables 1 and 2 and Supplementary Fig. 1 show the relevant quality control results. All RNA used in the subsequent experiments were of sufficient quality.

miRNAs expression profiles in exosomes after TBI

miRNAs were sequenced to study the sEV miRNAs expression profiles from the brain extracellular space after TBI. Compared with the sham group, 50 miRNAs were observed to be differentially expressed (fold change ≥1.5 and $P<0.05$) in sEVs after TBI. Of these, five were upregulated (Supplementary Table 3), and 45 were downregulated (Supplementary Table 4). The top 10 differentially expressed miRNAs are listed in Table 1. A heat map was used to illustrate the different miRNAs expression profiles between the two groups (Fig. 1A). The alterations in the differentially expressed miRNAs between the sham and TBI groups were identified using a volcano plot (Fig. 1B).

GO analysis and KEGG pathway analyses

A GO analysis (Fig. 2) and a KEGG pathway analysis (Fig. 3) were conducted for the target genes and pathways of the differentially expressed miRNAs to examine the physiological and pathological significance of miRNAs in sEVs after TBI. The results showed that in GO terms of biological process, differentially expressed miRNAs were significantly associated with metabolic processes. The major enriched GO terms in cell components were cell, intracellular, organelle, cytoplasm, and axon. As for molecular function, the most enriched GO terms were binding, protein kinase activity, protein binding, and protein dimerization activity.

The KEGG pathway dot plot shows the significant enrichment pathway with the top ten enrichment score (-log10 (*P*-value)) values. The KEGG pathway analysis predicted that the pathways affected by the variation of miRNAs in sEVs after TBI included the Wnt signaling pathway and NF-κB signaling pathway, reported to be involved in neuronal development, injury, and repair [33–36].

Validation of the accuracy of miRNAs expression by qRT-PCR

To verify the miRNA-seq data, five differentially expressed miRNAs (mmu-let-7d-5p, mmu-miR-451a, mmu-miR-335-3p, mmu-miR-296-3p, and mmu-miR-135b-5p) were randomly selected for qRT-PCR validation. The expression levels of these miRNAs are shown in Table 2. Compared with the sham group, mmu-miR-335-3p, mmu-miR-296-3p and mmu-miR-135b-5p were

Table 1. The top 10 differently expressed microRNAs in sEVs after traumatic brain injury

Mature miRNA	Pre-miRNA	Mature sequence	Fold change	*P*	Regulation
mmu-miR-451a	mmu-mir-451a	AAACCGUUACCAUUACUGAGUU	11.52380952	0.044831202	up
mmu-miR-375-3p	mmu-mir-375	UUUGUUCGUUCGGCUCGCGUGA	4.714285714	0.014259685	down
mmu-miR-134-5p	mmu-mir-134	UGUGACUGGUUGACCAGAGGGG	4.595744681	0.008726172	down
mmu-miR-129-5p	mmu-mir-129-1	CUUUUUGCGGUCUGGGCUUGC	4.557800224	0.033481804	down
mmu-miR-129-5p	mmu-mir-129-2	CUUUUUGCGGUCUGGGCUUGC	4.557800224	0.033481804	down
mmu-miR-379-5p	mmu-mir-379	UGGUAGACUAUGGAACGUAGG	4.380305603	0.022719703	down
mmu-miR-1298-5p	mmu-mir-1298	UUCAUUCGGCUGUCCAGAUGUA	4.267552182	0.0422415	down
mmu-miR-671-3p	mmu-mir-671	UCCGGUUCUCAGGGCUCCACC	4.25	0.004356822	down
mmu-miR-novel-chr5_30413	mmu-mir-novel-chr5_30413	UCCGGUUCUCAGGGCUCCACC	4.25	0.004356822	down
mmu-miR-106b-3p	mmu-mir-106b	CCGCACUGUGGGUACUUGCUGC	4.212121212	0.033117474	down

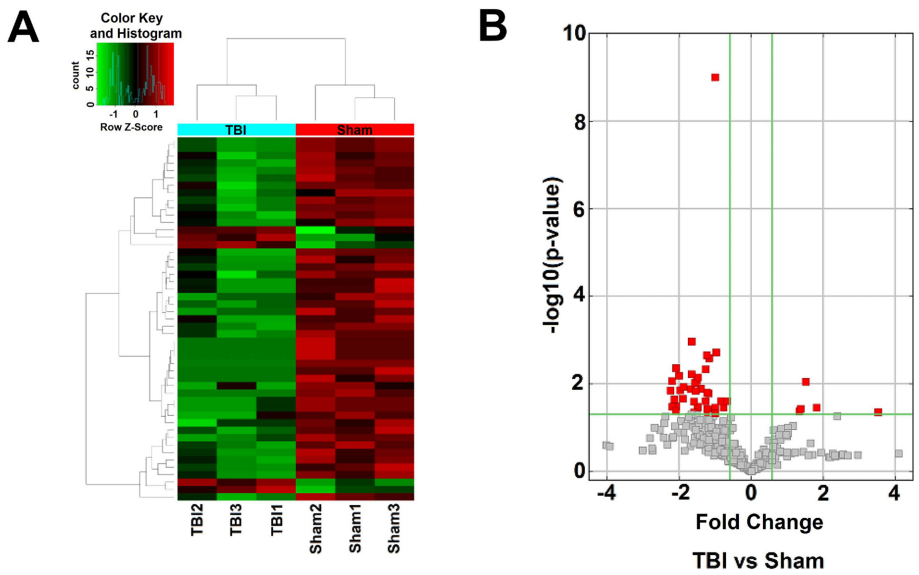

Fig. 1. Altered expression profile of microRNAs (miRNAs) in sEVs after traumatic brain injury (TBI) in mice. (A) Heat map of 50 differentially expressed miRNAs. Each column represents one sample; each row represents one probe set. The distributions of the total expression of miRNAs between the sham and TBI groups are nearly the same. (B) Volcano plot of the fold-change of miRNAs between the sham and TBI groups. The red dots represent the differentially expressed miRNAs.

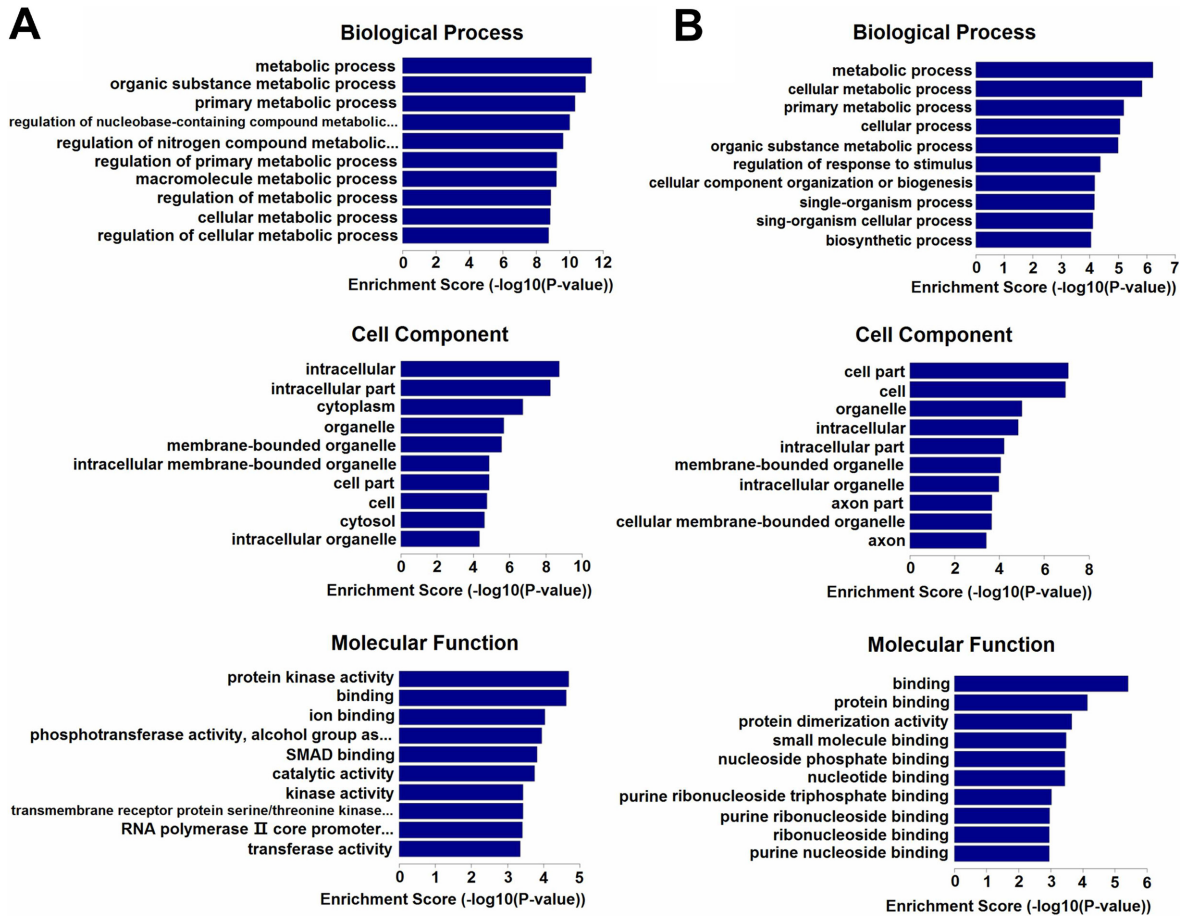

Fig. 2. Gene ontology (GO) analysis for the target genes of the differentially expressed miRNAs to examine the physiological and pathological significance of miRNAs in sEVs after TBI. The horizontal axis is the enrichment score for the GO terms, and the vertical axis is the GO terms. (A) Upregulated genes. (B) Downregulated genes.

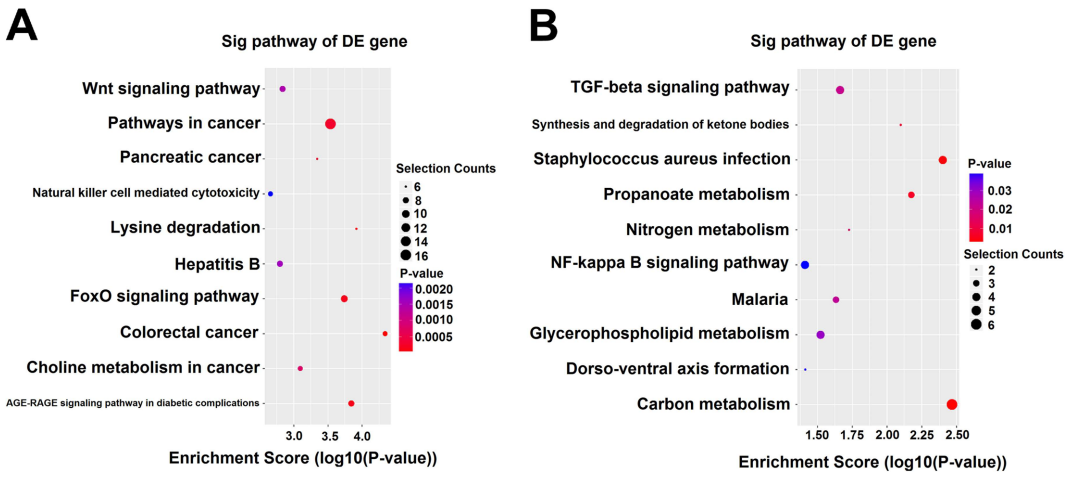

Fig. 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for the target pathways of the differentially expressed miRNAs to examine the physiological and pathological significance of miRNAs in sEVs after TBI. Selection counts represent the number of entities of the differentially expressed genes directly associated with the listed pathway ID. (A) Upregulated genes. (B) Downregulated genes.

Table 2. qRT-PCR validation results of five selected microRNAs

microRNA	Expression level	
	Sham group	TBI group
mmu-let-7d-5p	0.045 ± 0.009	0.188 ± 0.011**
mmu-miR-451a	1.475 ± 0.169	51.447 ± 6.895**
mmu-miR-335-3p	1.170 ± 0.239	0.303 ± 0.057**
mmu-miR-296-3p	0.287 ± 0.060	0.009 ± 0.003*
mmu-miR-135b-5p	0.180 ± 0.030	0.010 ± 0.002**

**P<0.01 vs. Sham, *P<0.05 vs. Sham, Student's t-test (two-tailed).

down-regulated, and mmu-let-7d-5p and mmu-miR-451a were up-regulated after TBI. The expression of these miRNAs was consistent with the miRNA-seq data.

Discussion

The present study is the first to examine the miRNA expression profiles in sEVs from the brain extracellular space after TBI in mice. The results showed that 50 miRNAs were differentially expressed in the early stage (3 h) after TBI. Of these, five were upregulated, and 45 were down-regulated. We identified the major significantly changed GO terms and most correlated KEGG pathway to gain insight into the potential function of the differentially expressed miRNAs. Owing to these altered miRNAs' potential roles in pathophysiologic processes after TBI, they might be used as biomarkers or attractive new therapeutic targets that could be the focus of future studies.

Trauma can lead to permanent or temporary injuries due to the neuronal insult. The primary neuronal damage is the direct effects of the force itself on neuroanatomy and function. In contrast, secondary damage consists of the biochemical responses to the injury resulting in physiological changes, neuronal repair, and cell death [37–40]. miRNAs are involved in the primary and secondary damage responses due to TBI [41]. The changes in gene expression after TBI is kind of a "black box" that can reveal what is happening at the cellular and molecular levels after TBI [42], including the complex changes in the three-dimensional network [43]. Indeed, TBI rapidly modulates the expression of many genes [44], and the subsequent changes in the expression of the upstream genes contribute to the expression changes in the downstream genes [45] and in the biochemical cascade that participates in the deterioration in brain function [46]. Thus cascade consists of mechanical insult, oxidative stress, apoptotic cell death, subacute repair, and chronic remodeling [47].

EVs are released by all major cells in the CNS, including neurons, astrocytes, microglia and oligodendrocytes [48–50]. In response to TBI, microglia, as well as astrocytes, become active, changing morphology and initiating an inflammatory cascade by secreting cytokines, chemokines, and growth factors [51, 52]. The inflammatory response following TBI starts within minutes of the injury [53]. Resident brain microglia are the first to activate and migrate toward the focal injury [54, 55]. Within hours of injury, neutrophils arrive at the injury site to begin clearance, followed by macrophages 1–2 days later [56]. In TBI, microglia and astrocyte activation is a double-edged sword. Microglia and astrocyte activation can elicit protective mechanisms, but their persistent activation can also trigger deleterious processes and worsen tissue injury [57, 58]. At a certain timepoint during disease progression, glial cells assume a useful role,

then progress into a dysfunctional cell that ultimately becomes harmful. In this study, we extracted EVs from the intercellular space of injured brain tissue three hours after TBI. We verified that the obtained EVs expressed the marker GFAP of astrocytes. However, these EVs may originate from activated microglia and astrocytes, as well as other cells in the CNS, which may involved in the regulation of the pathophysiological process of early stage after TBI.

EVs are produced by cells and contain nucleic acids, including miRNAs. Studying EVs in TBI is of significance because the EV can be uptaken by cells, in which the miRNAs carried by the EV can modulate cellular processes. Still, whether the EV contribute to the pathological or repair processes after TBI is currently unknown. In this study, we identified 50 differentially expressed miRNAs in sEVs from the extracellular brain space at 3 h after TBI in mice, which were involved in complex network regulation after TBI. It is worth noting that mmu-miR-451a changed by more than ten folds after TBI.

The GO project has developed a structured, controlled vocabulary for annotating genes, gene products, and sequences, and GO is a bioinformatic concept that unifies all species' genes and gene products [59]. We use the GO gene analysis of differentially expressed microRNA target genes (top-100 target genes of each microRNA, not all of them) to analyze and infer the functions of these differentially expressed miRNAs. The significant GO terms included metabolic process, binding, protein kinase activity, protein binding, and protein dimerization activity. Significant GO cell component terms of differentially expressed microRNAs showed that these microRNAs were associated with cell, intracellular, organelle, cytoplasm, and axon. Therefore, these differentially expressed microRNAs might be related to the metabolic and specific substance binding process, which might participate in the growth and repair of neurons and the transmission of nerve signals. TBI has been associated with specific GO terms after TBI in various studies, but those studies examined mRNAs across different models and used different timings after TBI [60–62]. The present study examined miRNAs in the early acute phase (3 h) after TBI.

KEGG pathway is the process of mapping molecular data sets in genomics, transcriptomics, proteomics, and metabolomics onto KEGG pathway maps to explain these molecules' biological functions. The KEGG analysis revealed that the Wnt and NF-κB signaling pathways were influenced by the differentially expressed miRNAs identified in this study. Previous studies have identified these two pathways as being involved in neuronal injury and repair [33–36]. The Wnt signaling pathway plays an important role in the proliferation and differentiation of neural stem cells by generating stem cell regulatory factors, hence promoting neural regeneration and repair [63]. The NF-κB signaling pathway is involved in the pathogenesis of infections, inflammation, immune responses, apoptosis, tumorigenesis, and cell cycle regulation and cell differentiation [64]. Activation of NF-κB signaling has been documented in the brain after TBI [64]. The NF-κB signaling pathway also plays a central role in developing astrocyte swelling and brain edema associated with TBI [65].

Still, how exactly the differentially expressed exosome miRNAs identified in this study participate in TBI remains to be elucidated in future *in vitro* and *in vivo* experiments. Therapeutic targets might also be revealed. Future studies should also include additional time points after TBI to determine the changes in exosome miRNAs in time after TBI and from acute to chronic TBI.

Conclusions

In conclusion, this study identified a series of dysregulated microRNAs related to the physiological and pathological processes that occur early after TBI. This study also predicted the potential roles of these aberrantly expressed microRNAs. The findings might provide novel targets for further investigation of both the molecular mechanisms of TBI and potential therapeutic interventions.

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Declarations of Interest

None.

Author Contributions

Ye Tian contributed to conception, design, interpreta-

tion of data, and critically revised the manuscript. Ruiting Zhao contributed to acquisition of data, analysis of data, interpretation of data, drafted and critically revised the manuscript. Xiaochun Li contributed to acquisition of data, analysis of data, and critically revised the manuscript. Ju Zhou contributed to acquisition of data, analysis of data, interpretation of data, and drafted the manuscript. Daqiang Zhan, Yuanzhi Wang, Yifan He, Jiacheng Zhang contributed to acquisition of data and analysis of data. Hengjie Yuan contributed to conception, design, interpretation of data, and critically revised the manuscript. All authors have read and approved the final manuscript.

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