Histol Histopathol (2022) 37: 159-168

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE

Open Access

MiR-124-3p attenuates brain microvascular endothelial cell injury *in vitro* by promoting autophagy

Jing Zhao[1,2*], Yan Wang[1,2*], Dong Wang[1,2*], Wei Yan[3], Shishuang Zhang[1,2], Dai Li[1,2], Zhaoli Han[1,2], Fanglian Chen[4,5] and Ping Lei[1,2]

[1]Laboratory of Neuro-Trauma and Neurodegenerative Disorders, Tianjin Geriatrics Institute, Tianjin Medical University General Hospital, [2]Department of Geriatrics, Tianjin Medical University General Hospital, [3]Pediatric Surgery, Tianjin Children's Hospital, [4]Key Laboratory of Injuries, Variations and Regeneration of Nervous System, Tianjin Neurological Institute, Tianjin Medical University General Hospital and [5]Key Laboratory of Post-Trauma Neuro-Repair and Regeneration in Central Nervous System, Ministry of Education, Tianjin, PR China

*Jing Zhao, Yan Wang and Dong Wang contributed equally to this work

Summary. Traumatic brain injury (TBI) can cause the pathological disruption of the blood-brain barrier (BBB) and associated neurological injury. Reducing the severity of such barrier disruption following TBI can decrease the degree of brain edema, suppress intracranial inflammation, and thereby protect against neurological damage. The BBB is made up of brain microvascular endothelial cells (BMVECs), neurons, pericytes, astrocytes, and extracellular matrix components. In prior analyses, we have demonstrated that miR-124-3p expression is enhanced in microglia-derived exosomes following TBI, with this miRNA being capable of promoting neural repair after such injury. Based upon these results, the present study was formulated to examine the impact of miR-124-3p on BMVEC function and to evaluatethe mechanistic basis for its activity by overexpressing miR-124-3p in these endothelial cells. We utilized a bEnd.3 cell scratch wound *in vitro* model to simulate TBI-associated brain microvascular endothelial cell injury. Lipofectamine3000 was used to transfect endothelial cells such that they overexpressed miR-124-3p. Fluorescence microscopy was used to observe the effects of miR-124-3p expression on these endothelial cells. TUNEL+CD31 immunofluorescence stainingwas employed to observe endothelial cell apoptosis. Tight junctions were observed via ionconductivity microscopy. Western blotting was used to detect the expression of tight junction proteins (occludin, ZO-1), autophagy-associated proteins (Beclin-1, p62, LC3-II/LC3-I), and mTOR-associated proteins (p-mTOR, PDE4B). Chloroquine was used to treat these injured endothelial cells overexpressing miR-124-3p, and endothelial cell apoptosis was assessed via TUNEL+CD31 immunofluorescence staining. We found that the upregulation of miR-124-3p was sufficient to suppress bEnd.3 cell apoptotic death following *in vitro* scratch injury while promoting the upregulation of the tight junction proteins ZO-1 and occludin in these cells, thereby reducing the degree of leakage across the cerebral microvascular endothelial barrier. These protective effects may be related to the ability of miR-124-3p to suppress mTOR signaling and to induce autophagic activity within BMVECs. These data support a model wherein miR-124-3p can inhibit mTOR signaling and promote autophagic induction in BMVECs, thereby protecting these cells against TBI-induced damage.

Key words: Autophagy, Brain microvascular endothelial barrier, Traumatic brain injury, MicroRNAs

Introduction

Traumatic brain injuries (TBIs) are potentially serious forms of damage to the central nervous system that result in key pathological changes associated with neuronal damage and the disruption of the blood-brain barrier (BBB) (Blennow et al., 2012). The BBB is composed of a range of cell types, including brain microvascular endothelial cells (BMVECs), pericytes, astrocytes and neurons, together with extracellular matrix components (Persidsky et al., 2006), and it protects the CNS against pathogens and other potentially damaging factors in circulation under physiological conditions (Zweakberger et al., 2006). Damage to the BBB results in inflammation and increased vascular permeability, in turn causing neuronal apoptosis or

Corresponding Author: Ping Lei, No.154, Anshan Road, Heping District, Tianjin, 300051, PR China. e-mail: leiping8866@126.com
DOI: 10.14670/HH-18-406

©The Author(s) 2022. **Open Access.** This article is licensed under a Creative Commons CC-BY International License.

necrosis, disrupted local circulation, aberrant metabolic activity, and consequent CNS damage (Edwards et al., 2005). Bolstering the integrity of the BBB following TBI thus represents a potentially potent and protective approach to improving patient clinical outcomes.

The roles of microRNAs (miRNAs) as regulators of neurological disease have been increasingly well-studied in recent years (Song et al., 2011). Such miRNAs can interact with complementary mRNAs, thereby suppressing translation in a sequence-specific fashion. A few prior studies have explored the roles of miRNAs in the context of TBI. Notably, we previously found that microglia exosome-derived miR-124-3p can suppress TBI-related neuroinflammation after TBI, thereby suppressing tissue damage and augmenting cognitive function (Huang et al., 2018; Xingtong et al., 2019).

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates diverse metabolic, survival, and growth-related processes in eukaryotic cells by signaling primarily through the PI3K/Akt/mTOR pathway (Slomovitz and Coloman, 2012). Candice et al. (2018) found that mTOR inhibition was sufficient to promote an increase in tight junction protein expression between endothelial cells, thereby promoting angiogenesis and decreasing vascular leakage such that treatment supported recovery from ischemic cerebrovascular disease. The activation of mTOR signaling has been shown to be suppressed by miR-124-3p in the context of both Parkinson's disease (Xintong et al., 2016) and hepatocellular carcinoma (Zhang et al., 2014), yielding distinct disease-related outcomes. We have also shown that miR-124-3p can suppress mTOR signaling in murine neuronal cells, suppressing neuroinflammation following TBI (Huang et al., 2018). Kim et al. (2015) demonstrated that high levels of miR-124 expression were sufficient to suppress PDE4B expression and to thereby increase cAMP levels within diffuse large B-cell lymphoma cells, thus suppressing mTOR signaling. Consistent with their reports, we have confirmed the ability of miR-124-3p to suppress PDE4B expression and thereby inhibit mTOR pathway activation (Huang et al., 2018).

Autophagy is a highly conserved intracellular process that can serve to promote homeostasis by degrading damaged organelles or proteins under physiological conditions. Indeed, the induction of an intermediate level of autophagy can protect cells against harm (Wu et al., 2014), and autophagic activity in the brain can control cellular survival following TBI (Rubinsztein et al., 2005). Specifically, autophagy has been shown to suppress vascular endothelial cell inflammation and apoptosis while preserving BBB integrity (Li et al., 2014). In addition to its above mentioned regulatory roles, mTOR is a key mediator of apoptotic signaling such that mTOR activation can directly inhibit the initiation of autophagy (Levine and Kroemer, 2008).

In light of the above results, we herein sought to evaluate whether the upregulation of miR-124-3p in BMVECs was sufficient to inhibit mTOR activation and to promote autophagy, thereby reducing the sensitivity of these cells to TBI-induced damage.

Materials and methods

Cell culture

The bEnd.3 BBB cell line was purchased from China Infrastructure of Cell Line Resources (Beijing, China), and cells were cultured in high-glucose complete endothelial cell medium containing 10% FBS, 1% penicillin, and 1%streptomycin (Thermo Fisher Scientific) in a 37°C 5% CO_2 incubator. CD31 immunostaining was used to positively identify endothelial cells.

Endothelial cell injury model establishment and Experimental grouping

For this study, TBI was simulated using a manual scratch wounding assay where in cells cultured in 60 mm culture dishes were scratched with a sterile plastic pipette to simulate damage to the BBB following TBI (Chaudhuri et al., 2006; Han et al., 2014). After aspirating a portion of the culture medium in each culture dish, a 10 uL pipette tip was used to scratch the culture surface in horizontal and vertical directions with 4 mm between lines such that the remaining cells were arranged in a grid. The culture dishes were then rinsed with PBS, and normal growth medium was added. Untreated cells served as a control.

Cultured cells were separated into the four following treatment groups: a control cell group, a scratch injury group, and scratch injury + miR-124-3p mimic transfection group, and a scratch injury + negative control transfection group.

Transfection

For functional studies, bEnd.3 cells were transfected with miR-124-3p mimic (5’-UAAGGCACGCGG UGAAUGCC-3’) and negative control (NC) (5’-UUCUCCGAACGUGUCACGUTT-3’, Gene Pharma, Shanghai, China) constructs. Briefly, these constructs were diluted to a 20 uM concentration. Cells were then starved for 40 min prior to transfection, after which cells were incubated in high-glucose media. Next, 500 uL of serum-free high-glucose medium was added together with 5 uL of appropriate mimic constructs and an equivalent volume of Lipofectamine3000, with these solutions being combined for 20 min at room temperature. This mixture was then added to cells for 6 h, after which a scratch wound model was established as above and media was replaced with complete serum-containing media. The FAM-miR-124-3p construct was transfected via this same approach, with FAM-positive cells being assessed via fluorescent microscopy at 48 h post-transfection.

qPCR

TRIzol (Thermo-Fisher Scientific) was utilized for the extraction of total RNA from experimental cell populations, after which an ND-2000 spectrophotometer (Thermo-Fisher Scientific) was employed to measure RNA quality and concentration. A Hairpin-it miRNA (miR-124-3p) RT-PCR quantification kit/U6 snRNA RT-PCR standardization kit (GenePharma) was then used in conjunction with appropriate primers (Table 1) to prepare cDNA as per provided directions, with U6 serving as a normalization control. All qPCR analyses were conducted using a CFX Connect RT-PCR instrument (Bio-Rad, CA, USA), with data being assessed via the $2^{-\Delta\Delta Ct}$ method.

Table 1. List of the primer sequences used for quantitative RT-PCR in this study.

Gene	Primer sequence	
	Forward	Reverse
miR-124-3p	5'-TCTTTAAGGCACGCGGTG-3'	5'-TATGGTTTTGACGACTGTGTGAT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

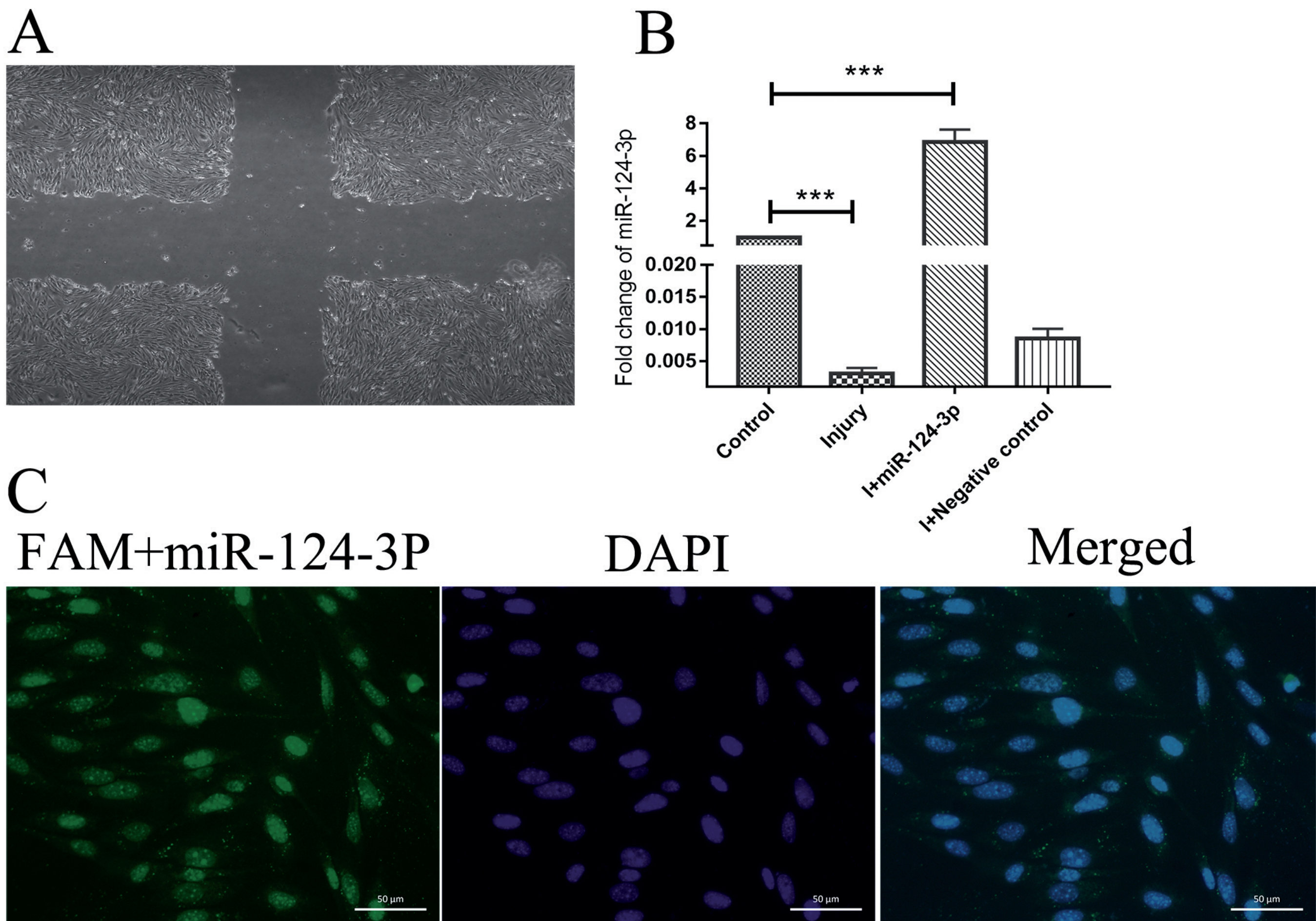

Fig. 1. A-C. The impacts of treatment on the bEnd.3 cell miR-124-3p expression. **A.** Scratch damage effects in cultured bEnd.3 cells as visualized via transmission electron microscopy. **B.** Changes in miR-124-3p expression in bEnd.3 cells following miR-124-3p mimic transfection at 48 h post-scratch injury, revealing significantly increased miR-124-3p expression in the injury+mimic group relative to the control group. **C.** FAM-miR-124-3p, DAPI, and merged plots. Successful transfection was achieved for almost all cells. Data are mean s± standard deviation, n=3 (*P<0.05, **P<0.01, ***P<0.001 vs. control group). Scale bars: 50 µm.

Western blotting

At 48 h post-scratch wounding,10% SDS-PAGE was used to separate proteins to detect Beclin 1, p62, mTOR, PDE4B (using antibodies from CST, USA, diluted 1:1000), occludin, and ZO-1 (using antibodies from Abcam, USA, diluted 1:1000), while 12% SDS-PAGE was usedtoseparate proteins to detect LC3-I and LC3- II (using antibodies from CST, USA, diluted 1:1000). Protein bands were analyzed with the ChemiDoc TM XRS + Imaging System (Bio-Rad) and the Quantity One (Bio-Rad) software, with GAPDH (CST, USA) serving as a loading control.

TUNEL staining

At 48 h after scratch wounding, endothelial cells were fluorescently labeled with a TUNEL kit (Abcam, UK), anti-CD31 (1:100; Abcam, USA), and DAPI (MilliporeSigma, USA). A fluorescent microscope was then used to quantitatively analyze stained cells under a 20x objective so as to identify positively labeled cells. Background signal in a given region was assessed with the NIS-Elements BR analysis system.

Drug Treatment

Initially, 0.515 g of chloroquine was dissolved in 10 ml of double-distilled water (DDW) to prepare solution A. Then, 100 ul of solution A was dissolved in 10 mL of DDW to generate solution B. At 6 hours after endothelial cell transfection, 10 ul of solution B was added per 1 ml of medium to yield afinalchloroquine concentration of 10 umol/L.

Hopping probe ion conductance microscopy (HPICM)

HPICM analyses were conducted based on improvements to the commercial ICNan type scanning ion conductance microscope (SICM) (Ionscope Ltd., UK). HPICM analyses were able to scan an 80×80 µm area at a 256×256 pixel resolution in 20-30 min at room temperature. Topographical data were analyzed with the SICM Image Viewer software (Ionscope Ltd, UK) to assess tight junction changes.

Statistical analysis

Data derived from at least three replicate

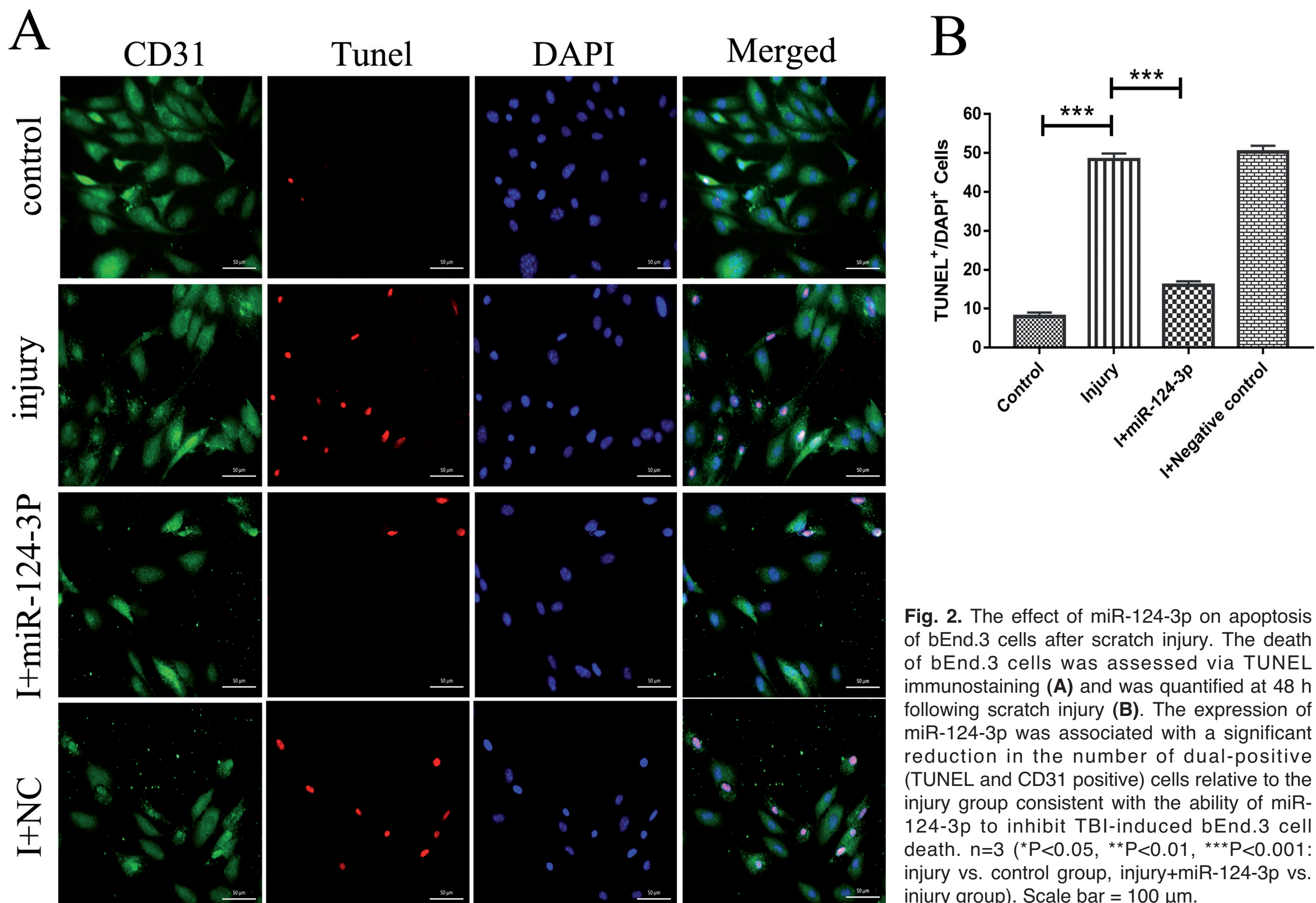

Fig. 2. The effect of miR-124-3p on apoptosis of bEnd.3 cells after scratch injury. The death of bEnd.3 cells was assessed via TUNEL immunostaining **(A)** and was quantified at 48 h following scratch injury **(B)**. The expression of miR-124-3p was associated with a significant reduction in the number of dual-positive (TUNEL and CD31 positive) cells relative to the injury group consistent with the ability of miR-124-3p to inhibit TBI-induced bEnd.3 cell death. n=3 (*P<0.05, **P<0.01, ***P<0.001: injury vs. control group, injury+miR-124-3p vs. injury group). Scale bar = 100 µm.

experiments are given as means ± standard deviations and were analyzed using SPSS v22.0 and GraphPad Prism 7. Data were compared using Dunnett's t-tests, with $P<0.05$ as the significance threshold.

Results

miR-124-3p upregulation suppresses the TBI-induced apoptotic death of BMVECs

The murine bEnd.3 cell line is commonly utilized as a commercial BMVEC model (Park et al., 2014). To explore the mechanisms whereby miR-124-3p influences BMVEC injury following TBI, we therefore utilized a scratch injury model wherein cultured bEnd.3 cells were manually wounded with a sterile pipette tip after growing to an appropriate density *in vitro* (Fig. 1A). The expression of miR-124-3p in these cells was upregulated via miR-124-3p mimic transfection at 6 h prior to scratch injury induction as confirmed by qPCR, which revealed a ~7-fold increase in the expression of this miRNA in the injury + miR-124-3p treatment group (Fig. 1B; ***$p<0.001$) and significantly decreased in the scratch damage group. FAM-labeled miR-124-3p was additionally used to confirm transfection efficiency (Fig. 1C), revealing the successful transfection of 87% of bEnd.3 cells, confirming that we were able to reliably upregulate miR-124-3p in bEnd.3 cells.

The endothelial cell marker CD31 was next analyzed via immunofluorescent staining, revealing the majority of cells to be CD31+ consistent with a pure endothelial cell population. We then conducted TUNEL staining to assess the impact of miR-124-3p expression on scratch injury-induced BMVEC apoptotic death (Fig. 2A,B). Relative to the control group, there were significantly more apoptotic cells in the injury and injury + negative control mimic groups without any difference between these groups, whereas significantly fewer apoptotic cells were evident in the injury+miR-124-3p mimic group relative to the injury group. This suggests that miR-124-3p upregulation can inhibit TBI-induced BMVEC apoptosis.

miR-124-3p upregulation may decrease the severity of TBI-induced endothelial barrier leakage.

Endothelial cell tight junctions, which are vital to the integrity of the vascular and BBB, are composed of proteins including ZO-1, occludin, and claudin-5. We have previously shown the expression of these tight junction proteins to be impaired following brain injury (Xintong et al., 2014). To establish the effect of miR-124-3p on endothelial barrier leakage after TBI, we measured ZO-1 and occludin expression in bEnd.3 cells at 48 h post-scratch injury (Fig. 3A), revealing that the expression of both of these proteins was reduced

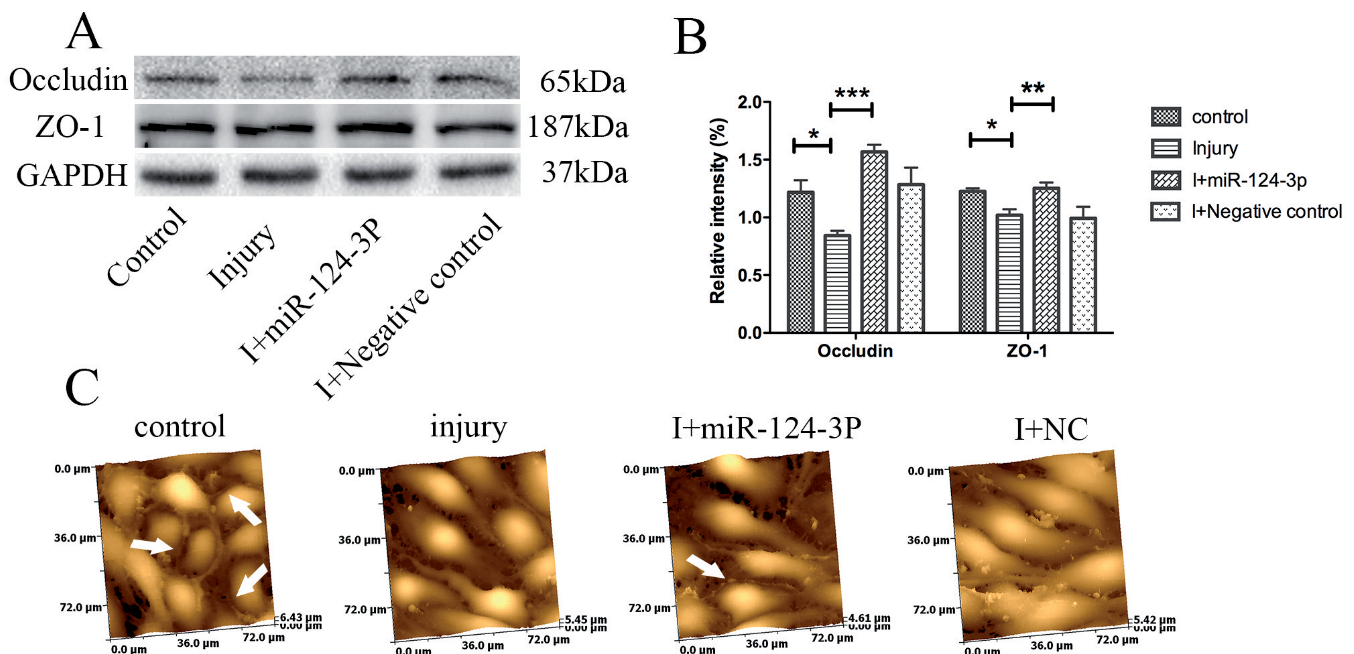

Fig. 3. The effect of miR-124-3p upregulation on bEnd.3 cell tight junctions following scratch injury. **A.** The expression of occludin and ZO-1 in bEnd.3 cells was assessed via Western blotting at 48 h following scratch injury and was quantified **(B)**. Scratch injury was associated with a loss of tight junction protein expression, while miR-124-3p upregulation was sufficient to reverse this effect. **C.** Hopping-probe ion conductivity microscopy (HPICM) scans of bEnd.3 cell tight junctions (white arrows) before and after scratch treatment. Following scratch treatment, no tightly connected structures were detectable, whereas miR-124-3p overexpression significantly enhanced the generation of such tightly connected structures following scratch injury. n=3 (*$P<0.05$, **$P<0.01$, ***$P<0.001$. injury vs. control group, injury+miR-124-3p vs. injury group). Scale bars: 50 µm.

following injury, whereas their expression was increased in cells following miR-124-3p mimic transfection. This thus suggested that the upregulation of miR-124-3p helps to protect BMVECs against TBI-associated damage.

Using an HPICM approach, we additionally evaluated microstructural alterations in bEnd.3 cell tight junctions at 5 days following scratch injury with or without miR-124-3p mimic transfection (Fig. 3C). This analysis revealed clear tight junctions between bEnd.3 cells that were disrupted by scratch injury, consistent with the disruption of the BMVEC barrier. Relative to the injury group, the generation of tight junctions in the injury + miR-124-3p mimic transfection group was significantly increased consistent with the ability of miR-124-3p to reduce the severity of the TBI-induced disruption of tight junctions between endothelial cells, thereby preserving the BBB following TBI.

miR-124-3p protects endothelial cells against TBI by promoting autophagic induction

To understand how miR-124-3p upregulation impacted BMVEC autophagic activity following TBI *in*

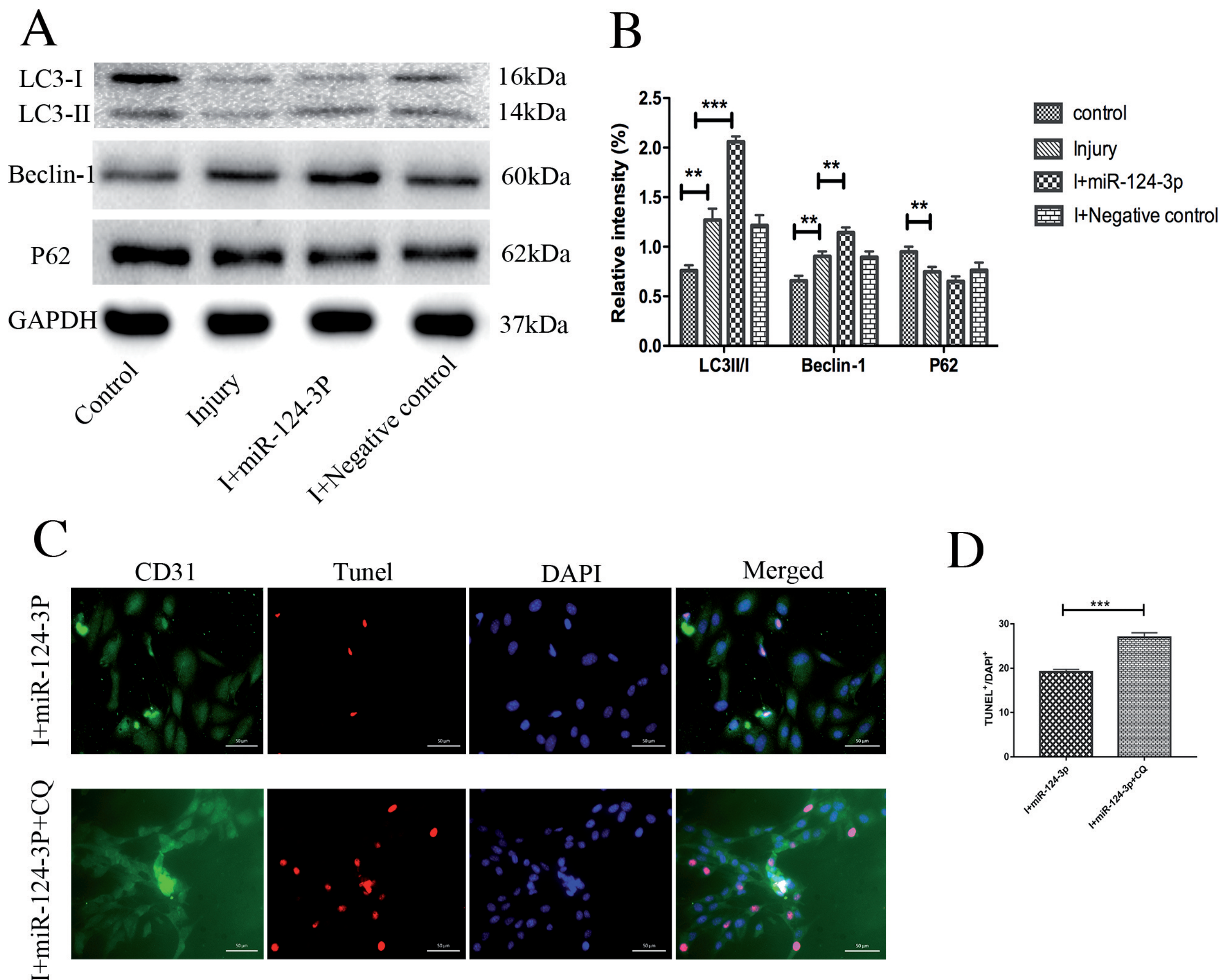

Fig. 4. The impact of miR-124-3p upregulation on endothelial cell autophagy following TBI. **A.** LC3-II / I, Beclin-1, and p62 levels were assessed in bEnd.3 cells via Western blotting at 48 h post-scratch injury, with the resultant data being quantified in **(B)**, revealing a significant increase in Beclin-1 and LC3 expression in the injury + miR-124-3p mimic group. **C.** Immunostaining and quantification of bEnd.3 cell death **(D)** as assessed via TUNEL staining. A significant increase in the frequency of dual-immunopositive (TUNEL and CD31 positive) cells was observed in the miR-124-3p mimic + CQ group relative to the control group, suggesting that the protective effect of miR-124-3p on bEnd.3 cells was markedly ablated following the inhibition of autophagy. n=3 (*P<0.05, ** P<0.01, ***P<0.001. injury vs. control group, injury+miR-124-3p vs. injury group, injury +miR-124-3pvs.injury + miR-124-3p + CQ group). Scale bars: 100 μM.

vitro, we next assessed the expression of three autophagosome marker proteins (LC3, Beclin-1, and p62) (Zhang et al., 2018) in these bEnd.3 cells via Western blotting (Fig. 4A,B). These data indicated that LC3-II/I and Beclin-1 expression was significantly enhanced in the injury + miR-124-3p mimic group relative to the injury model group, demonstrating that miR-124-3p upregulation induced autophagic activity in bEnd.3 cells following scratch injury. In contrast, no differences in p62 expression levels were observed in these different treatment groups.

To clarify whether the inhibition of autophagy was sufficient to attenuate the protective effects of miR-124-3p on BBB integrity, we next treated these cells with the autophagy inhibitor chloroquine (CQ) and evaluated apoptotic cell death via the TUNEL method (Fig. 4C). The results of this experiment indicated that significantly more bEnd.3 cell death was observed in the injury + miR-124-3p + CQ treatment group, consistent with the inhibition of autophagy having at least partially reversed the protective effects of autophagic induction in these BMVECs in the context of TBI. These data thus indicate that miR-124-3p expression protects against the apoptotic death of endothelial cells following TBI by promoting autophagy.

miR-124-3p suppresses mTOR signaling and promotes autophagy in BMVECs

The mTOR signaling pathway plays an essential role in the regulation of autophagic induction such that mTOR activation directly suppresses autophagy (Qi et al., 2019; Wang et al., 2019; Sanches et al., 2020). We have previously identified PDE4B as a miR-124-3p target gene that regulates mTOR signaling (Huang et al., 2018). To better understand how miR-124-3p protects the BBB following TBI, we assessed PDE4B and mTOR protein expression in bEnd.3 cells at 48 h post-TBI via Western blotting (Fig. 5A). The analysis revealed that PDE4B and mTOR levels were significantly reduced in the injury+miR-124-3p group relative to the injury model group, suggesting that miR-124-3p suppresses mTOR signaling activity, thereby activating autophagy and ultimately protecting BMVECs following TBI, potentially through a mechanism dependent on the downregulation of PDE4B expression.

Taken together, we found that the upregulation of miR-124-3p was sufficient to protect BMVECs barrier following *in vitro* scratch injury while promoting the upregulation of the tight junction proteins ZO-1 and occludin in these cells. These protective effects may be related to the ability of miR-124-3p to suppress mTOR signaling and to induce autophagic activity within BMVECs (Fig. 6).

Discussion

The BBB is one of the most robust barriers in the body, effectively preventing harmful macromolecules from entering the brain (Lochhead et al., 2020). BMVECs are the primary components of this BBB, with the tight junctions between endothelial cells playing an

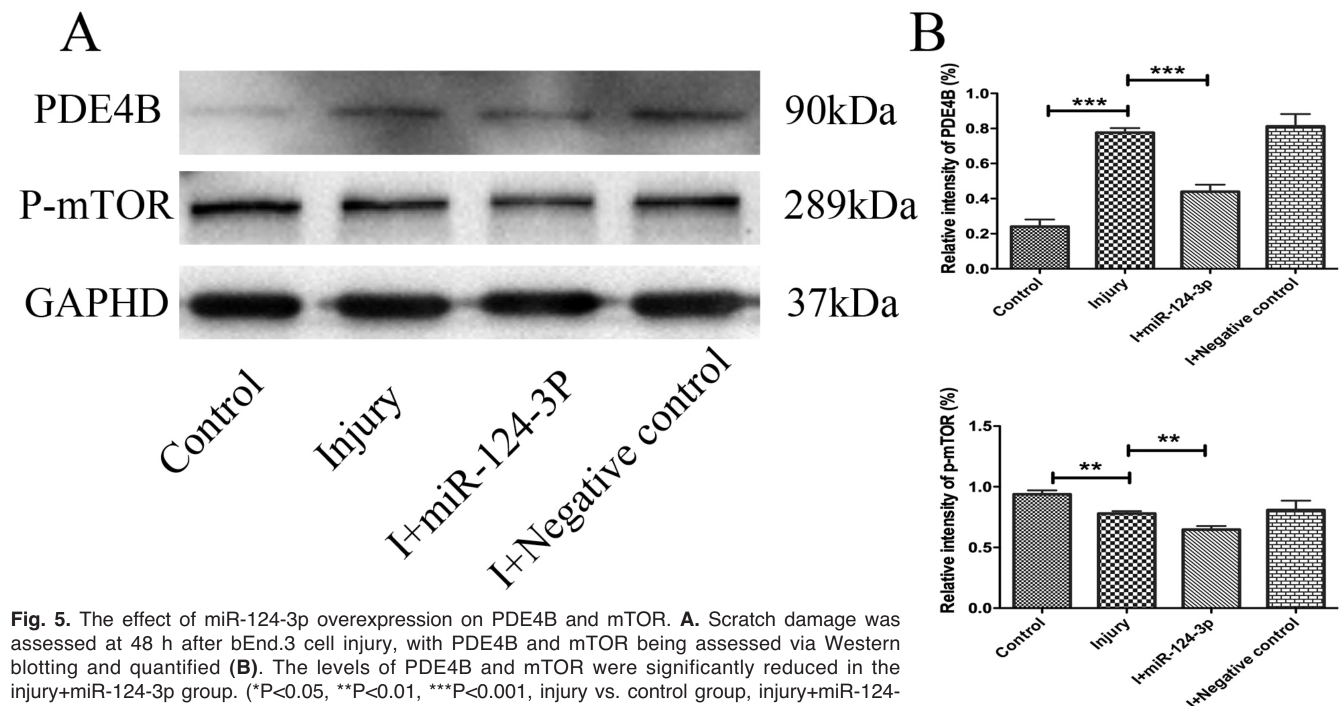

Fig. 5. The effect of miR-124-3p overexpression on PDE4B and mTOR. **A.** Scratch damage was assessed at 48 h after bEnd.3 cell injury, with PDE4B and mTOR being assessed via Western blotting and quantified **(B)**. The levels of PDE4B and mTOR were significantly reduced in the injury+miR-124-3p group. (*P<0.05, **P<0.01, ***P<0.001, injury vs. control group, injury+miR-124-3p vs. injury group).

essential role in the maintenance and functionality of this barrier. The regulation of tight junction function is mainly achieved through the phosphorylation of Claudin-5, Occludin, ZO-1, and VE-cadherin (Hashimoto et al., 2020).

Herein, we employedan abEnd.3 cell scratch wound *in vitro* model to explore the ability of miR-124-3p to protect BMVECs against damage following craniocerebral injury. We found that miR-124-3p was able to suppress the apoptotic death of these cells following scratch injury, promoting the upregulation of tight junction proteins including occludin and ZO-1, and thereby may decrease leakage through the cerebral microvascular endothelial barrier. These effects were associated with the ability of miR-124-3p to inhibit mTOR signaling and thereby promote autophagic activity in these BMVECs, suggesting that miR-124-3p may represent a viable therapeutic tool capable of preserving endothelial cell integrity following TBI.

Multiple prior reports have identified miR-124-3p as a key regulator of immunity and inflammation (Sun et al., 2013; Zhen et al., 2016). The expression of miR-124-3p is primarily evident in cells of the CNS and the immune system (Louw et al., 2016). However, there have been few studies regardingthe role of miR-124-3p in the context of traumatic brain injury. Li et al. found that BMVEC injury represents the initial stage of BBB rupture, with tight junction protein expression levels being decreased following brain injury (Li et al., 2014). Using the bEnd.3 cell *in vitro* scratch wound model and the miR-124-3p mimic transfection, we were able to demonstrate that the upregulation of this miRNA mitigated TBI-induced BMVEC death, attenuated TBI-induced tight junction protein downregulation, and enhanced BBB stability, indicating that increasing miR-124-3p expression in these vascular endothelial cells following TBI can protect them from injury and associated apoptotic death.

Recent reports suggest that the induction of intermediate levels of autophagy can protect endothelial cells against stress-induced injury (Liu et al., 2009; Xie et al., 2011). How TBI-induced autophagy shapes the responses of BMVECs to injury-related stress, however, remains to be fully clarified. In one recent report, autophagic induction was shown to enhance BMVEC survival in a glucose and oxygen deprivation/reoxygenation experimental model system (Li et al., 2017). Several reports have highlighted the ability of specific miRNAs to regulate autophagy in pathological contexts by interacting with mRNAs encoding key autophagy-related proteins (Frankel et al., 2012; Ying et al., 2012). In the present report, we measured the expression of LC3-II/I, Beclin-1, and p62, all of which are important for the initiation and/or termination of autophagy, and thereby revealed that miR-124-3p promoted autophagy and BMVEC survival following

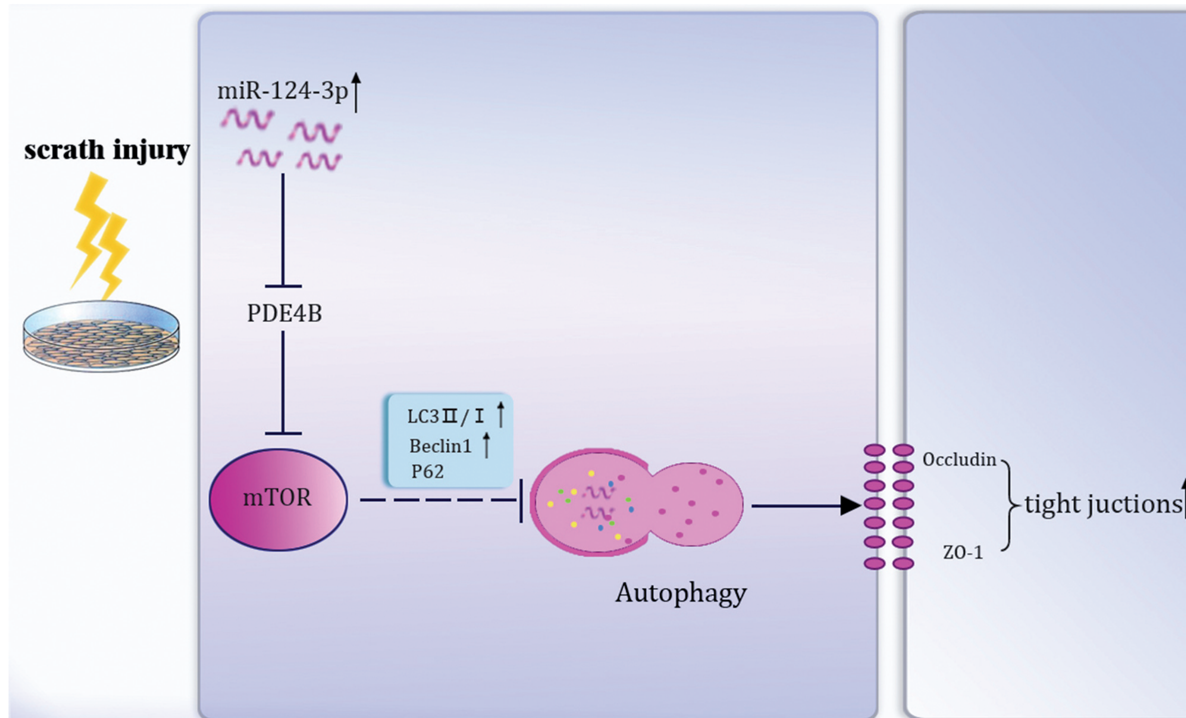

Fig. 6. The upregulation of miR-124-3p can protect the tight junction following *in vitro* scratch injury while promoting the upregulation of the tight junction proteins ZO-1 and occludin in BMVECs. These protective effects may be related to the ability of miR-124-3p to suppress mTOR signaling and to induce autophagic activity within BMVECs.

TBI. We observed no significant differences in p62 expression among the control, injury, and injury + miR-124-3p mimic groups in the present study, whereas Beclin 1 and LC3-II/I expression were upregulated following miR-124-3p mimic transfection. Given that p62 regulates autophagosome degradation while Beclin I and LC3-II/I control autophagosome formation (He et al., 2010; Narendra et al., 2010), this may suggest that miR-124-3p is important for the initiation of autophagy rather than its termination.

The activation of mTOR can suppress the initiation of autophagic processes (Levine and Kroemer, 2008), and elevated miR-124 expression has previously been shown by Kim et al. (2015) to suppress PDE4B expression in diffuse large B-cell lymphoma and to thereby increase intracellular cAMP levels, thereby inhibiting Akt/mTOR/MCL1 signaling. We have also previously demonstrated that miR-124-3p derived from microglia exosomes can target PDE4B to suppress mTOR pathway activation following TBI, thereby mitigating neuronal damage (Huang et al., 2018). Kim et al. (2019) found that inhibitors of PDE4B were able to improve the survival of colon cancer by inhibiting the mTOR-Myc axis to exert tumor suppressive effects (Kim et al., 2019). Consistent with these prior reports, we herein observed that significantly increasing the expression of miR-124-3p relative to levels observed in our injury model group was sufficient to suppress PDE4B expression and mTOR signaling. Together, our data suggest that high miR-124-3p levels can inhibit mTOR activation and thereby promote autophagy through a mechanism associated with PDE4B downregulation in BMVECs.

In the future, we plan to conduct detailed *in vivo* studies of the mechanisms whereby miR-124-3p upregulation can protect BMVECs following TBI in order to establish whether the activation of autophagy by this miRNA is similarly beneficial in a more complex animal model system. The blood-brain barrier is a tightly regulated structural and functional unit composed of endothelial cells, pericytes, neurons, glial cells, and a range of extracellular components. The upregulation of miR-124-3p in BMVECs following TBI may also indirectly influence other cells associated with this barrier system, although further research will be needed to test this possibility.

Conclusions

In conclusion, the results of the present analysis indicate that miR-124-3p may suppress PDE4B expression and mTOR signaling in brain microvascular endothelial cells, thereby promoting autophagy. This mechanism may protect BMVECs against damage following TBI, but needs further in-depth study. As such, we have provided novel experimental and theoretical basis to prevent secondary BBB damage following TBI.

Acknowledgements. This research was supported by grants from Tianjin Medical University General Hospital Youth Cultivation Foundation (grant no. ZYYFY2017015), Tianjin Science Foundation (grant no.19JCQNJC10200) and Tianjin Municipal Education Commission Scientific Research Program Project (grant no. 2020KJ164).

Conflicts of interest. There are no conflicts of interest to declare.

References

Blennow K., Hardy J. and Zetterberg H. (2012). The neuropathology and neurobiology of traumatic brain injury. Neuron. 76, 886-889.

Chaudhuri V. and Karasek, M.A. (2006). Mechanisms of microvascular wound repair II. Injury induces transformation of endothelial cells into myofibroblasts and the synthesis of matrix proteins. Vitr. Cell. Dev. Biol. Anim. 42, 314-319.

Candice E.V.S., Jordan B.J., Angela B.O., Naomi L.S., Stacy A.H., Zoltan I.U., James D.L and Veronica G. (2018). Inhibition of mTOR protects the blood-brain barrier in models of Alzheimer's disease and vascular cognitive impairment. Am. J. Physiol. Heart. Circ. Physiol. 314, H693-H703.

Edwards P., Arango M., Balica L., Cottingham R. and Yutthakasemsunt S. (2005). Final results of MRCCRASH, a randomized plaebo-controlled trial of intravenous corticosteroid in adults with head injury-outcomes at 6 months. Lancet. 365, 1957-1959.

Frankel L.B. and Lund A.H. (2012). MicroRNA regulation of autophagy. Carcinogenesis. 33, 2018-2025.

He C, and Levine B. (2010). The Beclin 1 interactome. Curr. Opin. Cell. Biol. 22,140-149.

Han Z., Chen F., Ge X., Tan J., Lei P. and Zhang J. (2014). MiR-21 alleviated apoptosis of cortical neurons through promoting PTEN-Akt signaling pathway *in vitro* after experimen-tal traumatic brain injury. Brain. Res. 1582, 12-20.

Huang, S., Xintong G., Jingwen Y., Zhaoli H., Zhenyu Y., Ying L., Fanglian C., Haichen W., Jianning Z. and Ping L. (2018). Increased miR-124-3p in microglial exosomes following traumatic brain injury inhibits neuronal inflammation and contributes to neurite outgrowth via their transfer into neurons. FASEB. J. 32, 512-528.

Hashimoto Y. and Campbell M. (2020). Tight junction modulation at the blood-brain barrier: Current and future perspectives. BBA – Biomembranes. 1862, 183298.

Kim J., Jeong D., Nam J., Aung T.N., Gim J.A., Park K.U. and Kim S.W. (2015). MicroRNA-124 regulates glucocorticoid sensitivity by targeting phosphodiesterase 4B in diffuse large B cell lymphoma. Gene. 558, 173-180.

Kim D.U., Kwak B., and Kim S-W. (2019). Phosphodiesterase 4B is an effective therapeutic target in colorectal cancer. Biochem. Biophys. Res. Commun. 508, 825-831.

Levine B. and Kroemer G. (2008). Macroautophagy. Cell. 132, 162.e1-162.e3.

Liu X.., Jing Z., Jiping X., Baoxiang Z., Yun Z., Shangli Z. and Junying M. (2009). Protective effects of a benzoxazine derivative against oxidized LDL-induced apoptosis and the increases of integrin beta4, ROS, NF-kappaB and P53 in human umbilical vein endothelial cells. Bioorg. Med. Chem. Lett. 19, 2896-900.

Li H., Gao A., Feng D., Wang Y. and Zhang L. (2014). Evaluation of the protective potential of brain microvascular endothelial cell autophagy on blood-brain barrier integrity during experimental cerebral

ischemia-reperfusion injury. Transl. Stroke Res. 5, 618-626.
Li Z., Li J. and Tang N. (2017). Long noncoding RNA Malat1 is a potent autophagy inducer protecting brain microvascular endothelial cells against oxygen-glucose deprivation/reoxygenation-induced injury by sponging miR-26b and upregulating ULK2 expression. Neuroscience. 354, 1-10.
Lochhead J. J., Yang J., Ronaldson P.T. and Davis T.P. (2020). Structure, Function and Regulation of the Blood-Brain Barrier Tight Junction in Central Nervous System Disorders. Front. Physiol. 11, 914.
Louw A.M., Kolar M.K., Novikova L.N., Kingham P.J., Wiberg M., Kjems J. and Novikov L.N. (2016). Chitosan polyplex mediated delivery of miRNA-124 reduces activation of microglial cells *in vitro* and in rat models of spinal cord injury. Nanomedicine. 12, 643-653.
Narendra D., Kane L. A., Hauser D. N., Fearnley I. M. and Youle R. J. (2010). p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy, 6, 1090-1106.
Park R., Kook S.Y., Park J.C. and Mook-jung I. (2014). Aβ1-42 reduces P-glycoprotein in the blood-brain barrier through RAGE-NF-κB signaling. Cell. Death. Dis. 5, e1299.
Persidsky Y., Ramirez SH., Haorah J. and Kanmogne G.D. (2006). Blood-brain barrier: structural components and function under physiologic and pathologic conditions. J. Neuroimmune. Pharmacol. 1, 223-236.
Qi H., Ren J., Ba L., Song C., Zhang Q., Cao Y., Shi P., Fu B., Liu Y. and Sun H. (2019). MSTN Attenuates Cardiac Hypertrophy through Inhibition of Excessive Cardiac Autophagy by Blocking AMPK /mTOR and miR-128/PPARγ/NF-κB. Mol. Ther. Nucleic. Acids. 19, 507-522.
Rubinsztein DC., DiFiglia M., Heintz N., Nixon RA., Qin ZH., Ravikumar B., Stefanis L. and Tolkovsky A. (2005). Autophagy and its possible roles in nervous system diseases, damage and repair. Autophagy. 1, 11-22.
Slomovitz B.M. and Coloman R.L. (2012). The PI3K/AKT/mTOR Pathway as a Therapeutic Target in Endometrial Cancer. Clin. Cancer. Res. 18, 5856-5864.
Song YJ., Tian XB., Zhang S., Zhang YX., Li X., Li D., Cheng Y., Zhang JN., Kang CS. and Wen Z. (2011). Temporal lobe epilepsy induces differential expression of hippocampal miRNAs including let-7e and miR-23a/b. Brain Res. 1387, 134-140.
Sun Y., Li Q., Gui H., Xu D.P., Yang Y.L., Su D.F. and Liu X. (2013). microRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. Cell Res. 23, 1270-1283.
Sanches-Silva A., Testai L, Nabavi S.F., Battino M., Devi K.P., Tejada S., Sureda A., Xu S., Yousefi B., Majidinia M., Russo G.L., Efferth T., Nabavi S.M. and Farzaei M.H. (2020). Therapeutic potential of polyphenols in cardiovascular diseases: regulation of mTOR signaling pathway. Pharmacol. Res. 152, 104626.
Wu Q., Yang Z., Nie Y., Shi Y. and Fan D. (2014). Multi-drug resistance in cancer chemotherapeutics: Mechanisms and lab approaches. CANCER LETTERS. 347, 159-166.
Wang H., Liu Y., Wang D., Xu Y., Dong R., Yang Y., Lv Q., Chen X. and Zhang Z. (2019). The Upstream Pathway of mTOR-Mediated Autophagy in Liver Diseases. Cells. 8, 1597.
Xingtong G., Ping L., Haichen W., Anling Z., Zhaoli H., Xin C., Shenghui L., Rongcai J., Chunsheng K. and Jianning Z. (2014). MiR-21 improves the neurological outcome after traumatic brain injury in rats. Sci. Rep. 4, 6718.
Xie, Y., You S.J., Zhang Y.L., Han Q. and Liu C.F. (2011). Protective role of autophagy in AGE-induced early injury of human vascular endothelial cells. Mol. Med. Rep. 4, 459-564.
Xintong G., Huiqing W., Yongyi Y., Yugao S., Yongwen D., Xiaozheng H., Guohui L. and Shizhong Z. (2016). miR-124 regulates cell apoptosis and autophagy in dopaminergic neurons and protects them by regulating AMPK/mTOR pathway in Parkinson's disease. Am. J. Transl. Res. 8, 2127-2137.
Xingtong G., Mengtian G., Tianpeng H., Wenzhu W., Shan H., Zhenyu Y., Ying L., Fanglian C., Luoyun Z., Chunsheng K., Rongcai J., Ping L. and Jianning Z. (2019). Increased Microglial Exosomal miR-124-3p Alleviates Neurodegeneration and Impoves Cognitive Outcome after rmTBI. Mol. Ther. 28
Ying C., Wei Y., Xingxing H., Lemeng Z., Chuanjiang L., Hai H., Gary N., David A.G., Jusheng L. and Allan T. (2012). MiR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. Gastroenterology. 143, 177-187.
Zweakberger K., Eros C., Zimmermann R., Kim S.W., Engel DC. And Plesnila N. (2006). Effect of early and delayed decompressive craniectory on secondary brain damage after controlled cortical impact in mice. J. Neurotrauma. 23, 1083-1093.
Zhang, Y., Guo, X., Xiong L., Yu L., Li Z., Guo Q., Li Z., Li B. and Lin N. (2014). Comprehensive analysis of microRNA-regulated protein interaction network reveals the tumor suppressive role of microRNA-149 in human hepatocellular carcinoma via targeting AKT-mTOR pathway. Mol. Cancer. 13, 253.
Zhen Q., Peng Y.W., Ding F.S. and Xia H. (2016). miRNA-124 in immune system and immune disorders. Front. Immunol. 7, 406.
Zhang, L. and Wang H. (2018). Autophagy in Traumatic Brain Injury: A New Target for Therapeutic Intervention. Font. Mol. Neurosci. 11, 190.

Accepted December 13, 2021