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Original Article

TUG1 aggravates intracerebral hemorrhage injury by inhibiting angiogenesis in an miR-26a-dependent manner

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Abstract: Long non-coding RNA taurine-upregulated gene 1 (TUG1) plays pivotal roles in angiogenesis, an important mechanism of neural repair after intracerebral hemorrhage (ICH). However, the role of TUG1 in angiogenesis following ICH is not clear. Therefore, in this study, we investigated the role and the underlying mechanism of TUG1 in neurologic impairment and cerebral angiogenesis following ICH. The ICH rat model was established and then rats were injected with TUG1-expressing plasmid (pcDNA-TUG1) or miR-26a mimic, a critical regulator of VEGF-mediated angiogenesis. We confirmed the overexpression of TUG1 and miR-26a by qRT-PCR. The neurological deficits of ICH rats were evaluated by modified neurological severity scores. The expression of angiogenesis markers VEGF and CD31 were examined by immunohistochemistry and western blot. The interaction between TUG1 and miR-26a was determined by luciferase reporter assay. Our results showed that ICH caused a marked upregulation of TUG1 and a significant downregulation of miR-26a. TUG1 overexpression led to the deterioration of neurologic function and inhibited cerebral angiogenesis in ICH rats. In contrast, overexpression of miR-26a alleviated the neurologic damage and promoted cerebral angiogenesis in ICH rats, but these could be attenuated by TUG1 overexpression. Furthermore, TUG1 directly bound to miR-26a and inhibited its expression. Importantly, TUG1 overexpression inhibited the expression of VEGF by targeting miR-26a. In conclusion, our results indicated that TUG1 aggravated ICH-mediated injury by suppressing angiogenesis by downregulating miR-26a. This suggests a rationale for targeting TUG1/miR-26a in the therapy of ICH.

Keywords: Angiogenesis, intracerebral hemorrhage, miR-26a, neurological function, taurine-upregulated gene 1

Introduction

Intracerebral hemorrhage (ICH) is one of the critical neurological diseases with high mortality and disability. There are at least 3 million new ICH cases worldwide every year, and the incidence of ICH is rising [1, 2]. Recent studies have reported that not only have 45.4% of the patients with ICH die within one year of its onset [3], but also the survivors often have residual disabilities and tend to experience progressive neurologic deterioration after a period of treatment [4]. However, effective treatments for ICH are limited. Thus, it is necessary to understand the mechanisms underlying the pathogenic events of ICH and identify novel therapeutic strategies.

Cerebral angiogenesis is an important mechanism of damaged brain tissue repair after ICH by supplying oxygen and various nutrients for neural repair [5]. Evidence has shown that angiogenesis can promote the remodeling of the injured neurons and ameliorate neurological deficits [6, 7], indicating that angiogenesis may be an effective therapeutic target for the treatment of ICH. Angiogenesis is regulated by many factors such as vascular endothelial growth factor (VEGF) which promotes angiogenesis [8]. MicroRNAs (miRNAs) are important modulators of protein synthesis by regulating gene expression post-transcriptionally [9]. Recently, miR-26a has been reported to regulate angiogenesis through modulating VEGF activity under various physiologic and pathologic states [10, 11]. However, the influences of miR-26a on angiogenesis are cell-type specific [12], and the role of miR-26a in angiogenesis following ICH remains to be determined.

Emerging data have shown that long non-coding RNAs (lncRNAs) participate in the regulation

of various pathophysiologic processes, including angiogenesis, by regulating the stability of target genes and chromatin remodeling [13, 14]. Mechanistically, lncRNAs can regulate the target gene expression by competing with endogenous RNA or natural miRNA sponges. Taurine-upregulated gene 1 (TUG1) is one of the lncRNAs widely expressed in various tissues and is involved in angiogenesis under different pathophysiological conditions [15]. However, the role of TUG1 in angiogenesis following ICH has not been clearly defined.

Crosstalk between TUG1 and miR-26a has been reported [16]; However, their functional relationship in ICH injury is unclear. In this study, we report that TUG1 was upregulated in rats brain after ICH, and its overexpression inhibited neurologic functional recovery in ICH rats possibly by suppressing angiogenesis through downregulating miR-26a.

Materials and methods

Plasmid construction

The design and construction of recombinant plasmids, including TUG1-expressing pcDNA-TUG1 and vector control pcDNA-NC, were done by Genechem (Shanghai, China). MiR-26a mimics and mimic-NC control were from RiboBio (Guangzhou, China).

Animals

This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. A total of 50 Sprague-Dawley male adult rats (body weight of 230-270 g), provided by the Experimental Animal Center of Wuhan University, were used in this study. All rats were fed with standard rodent chow and water ad libitum and were caged in individually ventilated caging racks under a SPF condition (22± 1°C, humidity of 50-60%, cycle of 12 h light and 12 h dark).

Rat model of ICH

An in vivo rat ICH model was generated as previously described [17]. After anesthesia with 2% pentobarbital sodium (intraperitoneal injection, 30 mg/kg), the rats were immobilized on a stereotaxic frame. Then, a scalp incision was performed, and the frontal bone was exposed. The right corpus striatum was selected as injection site (0.6 mm in front of the bregma, 3.5 mm right lateral to the midline), and a 1 mm diameter hole in the cranium was made. A microinjector (26-gauge) was inserted into the striatum (5.5 mm depth below the skull), and then 50 μl blood collected from femoral artery was infused (10 μl/minute). The needle was extracted after a 10 minute interval. In the sham control group, the blood was replaced with 50 μl of saline. The rats were kept under a warm pad at the temperature of 37±0.5°C until the rats woke up.

Grouping and treatment of rats

After the surgery, 48 surviving rats were randomly divided into 8 groups and received the injection as follows (n = 6/group):

(1) sham group without ICH induction; (2) ICH group without any treatment; (3) ICH + pcDNA-TUG1 group (received an intracerebroventricular injection of TUG1 overexpression plasmid after ICH induction); (4) ICH + pcDNA-negative control (NC) group (injected with empty plasmid into the lateral ventricle); (5) ICH + miR-26a mimics group (received tail vein injection of miR-26a mimics after ICH); (6) ICH + mimic-NC group (injected with miR-26a-mimic-NC through tail vein); (7) ICH + miR-26a mimics + pcDNA-TUG1 group (received an intracerebroventricular injection of TUG1 overexpression plasmid and miR-26a mimics through tail vein after ICH induction); (8) ICH + miR-26a mimics + pcDNA-NC group (injected with empty plasmid and miR-26a mimics).

Neurological function evaluation

The short-term neurologic function of rats was scored by modified Neurological Severity Scores (mNSS). On the 1st, 3rd, and 7th day after the induction of ICH, each rat was scored by a researcher who was unaware of the experimental design. The mNSS was graded on a scale of 0 to 18 (18 = maximal deficit score; 0 = normal score), in which lower score represents a less severe neurological deficit. On the 7th day after ICH, rats were sacrificed, and the brain specimens ipsilateral to the hematoma were dissected for further analysis.

Cell culture

HEK-293 cells, presented by the Central Laboratory of Renmin Hospital of Wuhan University,

were cultured in DMEM medium supplemented with 10% FBS and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Immunohistochemical staining

The brain tissue samples dissected from the rats in the ICH model were fixed with paraformaldehyde, embedded in paraffin, and sectioned (4 μm). The tissue sections were then deparaffinized and rehydrated, and the endogenous peroxidase activity was inactivated by 3% H_2O_2 solution. Next, antigen was retried, and the sections were blocked and incubated with primary antibody followed by secondary antibody. The sections were incubated in peroxidase substrate solution, treated with 3, 3-diaminobenzidine, and counterstained with hematoxylin. A light microscope was used to observe and capture images at × 400 magnification. CD31 staining was performed as an indicator of microvessel density (MVD).

QRT-PCR

Total RNA was extracted by using TRIzol Reagent and was used for qRT-PCR analysis. U6 RNA was used as an internal control for miR-26a, and GAPDH was used as the internal control for TUG1 and VEGF. The standard $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of genes.

The primer sequences used were as follows:

TUG1 Forward 5'-CCTTCCCAGACGACTTGACA-3', Reverse 5'-CCATCAGTTCTTCCGGCTTG-3'; GAPDH Forward 5'-ACAGCAACAGGGTGGTGG-AC-3', Reverse 5'-TTTGAGGGTGCAGCGAACTT-3'; miR-26a Forward 5'-TCCGTTGTTTCAAGTA-ATCCAGG-3', Reverse 5'-ATCAACCACACGTCAT-GTGACT-3'; VEGF Forward 5'-ATCTTCAAGCC-ATCCTGTGTGC-3', Reverse 5'-GCTCACCGCCTC-GGCTTGT-3'; U6 Forward 5'-CCTGCTTCGGCA-GCACAT-3', Reverse 5'-AACGCTTCACGAATTTG-CGT-3'.

Western blot analysis

Western blot analysis was performed with the brain tissues dissected from the rats in ICH model. Briefly, brain tissues were lysed, and the total proteins were quantified and separated by SDS-PAGE. The proteins were then transferred onto PVDF membranes and incubated with the corresponding primary antibodies overnight. After extensive washing, the membranes were incubated with appropriate secondary antibody, and the signal was developed by ECL reagents and detected by LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA). The western blotting images were analyzed by using AlphaEaseFc software. GAPDH was used as internal control.

Bioinformatics analysis

We employed Starbase databases (http://starbase.sysu.edu.cn/) to predict the miR-26a targeting site of TUG1. The selection criteria were supporting experiences > 1 and cancer types > 5.

Luciferase reporter assay

The 3'UTR of TUG1 was amplified by PCR and cloned into luciferase reporter pmirGlo vector (GenePharm, Shanghai, China) as TUG1 wild type plasmid (pmirGLO-TUG1-WT). A mutation at the 3'-UTR of TUG1 was generated and cloned into pmirGlo to construct TUG1 mutant plasmid (pmirGLO-TUG1-MUT). HEK-293T cells were co-transfected with pmirGLO-TUG1-WT or pmirGLO-TUG1-MUT and miR-26a mimics or mimic-NC. Forty-eight hours after transfection, the cells were collected, and the cell lysates were prepared and subjected to luciferase reporter assay following the manufacturer's instruction (Promega, WI, USA).

Statistical analysis

SPSS 20.0 was utilized to compute and analyze the data. Measured data are presented as mean ± standard deviation (SD) unless otherwise indicated. Student's *t*-test was applied to analyze the statistical difference between two groups. $P < 0.05$ was considered significant.

Results

Opposite expression patterns of lncRNA TUG1 and miR-26a in ICH rats

To investigate the roles of *lncRNA TUG1 and miR-26a in ICH,* we first assessed the expression levels of lncRNA TUG1 and miR-26a in the brain tissues of ICH rats. Compared to controls, lncRNA TUG1 was significantly upregulated (**Figure 1A**), while miR-26a level was markedly decreased in rats with ICH (**Figure 1B**).

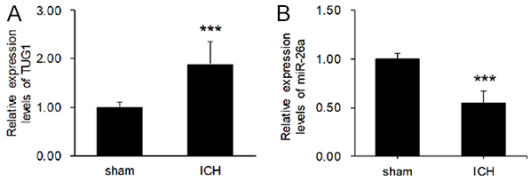

Figure 1. Upregulation of TUG1 and downregulation of miR-26a in ICH model in vivo. A. The expression of TUG1 in rat brain after ICH. B. The expression of miR-26a in rat brain after ICH. ***P < 0.001, *vs.* sham.

Opposite effects of TUG1 and miR-26a on the recovery of neural function of rats with ICH

Given the significant upregulation of TUG1 in response to ICH in vivo, we next examined the biological roles of TUG1 in ICH by overexpressing TUG1 in ICH rats. The overexpression of TUG1 was confirmed by qRT-PCR (**Figure 2A**). Our data showed that there was no difference in mNSS between the ICH and ICH + pcDNA-TUG1 group on the first day after ICH (**Figure 2B**); however, rats in ICH + pcDNA-TUG1 group exhibited a significantly more severe neurological function defect on the 3rd and 7th day after ICH, compared to those in ICH + pcDNA-NC group (**Figure 2C**, **2D**).

Since mirR-26a expression was negatively correlated with TUG1 expression, we also explored the effects of manipulating miR-26a expression on ICH rats. qRT-PCR confirmed the level of miR-26a was much higher in ICH + miR-26a mimics-injected rats than in mimic-NC-injected rats (**Figure 2E**). The mNSS was similar between the ICH and ICH + miR-26a mimics group on the first day after ICH (**Figure 2F**). However, on the 3rd and 7th day after the procedure, overexpressing miR-26a significantly reduced the mNSS in ICH rats (**Figure 2G**, **2H**).

Opposite roles of TUG1 and miR-26a in cerebral angiogenesis in the ICH region

Since angiogenesis is an important process during damaged brain tissue repair after ICH, we investigated the functions of TUG1 and miR-26a on cerebral angiogenesis following ICH. qRT-PCR and immunohistochemistry (IHC) were used to detect the expression of angiogenesis-related markers CD31 and VEGF in ICH rats. The results showed that miR-26a stimulated VEGF mRNA expression in the ICH region of rats (**Figure 3A**). Consistently, the IHC results confirmed this promoting effect of miR-26a on cerebral angiogenesis in ICH rats (**Figure 3B**). In contrast, the VEGF mRNA level in rat brain was significantly decreased by pcDNA-TUG1 injection (**Figure 3C**). Similarly, the IHC results also showed that TUG1 significantly inhibited the expression of VEGF and CD31 in the ICH region of rats (**Figure 3D**).

TUG1 can bind to miR-26a and inhibit its expression

To understand the functional relationship between TUG1 and miR-26a, we first employed Starbase databases to analyze the functional network of TUG1 and miR-26a and identified a potential miR-26a binding site on TUG1 (**Figure 4A**). Subsequently, we conducted a luciferase reporter assay in HEK-293T cells to verify the interaction between TUG1 and miR-26a. **Figure 4B** demonstrated that co-transfection of miR-26a mimics dramatically suppressed the luciferase activity of TUG1-WT reporter but had no effect on the luciferase activity of TUG1-MUT, in which the miR-26a-binding site was mutated, indicating a direct interaction between miR-26a TUG1. To further confirm whether miR-26a expression was regulated by TUG1, we measured the miR-26a expression in ICH rats treated with or without pcDNA-TUG1 by qRT-PCR. The results indicated that the expression of miR-26a in rat brain was very downregulated by the injection of pcDNA-TUG1 (**Figure 4C**).

TUG1 regulates neurologic functional recovery and cerebral angiogenesis following ICH, possibly by inhibiting miR-26a

Importantly, to understand the functional significance of TUG1 regulated miR-26a expression, we explored whether TUG1 affected neurologic functional recovery and angiogenesis after ICH by downregulation of miR-26a. We found overexpression of TUG1 attenuated the effect of miR-26a mimics on the mNSS of rats on the 3rd and 7th day after ICH (**Figure 5A-C**). In addition, the expression of VEGF mRNA in the ICH + miR-26a mimics group was significantly decreased after treatment with pcDNA-TUG1 (**Figure 5D**). As shown in **Figure 5E** and **5F**, the enhanced expression of VEGF induced by miR-26a was abolished by TUG1 co-expression, as determined by IHC and western blot analysis. Consistent with this finding, TUG1 overexpression also partially attenuated the

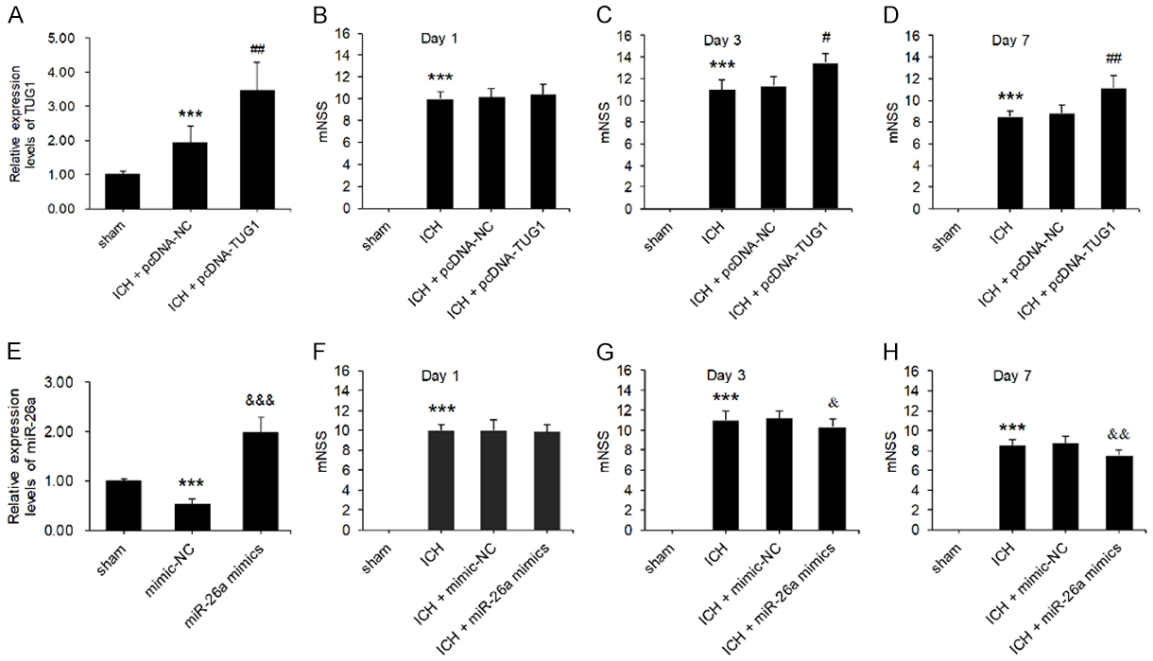

Figure 2. Roles of TUG1 or miR-26a in neurological deficits in rats with ICH. (A) pcDNA-TUG1 expression in vivo. (B-D) The effects of TUG1 overexpression on mNSS in rats on the 1st (B), 3rd (C), and 7th (D) day after ICH. (E) The expression of miR-26a mimics in rats. (F-H) The effects of miR-26a mimics on mNSS in ICH rats on the 1st (F), 3rd (G) and 7th (H) day after ICH. ***P < 0.001, *vs.* sham; #P < 0.05, *vs.* pcDNA-NC; ##P < 0.01, *vs.* pcDNA-NC; &P < 0.05, *vs.* mimic-NC; &&P < 0.01, *vs.* mimic-NC; &&&P < 0.001, *vs.* mimic-NC.

promoting effect of miR-26a on MVD in pericerebral hemorrhage foci. Taken together, these data indicated that TUG1 inhibited the cerebral angiogenesis and the recovery of neural function in ICH rats through downregulating miR-26a.

Discussion

Intracerebral hemorrage (ICH), the most destructive type of stroke, has a poor prognosis. Although there are many studies on the mechanism of ICH, the therapeutic effect of ICH treatment is still unsatisfactory. Therefore, it is crucial to explore new treatment targets for this disorder. Angiogenesis is an important mechanism to promote neurological function recovery following ICH [18, 19]. However, the regulatory mechanism of cerebral angiogenesis following ICH has not been fully understood. In this study, we explored the function and the underlying molecular mechanisms of TUG1 in neurologic impairment and cerebral angiogenesis after ICH.

TUG1 regulates angiogenesis in various diseases [20, 21]. A previous study has shown that TUG1, which is upregulated in the mouse model of oxygen-induced retinopathy, may regulate retinal angiogenesis by regulating miR-145-5p [22]. In addition, TUG1 was found to be important in promoting tumor angiogenesis by sponging miR-29c-3p [23]. Another study also indicated that TUG1 was highly expressed in glioma and promoted glioma angiogenesis [24]. However, the effect of TUG1 on angiogenesis after ICH is unclear. In this study, we found that overexpression of TUG1 contributed to the deterioration of neural function and the decreased cerebral angiogenesis in ICH rats.

VEGF, a pleiotropic angiogenic growth factor, is widely reported to promote angiogenesis by stimulating vascular endothelial cell proliferation [25, 26]. In our study, significant downregulation of VEGF was observed in the ICH region of pcDNA-TUG1-treated rats, suggesting that TUG1 inhibited angiogenesis following ICH in rats by downregulating VEGF.

It has been reported that miR-26a may increase the expression of VEGF to enhance angiogenesis in glioma [27]. In addition, miR-26a could prevent secondary cerebral injury following ICH

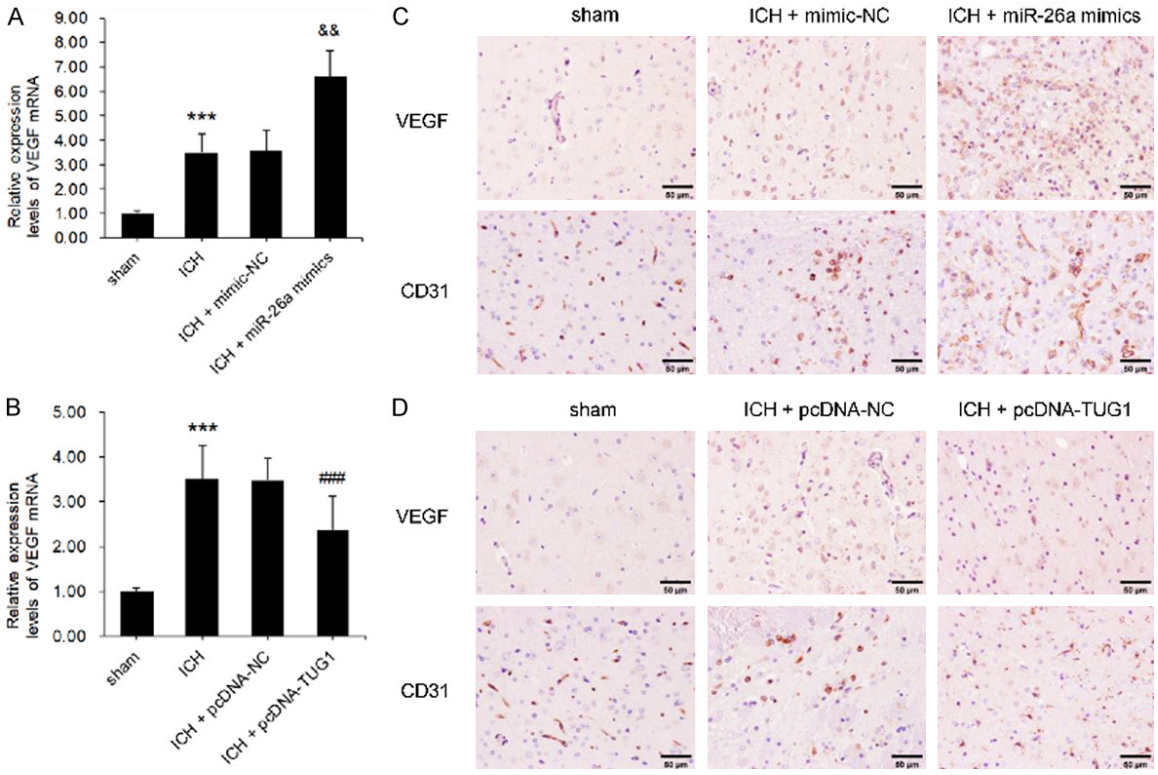

Figure 3. Effects of TUG1 or miR-26a on cerebral angiogenesis in rats with ICH. A. VEGF mRNA levels in ICH rats treated with mimic-NC or miR-26a mimics. B. The expression of VEGF and CD31 in ICH rats injected with miR-26a mimics. Original magnification, × 400. C. VEGF mRNA levels in ICH rats injected with pcDNA-NC or pcDNA-TUG1. D. IHC was performed to detect the expression of VEGF and CD31 in ICH rats injected with pcDNA-TUG1. Original magnification, × 400. ***P < 0.001, *vs.* sham; &&P < 0.01, *vs.* mimic-NC; ###P < 0.001, *vs.* pcDNA-NC.

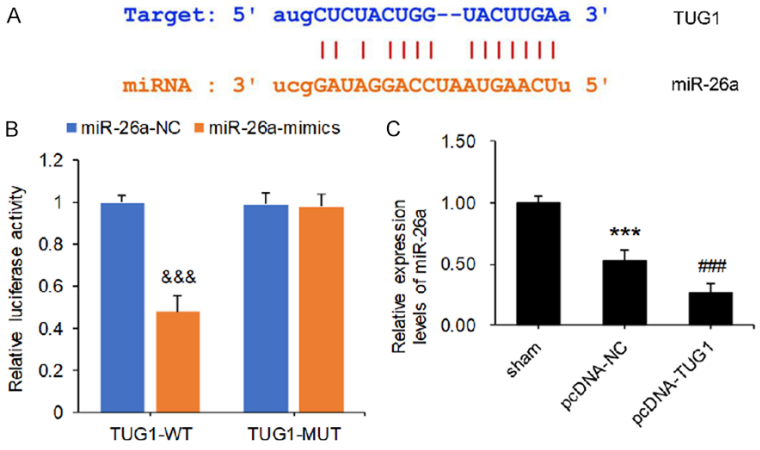

Figure 4. TUG1 directly interacts with miR-26a. A. The miR-26a-binding site on TUG1 was analyzed. B. The WT-TUG1 or MUT-TUG1 luciferase reporter activities were analyzed. C. miR-26a expression in ICH rats treated with pcDNA-TUG1. &&&P < 0.001, *vs.* mimic-NC; ***P < 0.001, *vs.* sham; ###P < 0.001, *vs.* pcDNA-NC.

by inhibiting the inflammatory response of microglia [28], suggesting that miR-26a could protect neurons after ICH in rats. However, it is unknown what effect miR-26a has on angiogenesis in ICH rats. In the current study, we demonstrated that miR-26a contributed to the recovery of neurologic function and the angiogenesis of brain tissue in rats with ICH.

LncRNAs participate in a diversity of physiological and pathophysiological processes by competitively binding to miRNAs [29]. Moreover, it has been demonstrated that lncRNA, microRNA, and circular RNA are associated with ICH [30]. In this study, we found that miR-26a was downregulated in ICH rats, in contrast to the increased expression of TUG1. Therefore, we explored a possible association between TUG1 and miR-26a. Our initial bioinformatics analysis predicted TUG1 could bind to miR-26a. We then experimentally confirmed the interaction between TUG1 and miR-26a by using

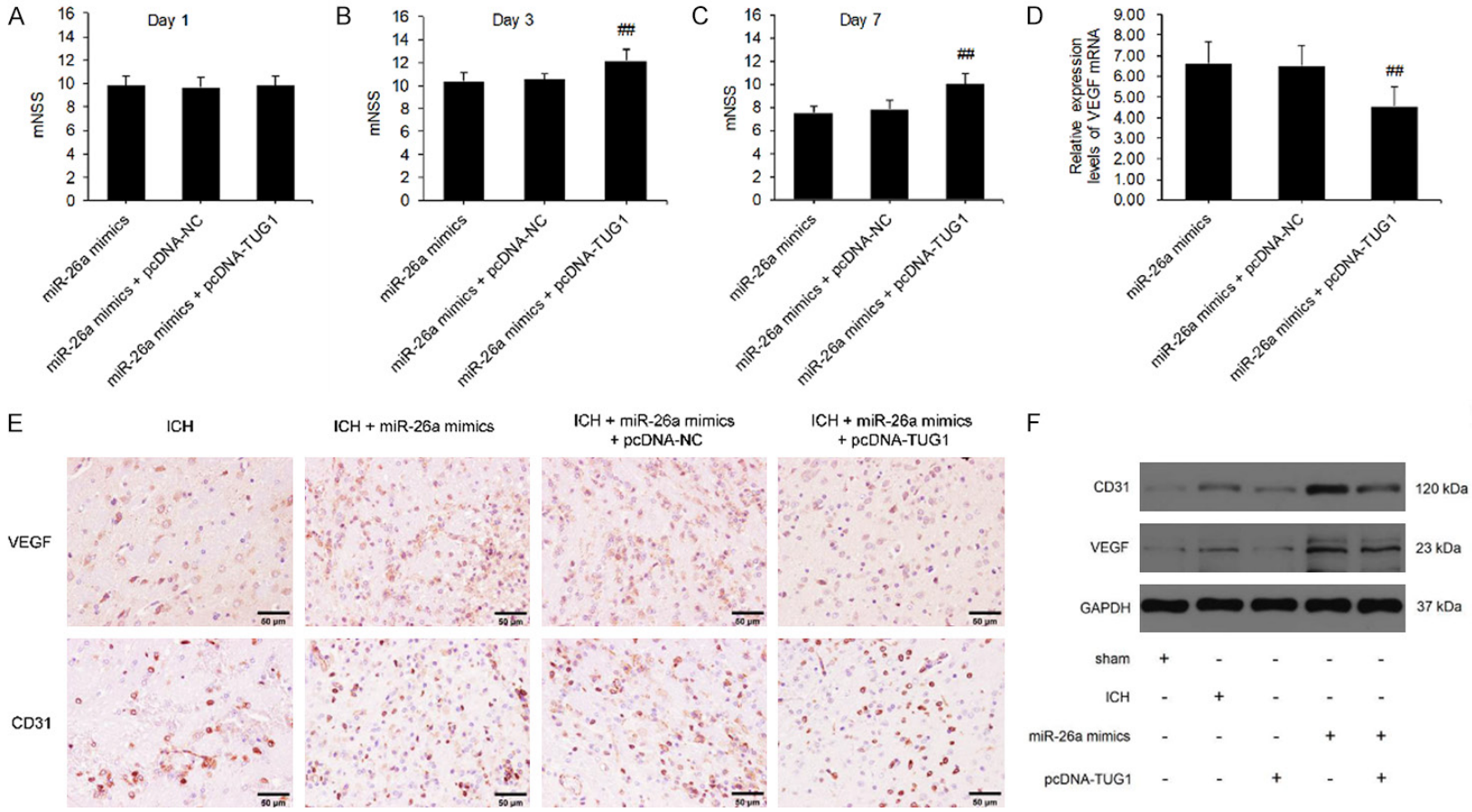

Figure 5. TUG1 overexpression abolished the roles of miR-26a in the neurobehavioral recovery and the cerebral angiogenesis of ICH rats. (A-C) The effects of TUG1 overexpression on mNSS in rats treated with miR-26a mimics on the 1st (A), 3rd (B) and 7th (C) day after ICH. (D) pcDNA-TUG1 attenuated the promoting effect of miR-26a on VEGF mRNA level. (E) The expression of VEGF and CD31 in ICH rats treated with miR-26a mimics + pcDNA-NC or miR-26a mimics + pcDNA-TUG1. Original magnification, × 400. (F) Western blot of VEGF and CD31 expression in different groups. ##P < 0.01, *vs.* pcDNA-NC.

luciferase reporter assay. We further demonstrated that the miR-26a level in ICH rats could be markedly downregulated by overexpressing TUG1. Moreover, overexpression of TUG1 could effectively antagonize the effect of miR-26a on accelerating the recovery of neurological function in ICH rats and abolish the promoting effect of miR-26a on the cerebral angiogenesis following ICH. These data suggest that overexpression of TUG1 inhibits angiogenesis after ICH through suppressing the expression of VEGF by targeting miR-26a in rats. Nevertheless, our study has certain limitations. First, our conclusions froman in vivo experimental model need to be validated with clinical samples. Second, the role and the mechanism of TUG1 in angiogenesis following ICH require further investigation with human tissue samples.

Conclusion

Our studies show that TUG1 can aggravate cerebral hemorrhage injury through suppressing angiogenesis in a miR-26a-dependent manner, indicating that lncRNA-TUG1 and miR-26a may serve as molecular targets in the treatment of ICH.

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Disclosure of conflict of interest

None.

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