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ORIGINAL PAPER

Knockdown of microRNA-17-5p Enhances the Neuroprotective Effect of Act A/Smads Signal Loop After Ischemic Injury

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Abstract

Cerebral ischemic injury is a leading cause of human mortality and disability, seriously threatening human health in the world. Activin A (Act A), as a well-known neuroprotective factor, could alleviate ischemic brain injury mainly through Act A/Smads signaling. In our previous study, a noncanonical Act A/Smads signal loop with self-amplifying property was found, which strengthened the neuroprotective effect of Act A. However, this neuroprotective effect was limited due to the self-limiting behavior mediated by Smad anchor for receptor activation (SARA) protein. It was reported that microRNA-17-5p (miR-17-5p) could suppress the expression of SARA in esophageal squamous cell carcinoma. Thus we proposed that knockdown of miR-17-5p could strengthen the neuroprotective effect of Act A/Smads signal loop through SARA. To testify this hypothesis, oxygen–glucose deficiency (OGD) was introduced to highly differentiated *rattus* pheochromocytoma (PC12) cells. After the transfection of miR-17-5p mimic or inhibitor, the activity of Act A signal loop was quantified by the expression of phosphorylated Smad3. The results showed that suppression of miR-17-5p up-regulated the expression of SARA protein, which prolonged and strengthened the activity of Act A signaling through increased phosphorylation of downstream Smad3 and accumulation of Act A ligand. Further luciferase assay confirmed that SARA was a direct target gene of miR-17-5p. These practical discoveries will bring new insight on the endogenous neuroprotective effects of Act A signal loop by interfering a novel target: miR-17-5p.

Keywords Ischemic injury · MiR-17-5p · Smad anchor for receptor activation · Act A/Smads signal loop

Introduction

Stroke is the second leading cause of death worldwide [1]. Ischemic stroke is its predominant type, yet clinical therapies for it remained elusive [2]. Cerebral blood vessel occlusion results in oxygen and glucose deprivation, which leads to rapid, irreversible necrotic loss of neurons in center and ischemic penumbra in the surrounding [3]. Due to limited blood flow, a serious of pathological process occurs in the penumbra cells promoting progressive cell death through apoptosis [4]. Meanwhile, multiple neuroprotective responses in the penumbra are activated to alleviate damage and promote recovery from ischemia [5, 6].

Among these endogenous signals, Activin A (Act A), has been found to play a positive role in neuroprotection. As an early gene that responded to cerebral ischemia, Act A activated Smad2 and Smad3 intracellular signals through transmembrane serine/threonine kinase receptors [7–10]. With the assistance of Smad anchor for receptor activation (SARA), Smad2/3 phosphorylated and assembled into a complex with Smad4. After translocating into the nucleus, Smads further affected target genes transcription. Thus, Act A can effectively convert transient biochemical signal to a relatively sustained Smads signal activation, which served as a transcription factor and regulated biological outcomes. In our previous study, a noncanonical Act A/Smads signal loop with self-amplifying property was found, which was initiated by the up-regulation of *INHBA* gene in response to

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ischemic injury and activated in extra Act A concentration dependent manner. After Act A/Smads signal activation, the excellular level of Act A accumulated rapidly due to the positive regulation effect of Smads complexes to *INHBA* gene, which further strengthened the neuroprotective effect of Act A. However, the activation stage of Act A signaling was short, limited by the down-regulation of SARA [11]. Thus the neuroprotective effect of Act A was finite to a limited period and strength.

MicroRNAs (miRNAs) are a class of small non-coding endogenous RNAs (~22 nt), that modulate gene expression at the post-transcriptional level by binding to the 3′-untranslated region (3′-UTR) of target messenger RNA (mRNA) [12]. They are highly conserved and able to regulate a wide range of biological processes including cell proliferation, differentiation, metabolism, and apoptosis [13]. It was reported that miR-17-5p could impede migration and invasion of esophageal squamous cell carcinoma (ESCC) via depressing the expression of SARA [14]. Thus, we supposed that suppression of miR-17-5p could strengthen the neuroprotective effect of Act A/Smads signal loop through increasing the level of SARA. To verify this hypothesis, oxygen–glucose deficiency (OGD) was introduced to highly differentiated *rattus* pheochromocytoma (PC12) cells to simulate the ischemic brain injury in vitro. Through the use of miR-17-5p mimic or inhibitor, we confirmed the regulation effects of miR-17-5p to SARA in Act A/Smads signal loop. Knockdown of miR-17-5p alleviated the OGD injury to cells through enhanced Act A signal activity, suggesting that miR-17-5p was a novel target to prolong and strengthen the neuroprotective effect of Act A/Smads signal loop.

Experimental Procedure

Cell Culture and the Establishment of OGD Model

The highly differentiated rat pheochromocytoma cell line (PC12 cells) (Cell Bank of the Chinese Academy of Sciences) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum(Biological Industries, Kibbutz Beithaemek, Israel) in a humidified environment of 5% CO_2 at 37 °C [15]. To induce OGD, PC12 cells were washed three times, cultured in Glucose free DMEM containing 10 mM $Na_2S_2O_4$, and then placed in a tri-gas incubator (HF100, China) aerated with an anaerobic gas mixture (5% CO_2 and 95% N_2) for different time (0, 1.5, 3, 6, 12 h) [16].

Transfection

MiR-17-5p mimic and mimic negative control (mimic NC), miR-17-5p inhibitor and inhibitor negative control (inhibitor NC) were purchased from RiboBio Co. Ltd. (Guangzhou, China). Cells cultured in complete medium without antibiotics at least twenty four hours prior to transfection. After washed with 1 × PBS (pH 7.4), cells were transiently transfected with mimic, mimic NC, inhibitor or inhibitor NC using riboFECT™ CP Transfection Kit (RiboBio, C10511-05, China), according to the manufacturer's instructions.

Real-Time RT–PCR Quantification

To detect miR-17-5p expression, total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacture's protocol. Then they were reverse transcribed to complementary DNA (cDNA) with the miDETECT A Track™ miRNA qRT-PCRStarter Kit(RiBoBio,C10712-1, China). qPCR was performed on the CFX96™ Real-Time System (Bio-Tek InstrumentInc, USA) using the miDETECT A Track™ miRNA qRT-PCR Starter Kit (RiBoBio, C10712-1,China). The primers for miR-17-5p (Reverse-transcribed primer: 5′-CTCAACTGGTGTCG TGGAGTCGGCAA TTCAGTTGAGCTACCTGC-3′, Forward primer: 5′-ACA CTCCAGCTGGGCAAAGTGCT TACAGTGC-3′, Reverse primer: 5′-TGGTGTCGTGGAGTCG-3′) and U6 (Reverse-transcribed primer 5′-AAC GCTTCACGAATTTGCGT-3′, Forward primer: 5′-CTCGCTTCGGCAGCACA-3′, Reverse primer: 5′-AACGC TTCACGAATTTGCGT-3′) were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). U6 was introduced as a control and each group was measured in triplicate by qRT-PCR. The relative mRNA expressions were calculated via relative quantification and analyzed by $2^{-\Delta\Delta CT}$ formula.

Western Blot

Total protein was extracted from cells using RIPA buffer (Beyotime Institute of Biotechnology, P0013B, China) with 1% phenylmethylsulfonyl fluoride (Beyotime, ST506, China) and 1% protein phosphatase inhibitor (CST,5870S,USA). The protein concentration was examined by a BCA Protein Assay Kit (Beyotime, P0010, China). Samples containing equal amounts of protein were processed for SDS–PAGE and immunoblotted as published. The following primary antibodies were used: Anti-SARA (180 kDa, abcam, ab124875, 1:10,000, USA), Anti-Smad3 (55 kDa, abcam, ab40854, 1:5000, USA), Anti-p-Smad3 (48 kDa, bioss, bs-3425R, 1:500, China), Anti-Act A (56 kDa, abcam, ab89307, 1:1000, USA), Anti-β-actin (42 kDa, abcam, ab8226, 1:2000, USA). Protein densitometry was normalized against β-actin and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell Counting Kit-8 Assay

Cell viability was measured by the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocols. Briefly, PC12 cells were plated at a density of 5×10^3cells/well in 96-well plates. 24 h after transfection, they were treated with OGD for 0, 3, 6 h. The media was replaced with 90 µl of normal medium and 10 µl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan). 2 h later, the optical density (OD) of each well was measured at 450 nm using a Universal Microplate reader (Bio-TEK Instrument. Inc, USA). The experiment was repeated three times. The percentage of survival cells in each group was calculated as follows: (A of OGD X h experimental group-A of blank control group)/(A of OGD 0 h group from the corresponding group-A of blank control group) × 100%.

Hoechst 33,342 Fluorescence Staining

Cells were seeded in the 24-well plates for 24 h, then transfected with mimic, mimic NC, inhibitor, or inhibitor NC respectively. 24 h later, they were explored to different time of OGD injury. Then 1 mg/ml Hoechst 33,342 (Invitrogen, Carlsbad, CA, USA) staining solution was introduced to each well for 30 min. After washed with PBS, cells were immediately photographed under a fluorescence microscope (Olympus 600 auto-biochemical analyzer, Japan).

Luciferase Assay

The 3′-UTRs of the predicted target genes forecasted by miRDB (https://www.mirdb.org/miRDB/index.html) and TargetScan (https://www.targetscan.org/mamm_31/) were amplified from rattus genomic DNA and individually inserted into the pmiR-RB-REPORT™ (Ribobio, China) using the XhoI and NotI sites. Similarly, the fragment of R-Zfyve9 3′-UTR mutant was inserted into the pmiR-RB-REPORT™ vector. Zfyve9 is known as SARA. The sequences (RiboBio, China) of the primers were available upon request. 250 ng per well of plasmid and 50 nM mimic were co-transfected into 293 T cells. 48 h after transfection, luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega, USA). Luminescence intensity was read with a Microplate Luminometer.

Statistical Analysis

Analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Student's *t*-test or ANOVA was employed to determine statistical significance as appropriate. A difference was considered statistically significant at $P < 0.05$.

Results

The Periodic Behavior of Act A/Smads Signal Loop After OGD Injury

To investigate the dynamic changes of Act A/Smads signal, different time of OGD treatment was introduced to PC12 cells. The expressions of Act A, SARA, total and phosphorylated Smad3 (p-Smad3) were detected by Western blot. As shown in Fig. 1a, b, the level of Act A protein increased by 37.06% and 86.47% in OGD 1.5 h and 3 h group, compared to that in OGD 0 h group. While it gradually reduced with the extending of OGD treatment. At OGD 12 h, the expression of Act A protein was almost at the same level as that in OGD 0 h. With the rise of Act A protein after OGD injury, Act A/Smads signal was activated. As shown in Fig. 1c, d, at OGD 3 h, the levels of total and phosphorylated Smad3 raised by 2.87, 2.93 times respectively, compared to that at OGD 0 h. However, the activation stage of Act A/Smads signaling was short. At OGD 6 h, the strength of Act A signaling dramatically attenuated, and vanished at OGD 12 h. Moreover, the expression of SARA also dynamically changed with OGD injury. As an essential phosphorylation assistor for Smad2/3 protein, it increased and promoted the activation of Act A/Smads signal at OGD 1.5 h and 3 h. While it damped rapidly after extending the OGD treatment to 6 h (Fig. 1e).

Dynamic Changes of miR-17-5p After OGD Injury

To detect the dynamic changes of miR-17-5p after OGD injury, RT-PCR was used with U6 as an endogenous control. The results showed that miR-17-5p expression increased to 200.56% after 1.5 h of OGD treatment, and peaked at OGD 3 h, which was over 10 times compared to that at OGD 0 h (Fig. 2). The up-regulation of miR-17-5p dramatically decreased at 6 h and 12 h. At OGD 6 h, it was only 1.12 times compared to that at OGD 0 h. Thus the expression changes of miR-17-5p concurred with the activity of Act A/Smads signaling after OGD injury.

Decreased SARA Expression by miR-17-5p

To explore the regulation effect of miR-17-5p to SARA, the levels of SARA protein were detected after 100 nM of miR-17-5p mimic transfection. As show in Fig. 3a, b, miR-17-5p mimic inhibited SARA protein by 52.18% after 24 h of transfection. In mimic group, the suppression effect of miR-17-5p mimic on SARA protein sustained after OGD injury. At OGD 3 h, the level of SARA protein decreased by 62.71% in mimic-OGD 3 h compared to that in mimic

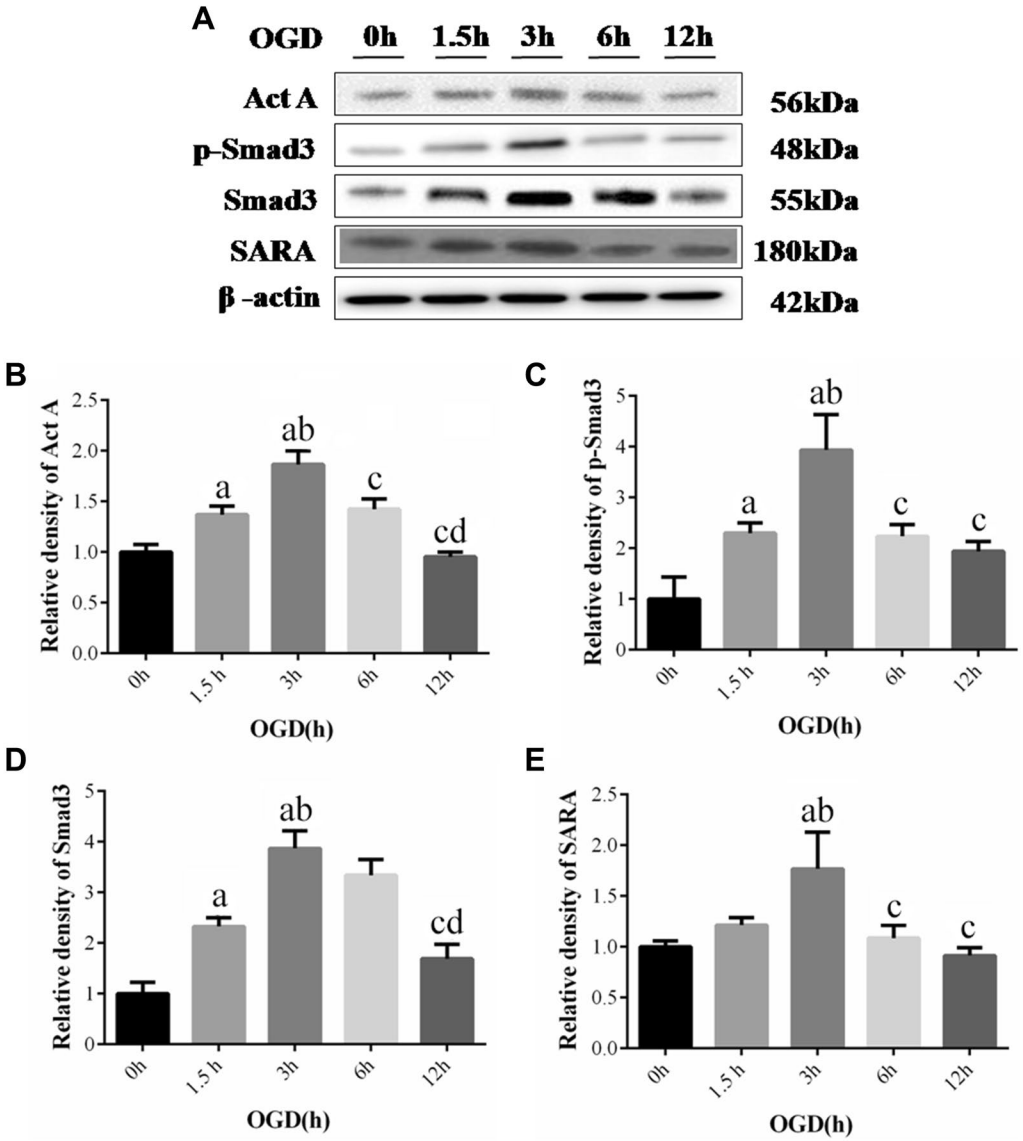

Fig. 1 Short-term Act A/Smads signal activation after OGD injury. **a** Protein levels of Act A, p-Smad3, Smad3 and SARA by western blot. To analyze loading, the same blot was stripped and reanalyzed with a probe for β-actin (lower panels). **b**–**e** Relative density of proteins. Bars indicated values which were the mean ± SEM from three independent experiments. ($^{a}P < 0.05$ vs OGD 0 h group, $^{b}P < 0.05$ vs OGD 1.5 h group, $^{c}P < 0.05$ vs OGD 3 h, $^{d}P < 0.05$ vs OGD 6 h)

NC-OGD 3 h. Then in OGD 6 h groups, it downed to 57.69% in mimic group compared with the corresponding mimic NC group. Thus 100 nM was taken as the effective dose of mimic in all the subsequent studies. Furthermore, the dosage of inhibitor was chosen as 200 nM, twofold of mimic, according to the directions. With the introduction of miR-17-5p inhibitor, the expression of SARA protein increased 94.63% at OGD 0 h compared to the corresponding inhibitor NC group (Fig. 4a, b). After 3 h of OGD injury, the level of SARA protein in inhibitor group increased by 117.18%, due to the suppression effect of inhibitor to miR-17-5p (Fig. 4a, b).

Regulation of Act A/Smads Signal Loop Through miR-17-5p

To explore the effect of miR-17-5p on Act A/Smads signaling, miR-17-5p mimic was first introduced to highly differentiated PC12 cells. The activity of Act A/Smads signal loop was measured through the expression of p-Smad3 protein. As shown in Fig. 3a, d, at OGD 0 h, the expression of p-Smad3 in mimic group decreased to 25.93% compared to that in mimic NC group. With the extension of OGD, it stained lower than that in the corresponding mimic NC groups. These results suggested the suppression effect of

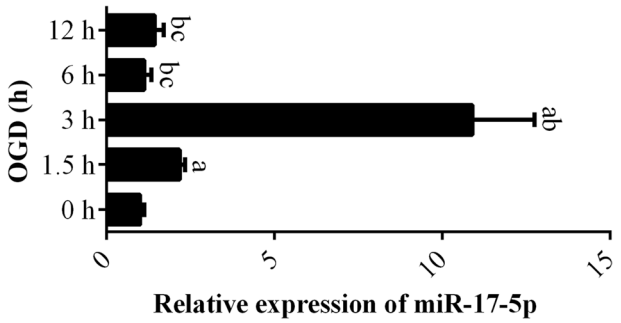

Fig. 2 The dynamic changes of miR-17-5p. Bars indicated values which were the mean ± SEM from three independent experiments. ([a]$P < 0.05$ vs OGD 0 h group, [b]$P < 0.05$ vs OGD 1.5 h group, [c]$P < 0.05$ vs OGD 3 h)

miR-17-5p to Act A signaling. Meanwhile, the general trend of total and phosphorylated Smad3 at different time of OGD treatment was independent of miR-17-5p mimic. Moreover, miR-17-5p mimic could decrease the expression of Act A protein. In the mimic group at OGD 0 h, the expression of Act A decreased by 35.68% in comparison with the corresponding mimic NC group. At OGD 3 h, it fell to 46.07% in the mimic group compared with the corresponding mimic NC group. At OGD 6 h, it decreased to 65.14% in the mimic group (Fig. 3a, c). Thus, through repression of SARA, miR-17-5p mimic could negatively regulate Act A/Smads signal.

Furthermore, miR-17-5p inhibitor was transfected to cells before OGD injury. With increased expression of SARA protein (Fig. 4a, b), the activity of Act A/Smads signaling was enhanced. As shown in Fig. 4a, d, e, the basal levels of total and phosphorylated Smad3 in miR-17-5p inhibitor group were higher than that in inhibitor NC group. After OGD 3 h, they increased to 116.71% and 149.79%, compared to that in the corresponding inhibitor NC group. At OGD 6 h, they were 118.70% and 230.16% comparing with the corresponding inhibitor NC groups. Meanwhile, the level of Act A protein also up-regulated due to miR-17-5p inhibitor. Before OGD exposure, the expression of Act A in the inhibitor group was 1.41 times of that in inhibitor NC group. With the activation of Act A signal, the level of Act A in inhibitor group increased at OGD 3 h, which was 1.20 times of that in the corresponding inhibitor NC group. Although the expression of Act A protein in inhibitor group began to reduce at OGD 6 h, it was still higher than that in the inhibitor NC group at OGD 3 h (Fig. 4c). These results indicated the positive effect of miR-17-5p inhibitor in prolonging and enhancing activity of Act A signaling.

Alleviated OGD Injury by miR-17-5p Inhibitor

To investigate the biological effect of miR-17-5p in OGD injury, PC12 cells were transfected with miR-17-5p mimic or inhibitor respectively. CCK-8 and Hoechst33342 staining were used after different time of OGD exposure. As shown in Fig. 5a, CCK-8 assay showed that cell viability decreased with the prolongation of OGD time. With miR-17-5p mimic treatment, it dramatically declined compared to that in the corresponding mimic NC group. At OGD3 h, it was 81.38% in the mimic group, 7.95% lower than that in the mimic NC group. Then it fell to 74.03% at OGD 6 h, significantly lower than that in the mimic NC group. On the other hand, miR-17-5p inhibitor enhanced the cell viability. The survival rates of cells in inhibitor group were dramatically higher than that in inhibitor NC group after 3 or 6 h of OGD exposure (Fig. 5b).

Hoechst33342 staining was used to observe the nuclear morphological changes. The blue-stained nucleus can be seen under the excitation of ultraviolet light. As shown in Fig. 5c, the nucleus of normal PC12 cells was round-shaped with homogeneous fluorescence intensity. After 3 h of OGD exposure, part of the nuclei were highlighted and compact. At OGD 6 h, the number of apoptotic nuclei increased, which was characterized by heterogeneous intensity, chromatin condensation and fragmentation. MiR-17-5p mimic enhanced the intensity of apoptosis after OGD treatment. As shown in Fig. 5c, the proportion of highlighted and compact nuclei in the mimic groups was significantly higher than that in the corresponding mimic NC group. While the classical apoptosis nuclei mitigated after miR-17-5p inhibitor introduction (Fig. 5c).

MiR-17-5p Yargets *rattus* Zfyve9

We then explored the underlying molecular mechanism behind miR-17-5p in Act A/Smads signaling after ischemic injury. With TargetScan and miRDB, one conserved and one poorly conserved miR-17-5p cognate sites were found in the 3′-UTR region of SARA mRNA. Zfyve9 is also known as SARA. Conserved sites 57–64 and poorly conserved sites 403–409 were cloned to the downstream of report gene hRluc in pmiR-RB-Report™ carrier (Fig. 6a), formed wide type luciferase reporter plasmid 1, named R-SARA-WT. Three mutated clones of binding sites were constructed, including Mut1 for nts 57–64, Mut2 for nts 403–409, and Mut1+Mut2 for both sites (Fig. 6b). The ability of miR-17-5p to inhibit expression of the adjacent hRluc coding region was quantified, after the luciferase reporter plasmid or a mutant reporter plasmids co-transfected with miR-17-5p mimic or mimic NC separately. The results showed that miR-17-5p mimic suppressed luciferase activity to approximately 28% when the reporter plasmid carried the wild type R-SARA-WT (Fig. 6c), and significant recoveries were observed when the reporter plasmid carried a mutant SARA 3′-UTR

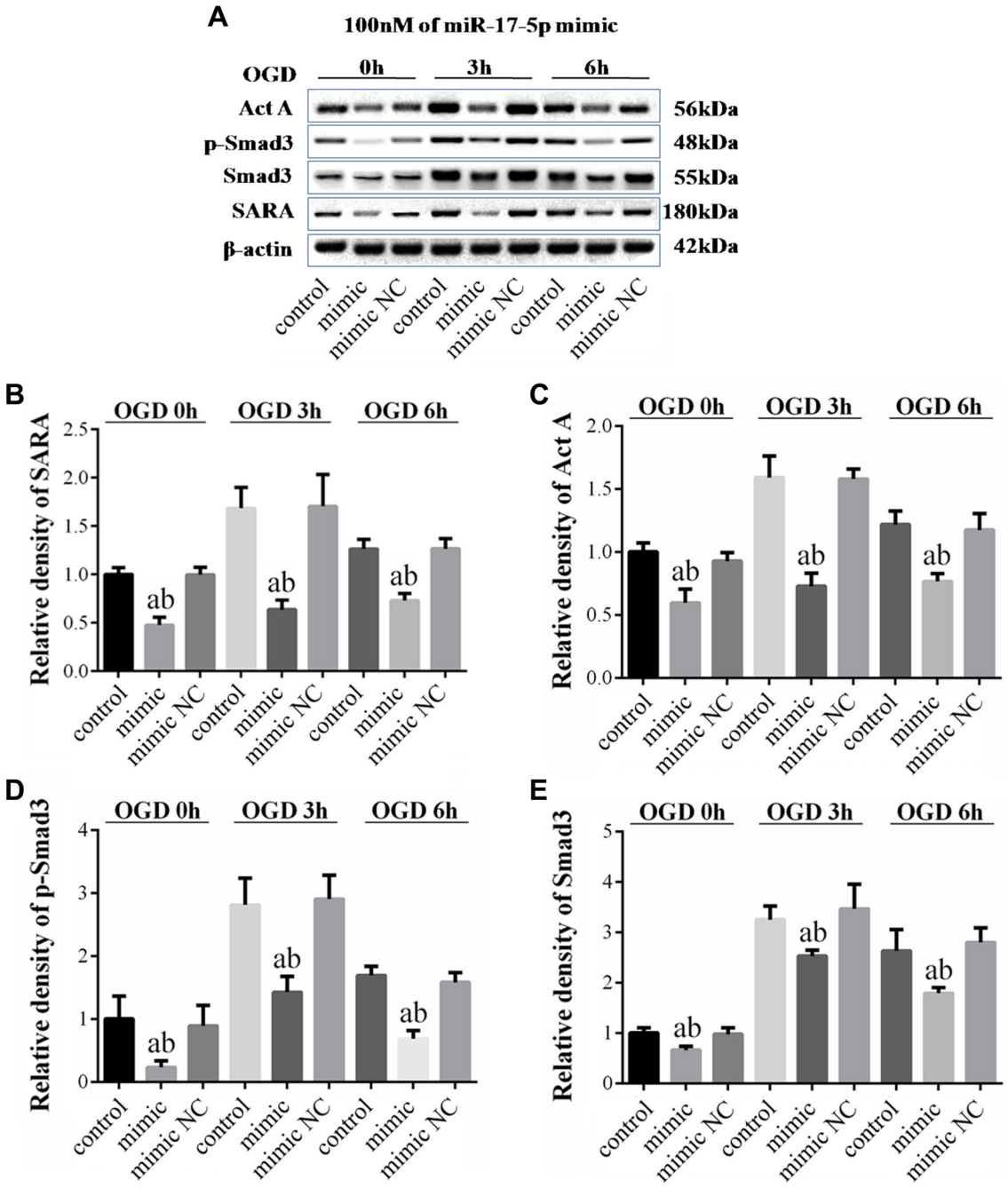

Fig. 3 Decreased activities of Act A/Smads signal loop after miR-17-5p mimic transfection. **a** Protein levels of Act A, p-Smad3, Smad3 and SARA by Western blot after 100 nM miR-17-5p mimic transfection. To analyze loading, the same blot was stripped and reanalyzed with a probe for β-actin (lower panels). **b–e** Relative density of proteins. Bars indicated values which were the mean ± SEM from three independent experiments. ([a]$P < 0.05$ vs corresponding control group, [b]$P < 0.05$ vs corresponding mimic NC group)

(pmiR-RB-REPORT™-R-SARA-mut1, 2, 1+2). These results suggested that miR-17-5p could directly bind to the predicted sites in the 3′-UTR region of SARA mRNA and negatively regulate SARA expression. Remarkably, the relative Rluc/Luc ratio of R-SARA-mut1+mut2 was obviously higher than either R-SARA-mut1 or R-SARA-mut2.

Discussion

Stroke is one of the leading causes of disability and mortality in the word [1]. Ischemic stroke caused by the occlusion of brain blood vessel induces pathophysiological cascades that lead to brain damage and corresponding neurological deficit [3]. Since neurons in ischemic penumbra remained

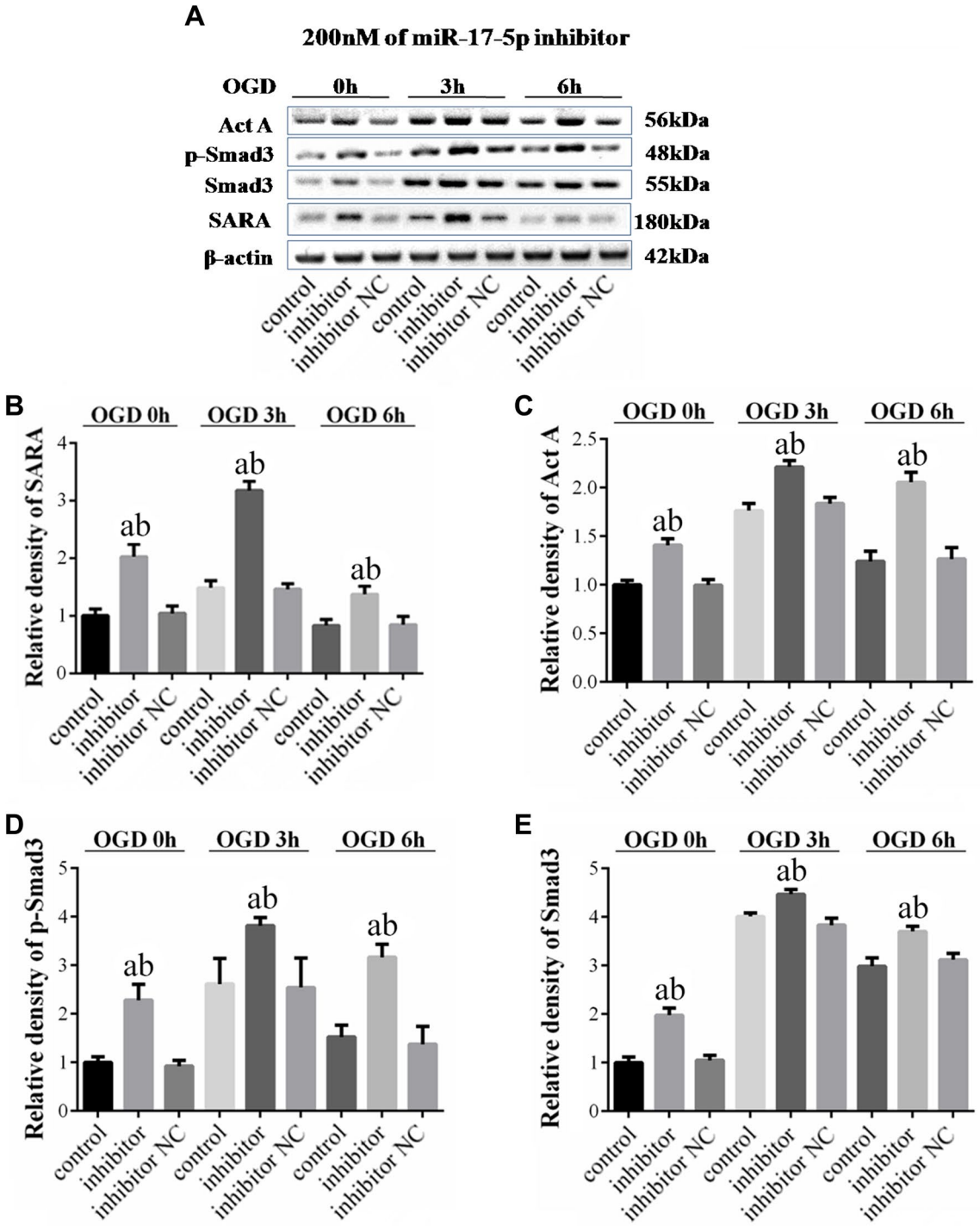

Fig. 4 Increased activities of Act A/Smads signal loop after miR-17-5p inhibitor transfection. **a** Protein levels of Act A, p-Smad3, Smad3 and SARA by Western blot after 200 nM miR-17-5p inhibitor transfection. To analyze loading, the same blot was stripped and reanalyzed with a probe for β-actin (lower panels). **b**–**e** Relative density of proteins. Bars indicated values which were the mean ± SEM from three independent experiments. ($^{a}P < 0.05$ vs corresponding control group, $^{b}P < 0.05$ vs corresponding inhibitor NC group)

metabolically active at the early time of ischemia. Surviving of them has been the main target for the current clinical therapy. Although thrombolytic therapy and endovascular intervention herald the spring of ischemic therapy [17], only a few of patients could benefit from those strategies due to the long delay from stroke onset to clinical intervention [18]. Thus strategies for extending the therapeutic time window for acute ischemic stroke have been explored all the time.

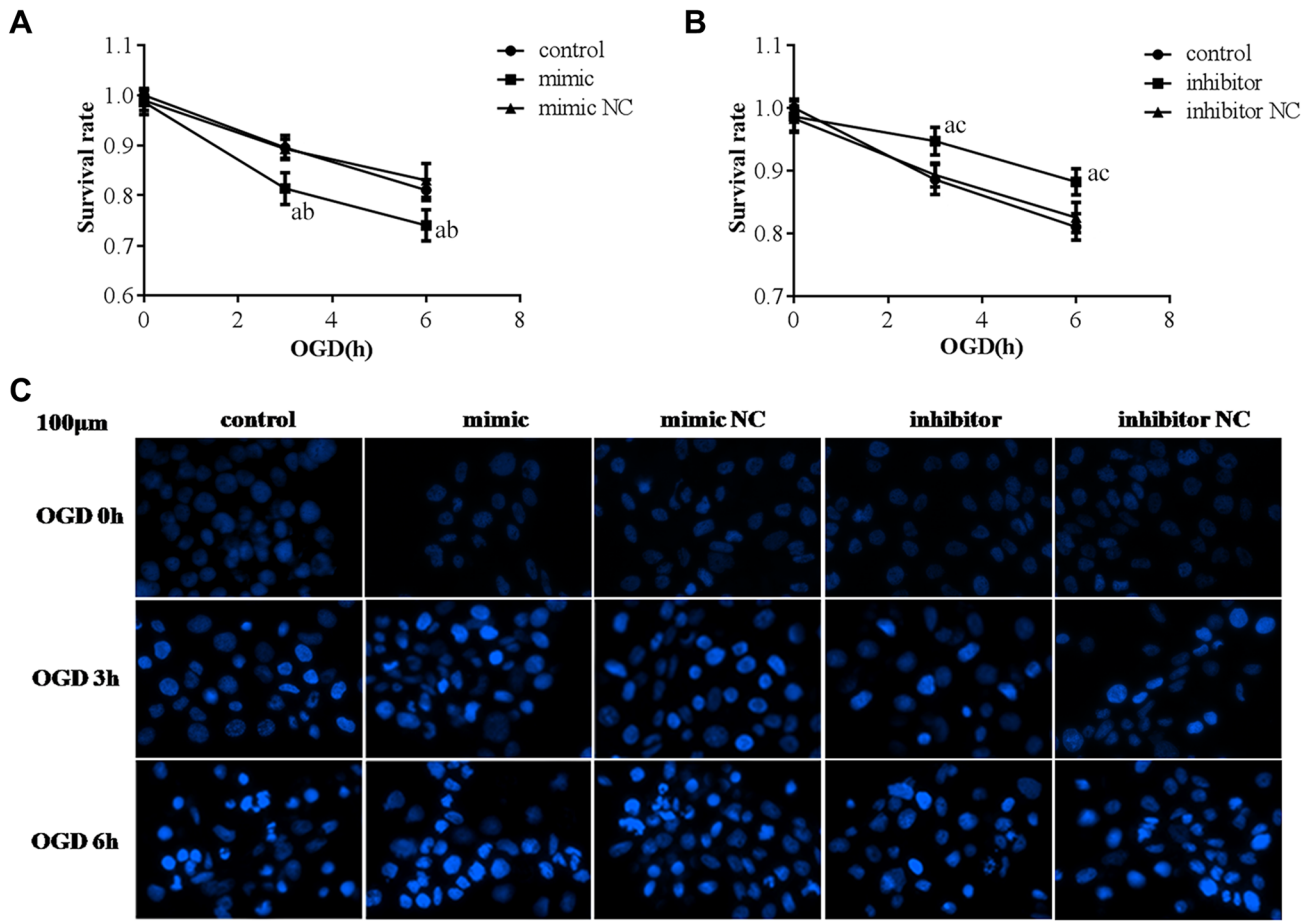

Fig. 5 Alleviated OGD injury by miR-17-5p inhibitor. The survival rates of cells after different time of OGD injury were measured following miR-17-5p mimic (**a**) or inhibitor (**b**) transfection. Bars indicated values which were the mean ± SEM from three independent experiments. ($^{a}P < 0.05$ vs corresponding control group, $^{b}P < 0.05$ vs corresponding mimic NC group, $^{c}P < 0.05$ vs corresponding inhibitor NC group). **c** The morphological features of apoptosis were monitored by fluorescence microscopy after staining with Hoechst 33,342. Scale bars: 100 μm

Neuroprotection, as a kind of traditional approach, could antagonize, interrupt, or slow the sequence of injurious biochemical and molecular events that eventuate in irreversible ischemic brain injury [12]. It was found that early initiation of neuroprotection could increase the number of patients who can be treated despite long transport time and to ameliorate the consequences of reperfusion injury [2, 19]. Even though emerging endogenous neuroprotection approaches have been found these years, the effect and duration of endogenous neuroprotection were limited, which cannot conceal the ischemic injury effectively. Under the circumstances, it is critical to explore the limitation of endogenous neuroprotection in strengthening the neuroprotection.

Act A is a well-known endogenous neuroprotective factor that participated in hypoxic–ischemic brain injury, neurodegenerative disorder, neuropsychiatric diseases and CNS demyelination [20–24]. Its receptor Acvr2 was reported to express in CC1+oligodendrocytes (Acvr2B+) and CD68+microglia/macrophages (Acvr2A+, Acvr2B+) [24]. A molecular mechanism for M2 microglia/macrophages driven oligodendrocyte differentiation was confirmed via secretion of Act A during remyelination [24]. Thus the wide expression of Act A and its receptors in CNS suggested the paracrine pattern of Act A signal. In our previous study, the autocrine pattern of Act A was illustrated through a noncanonical loop signaling, with self-amplifying property and spontaneous attenuation behavior [11]. As a rapid response factor, Act A could attenuate cerebral damage and promote neuronal survival through Act A/Smads signal [7, 21, 25–27]. Our further experiments suggested that the temporary activation of Act A/Smads signaling was closely associated with the down-regulation of SARA [11]. Therefore, a serious of experiments was performed in this study, in order to enhance the neuroprotective effect of Act A/Smads signaling. It was reported that miR-17-5p could impede migration and invasion of esophageal squamous cell

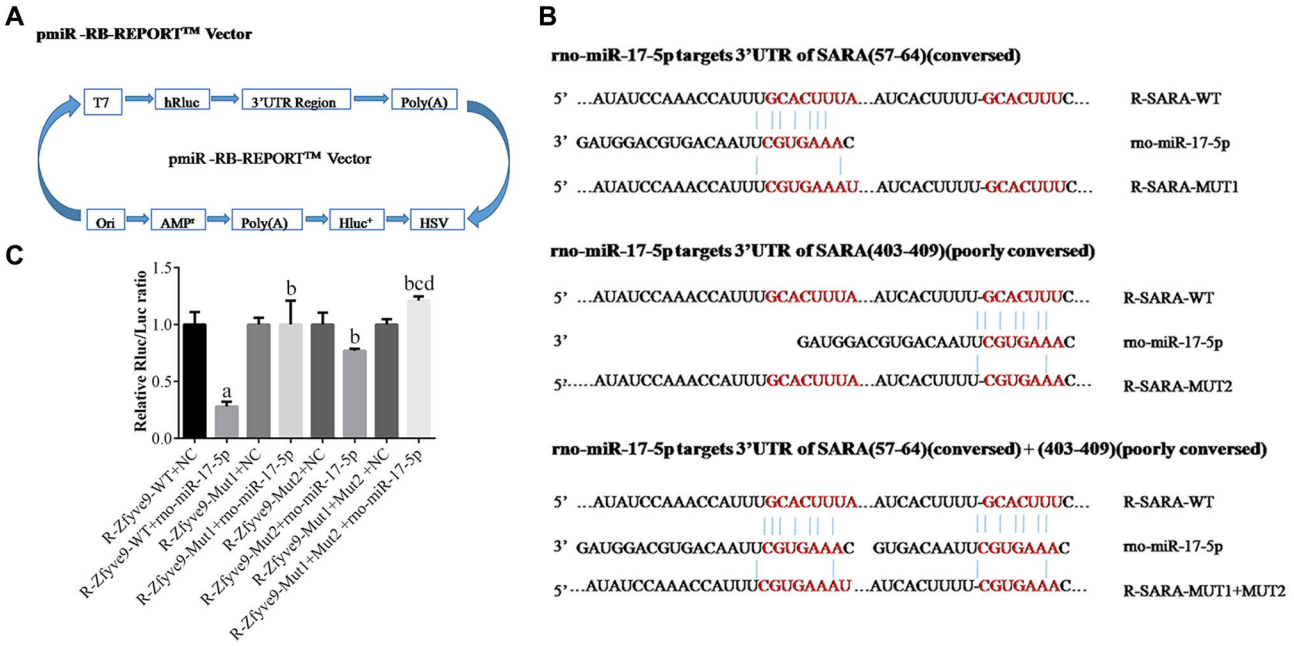

Fig. 6 SARA was target of miR-17-5p at specific 3′-UTR site. SARA also named Zfyve9. **a** pmiR-RB-REPORT™ dual-luciferase reporter vector. **b** The 3′-UTR of SARA harbors one miR-17-5p cognate site. **c** Relative luciferase activity of reporter plasmids carrying wild-type or mutant SARA 3′-UTR in 293 T cells co-transfected with NC or miR-17-5p mimic. ($^{a}P<0.05$ vs R-Zfyve9-WT+NC, $^{b}P<0.05$ vs R-Zfyve9-WT+rno-miR-17-5p, $^{c}P<0.05$ vs. R-Zfyve9-Mut1+rno-miR-17-5p, $^{d}P<0.05$ vs R-Zfyve9-Mut2+rno-miR-17-5p)

carcinoma (ESCC) via depressing the expression of SARA [14]. Thus, we supposed that suppression of miR-17-5p could strengthen the neuroprotective effect of Act A/Smads signal loop through increasing the level of SARA.

In fact, miR-17-5p has been found to participate in human ischemic stroke [28]. After cerebral ischemia, the serum level of miR-17-5p was up-regulated by 9.ninefold [28]. Multivariate logistic regression demonstrated that serum miR-17-5p level was strongly associated with acute ischemic stroke as a promising serum biomarker [28]. Knockdown of miR-17-5p ameliorated atherosclerotic lesions and restored the expression of very low density lipoprotein receptor [29]. Down-regulation of miR-17-5p could improve cardiac function after myocardial infarction [27]. However, the function and mechanism of miR-17-5p in Act A/Smads signal remains poorly understood. To explore this problem, miR-17-5p mimic was transfected into PC12 cells in this study. The expression of SARA was suppressed after 100 nM of miR-17-5p mimic transfection (Fig. 3b). Furthermore, the introduction of miR-17-5p inhibitor significantly increased the level of SARA protein before and after OGD injury, compared to the corresponding inhibitor NC group. All these results suggested that miR-17-5p could serve as a novel target in regulating the expression of SARA.

Since SARA could regulate the activity of Act A/Smads signal loop in ischemic injury [11, 30], the dynamic activity of Act A/Smads signal loop was detected after miR-17-5p mimic or inhibitor treatment. It was reflected by the level of p-Smad3 protein. In miR-17-5p mimic group, the levels of p-Smad3 protein decreased before and after OGD injury. Besides, the increased expression of Act A after OGD 3 h injury was also suppressed by miR-17-5p mimic. Thus, miR-17-5p mimic interfered the self-amplifying effect of Act A signal loop, as well as suppressed its signal activity. With miR-17-5p inhibitor transfection, the activity of Act A/Smads signal loop revised by the up-regulation of total and phosphorylated Smad3. Especially at OGD 6 h, the expressions of total and phosphorylated Smad3 were at the same level as that in inhibitor NC group at OGD 3 h, suggesting that miR-17-5p inhibitor could prolong the activation duration of Act A/Smads signaling. Moreover, the level of Act A before and after OGD injury also rose due to miR-17-5p inhibitor introduction. Therefore miR-17-5p inhibitor prolonged and strengthened Act A signal through increased downstream effector p-Smad3 and upstream ligand Act A, based on the self-amplified characteristic of Act A loop signaling. A graphic diagram was used to illustrate the direct and indirect regulation effect of miR-17-5p in Act A/Smads signal loop (Fig. 7).

Apoptosis is a main process after ischemic injury in penumbra. Hoechst33342 staining was used to observe the nuclear morphological changes. After OGD treatment, the number of apoptosis cells increased with condensed and fragmented nuclei. Meanwhile, the apoptotic morphology

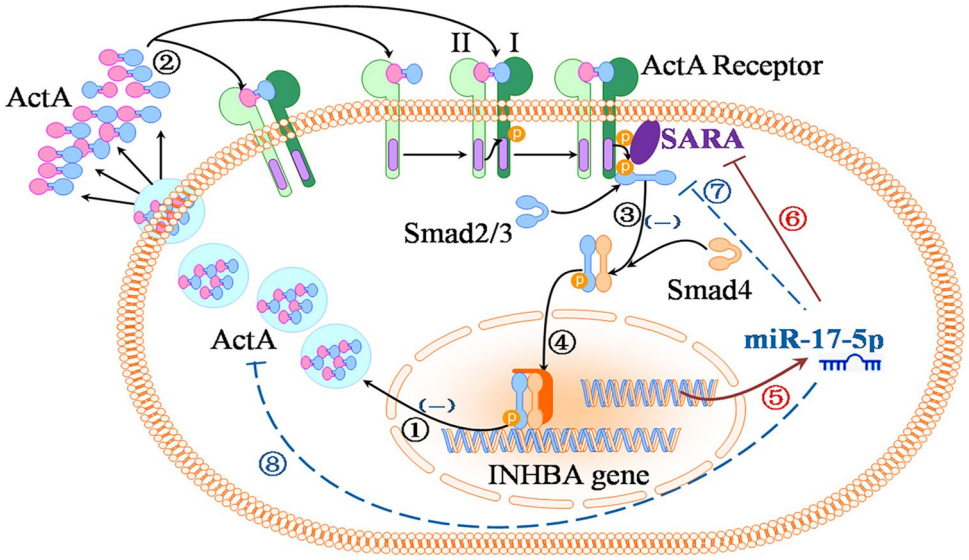

Fig. 7 MiR-17-5p regulated the activity of the Act A/Smads signal loop. ①–④ A self-amplifying feedback loop of Act A signaling: ① increased Act A gene (*INHBA*) expression in response to OGD injury. ② Accumulation of extracellular Act A protein. ③ Activation of Smads signal through R-Smads phosphorylation and heterodimer formation. ④ Enhanced transcription of *INHBA* gene after heterodimer translocation. ⑤–⑧ The negative regulation effect of miR-17-5p in Act A signaling: ⑤ OGD induced up-regulation of miR-17-5p. ⑥ The direct suppression of SARA protein by miR-17-5p. ⑦ The indirect down-regulation of p- Smad3 by miR-17-5p. ⑧ The indirect depression of Act A by miR-17-5p

due to OGD injury was alleviated with the introduction of miR-17-5p inhibitor. Moreover, with miR-17-5p inhibitor introduction, the cell mortality rate declined compared with that in mimic NC group at the same time of OGD exposure. The results revealed that suppression of miR-17-5p promoted the survival of cells in ischemia.

Since miR-17-5p mimic introduction could interfere in the expression of SARA protein, the underlying regulation mechanism was explored through luciferase assay. Wide type luciferase reporter plasmid R-SARA-WT was synthesized with one conversed site (57–64) and one poorly conversed site (403–409). Then three mutation plasmids carrying point mutations in the putative miR-17-5p binding sites R-SARA-mut1, R-SARA-mut2 and R-SARA-mut1+mut2 were designed. MiR-17-5p suppressed luciferase activity to approximately 28% when the reporter plasmid carried the wild type R-SARA-WT in 293 T cells. The fluorescence of mutant vectors showed obvious recovery. These results suggested that miR-17-5p could directly bind to the predicted sites in the 3′-UTR region of SARA mRNA and negatively regulate SARA expression. Interestingly, the relative Rluc/Luc ratio of R-SARA-mut1+mut2 reversed to 1.21, which is obviously higher than either R-SARA-mut1 or R-SARA-mut2. As expected, the combination of mutations disrupting both binding sites completely blocked the down-regulation effect of miR-17-5p mimic on luciferase activity. Taken together, MiR-17-5p could negatively regulate SARA expression by binding to the predicted 3′-UTR region of SARA mRNA.

Conclusions

In this study, we provided significant insight into miR-17-5p role in ischemic stroke. The investigation demonstrated that miR-17-5p was a novel target to prolong the strength of Act A/Smads signal loop and alleviate cell apoptosis in response to ischemic injury by targeting SARA. Our study suggested the potential for using miR-17-5p as a therapeutic target to enlarge the Act A signal loop effect of endogenous neuroprotection.

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Compliance with Ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Feigin VL, Norrving B, Mensah GA (2017) Global burden of stroke. Circ Res 120(3):439–448
2. Catanese L, Tarsia J, Fisher M (2017) Acute ischemic stroke therapy overview. Circ Res 120(3):541–558
3. Doyle KP, Simon RP, Stenzel-Poore MP (2008) Mechanisms of ischemic brain damage. Neuropharmacology 55(3):310–318
4. Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22(9):391–397
5. Namura S, Ooboshi H, Liu J, Yenari MA (2013) Neuroprotection after cerebral ischemia. Ann N Y Acad Sci 1278:25–32
6. Liu R, Yuan H, Yuan F, Yang SH (2012) Neuroprotection targeting ischemic penumbra and beyond for the treatment of ischemic stroke. Neurol Res 34(4):331–337
7. Mukerji SS, Katsman EA, Wilber C, Haner NA, Selman WR, Hall AK (2007) Activin is a neuronal survival factor that is rapidly increased after transient cerebral ischemia and hypoxia in mice. J Cereb Blood Flow Metab 27(6):1161–1172
8. Schubert D, Kimura H, Lacorbiere M, Vaughan J, Karr D, Fischer WH (1990) Activin is a nerve cell survival molecule. Nature 344(6269):868–870
9. Mukerji SS, Rainey RN, Rhodes JL, Hall AK (2009) Delayed activin A administration attenuates tissue death after transient focal cerebral ischemia and is associated with decreased stress-responsive kinase activation. J Neurochem 111(5):1138–1148
10. Mang J, Mei CL, Wang JQ, Li ZS, Chu TT, He JT, Xu ZX (2013) Endogenous protection derived from activin A/Smads transduction loop stimulated via ischemic injury in PC12 cells. Molecules 18(10):12977–12986
11. Wang JQ, Liang WZ, Cui Y, He JT, Liu HY, Wang Y, Xue LX, Ji QY, Shi W, Shao YK, Mang J, Xu ZX (2016) Noncanonical activin A signaling in PC12 cells: a self-limiting feedback loop. Neurochem Res 41(5):1073–1084
12. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294(5543):853–858
13. He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5(7):522–531
14. Jing C, Ma G, Li X, Wu X, Huang F, Liu K, Liu Z (2016) MicroRNA-17/20a impedes migration and invasion via TGF-β/ITGB6 pathway in esophageal squamous cell carcinoma. Am J Cancer Res 6(7):1549–1562
15. Li J, Zhang S, Liu X, Han D, Xu J, Ma Y (2018) Neuroprotective effects of leonurine against oxygen-glucose deprivation by targeting Cx36/CaMKII in PC12 cells. PLoS ONE 13(7):e0200705
16. Li CT, Zhang WP, Lu YB, Fang SH, Yuan YM, Qi LL, Zhang LH, Huang XJ, Zhang L, Chen Z, Wei EQ (2009) Oxygen-glucose deprivation activates 5-lipoxygenase mediated by oxidative stress through the p38 mitogen-activated protein kinase pathway in PC12 cells. J Neurosci Res 87(4):991–1001
17. Sheth KN, Smith EE, Grau-Sepulveda MV, Kleindorfer D, Fonarow GC, Schwamm LH (2015) Drip and ship thrombolytic therapy for acute ischemic stroke: use, temporal trends, and outcomes. Stroke 46(3):732–739
18. Alkhalili K, Chalouhi N, Tjoumakaris S, Hasan D, Starke RM, Zanaty M, Rosenwasser RH, Jabbour P (2014) Endovascular intervention for acute ischemic stroke in light of recent trials. Sci World J 2014:429549
19. Fisher M, Saver JL (2015) Future directions of acute ischaemic stroke therapy. Lancet Neurol 14(7):758–767
20. Hughes PE, Alexi T, Williams CE, Clark RG, Gluckman PD (1999) Administration of recombinant human Activin-A has powerful neurotrophic effects on select striatal phenotypes in the quinolinic acid lesion model of Huntington's disease. Neuroscience 92(1):197–209
21. Wu DD, Lai M, Hughes PE, Sirimanne E, Gluckman PD, Williams CE (1999) Expression of the activin axis and neuronal rescue effects of recombinant activin A following hypoxic-ischemic brain injury in the infant rat. Brain Res 835(2):369–378
22. Krieglstein K, Zheng F, Unsicker K, Alzheimer C (2011) More than being protective: functional roles for TGF-β/activin signaling pathways at central synapses. Trends Neurosci 34(8):421–429
23. Hasegawa Y, Mukai H, Asashima M, Hojo Y, Ikeda M, Komatsuzaki Y, Ooishi Y, Kawato S (2014) Acute modulation of synaptic plasticity of pyramidal neurons by activin in adult hippocampus. Front Neural Circuits 8:56
24. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, van Wijngaarden P, Wagers AJ, Williams A, Franklin RJM, Ffrench-Constant C (2013) M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci 16(9):1211–1218
25. Dijkerman HC, Wood VA, Hewer RL (1996) Long-term outcome after discharge from a stroke rehabilitation unit. J R Coll Phys Lond 30(6):538–546
26. Tretter YP, Hertel M, Munz B, ten Bruggencate G, Werner S, Alzheimer C (2000) Induction of activin A is essential for the neuroprotective action of basic fibroblast growth factor in vivo. Nat Med 6(7):812–815
27. Yang S, Fan T, Hu Q, Xu W, Yang J, Xu C, Zhang B, Chen J, Jiang H (2018) Downregulation of microRNA-17-5p improves cardiac function after myocardial infarction via attenuation of apoptosis in endothelial cells. Mol Genet Genom 293(4):883–894
28. Wu J, Du K, Lu X (2015) Elevated expressions of serum miR-15a, miR-16, and miR-17-5p are associated with acute ischemic stroke. Int J Clin Exp Med 8(11):21071–21079
29. Tan L, Meng L, Shi X, Yu B (2017) Knockdown of microRNA-17-5p ameliorates atherosclerotic lesions in $ApoE^{-/-}$ mice and restores the expression of very low density lipoprotein receptor. Biotechnol Lett 39(7):967–976
30. Wang JQ, He JT, Du ZW, Li ZS, Liu YF, Mang J, Xu ZX (2013) Effects of SARA on oxygen-glucose deprivation in PC12 cell line. Neurochem Res 38(5):961–971