European Review for Medical and Pharmacological Sciences 2018; 22: 5270-5277

High expression of GAS5 promotes neuronal death after cerebral infarction by regulating miR-365a-3p

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Abstract. – OBJECTIVE: To investigate whether growth arrest specific5 (GAS5) could regulate the expression of DCDC2 through competitive binding to miR-365a-3p, thus leading to increased neuronal death in cerebral cortex.

MATERIALS AND METHODS: The expression levels of GAS5 and DCDC2 in cerebral cortical neurons of mice with mouse middle cerebral artery occlusion (MCAO) cerebral infarction and in control mice were detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Meanwhile, the expression levels of GAS5 and DCDC2 in primary neurons cultured in vitro were also detected by qRT-PCR. The effects of GAS5 and DCDC2 on neuronal death were evaluated by calculating the cerebral infarct area by Tunel assay and TTC staining. Dual luciferase reporter assays were performed to detect the binding of miR-365a-3p to GAS5 and DCDC2. Western blot was applied to detect the protein expression of DCDC2, Bcl-2 and Bax after overexpression and knockdown of GAS5.

RESULTS: The expression of GAS5 and DCDC2 were significantly higher in cerebral cortical neurons and primary cultured neurons in vitro than those in control mice, respectively. Inhibition of GAS5 and DCDC2 in primary neurons decreased the neuronal cells death rate, while overexpression of GAS5 and DCDC2 increased the cell death rate. The dual luciferase reporter gene results showed that GAS5 regulated the expression of DCDC2 through competitive binding of miR-365a-3p thus forming a GAS5/miR-365a-3p/DCDC2 regulatory network. In addition, GAS5 inhibited Bcl-2 while promoting the expression of Bax.

CONCLUSIONS: High expression of GAS5 could promote neuronal death after cerebral infarction in mice, possibly through competitive binding to miR-365a-3p and promoting the expression of DCDC2.

Key Words:
GAS5, Cerebral infarction, Neuronal death, ceRNA.

Introduction

Acute cerebral infarction is a common clinical cerebrovascular disease. Atherosclerosis is the pathophysiological basis of the disease as atheromatous plaque rupture and intraarterial thrombosis can lead to blood flow disruption, cerebral ischemia and hypoxia injury[1]. Delayed neuronal death is closely related to cerebral ischemia and degenerative brain diseases. Apoptosis caused by acute hypoxic-ischemic brain damage is the main form of delayed neuronal death[2]. Bcl-2 is the most important apoptosis-inhibitory gene, while bax is the most important apoptosis-promoting gene. The ratio of Bcl-2/Bax affects the occurrence of apoptosis[3].

Non-coding RNA (ncRNA) includes microRNA (miRNA) and long noncoding RNA (lncRNA). miRNAs have been shown to play important roles in the pathogenesis of various inflammatory diseases, neoplastic diseases, cardiovascular and cerebrovascular diseases[4,5]. Long-chain non-coding RNAs are widely present in the nucleus and cytoplasm with a length greater than 200 nt[6]. LncRNAs play important roles in various biological processes, such as cell proliferation, cell cycle, differentiation, and apoptosis through various molecular mechanisms[7-9]. MALAT1 inhibits apoptosis in tumors such as pancreatic cancer and prostate cancer[10]. Decreased HOTAIR expression leads to cell cycle arrest in G0/G1 and *in situ* tumor growth inhibition[11]. LncRNA PVT1 oncogenes can promote cell proliferation, cell cycle progression, and stem cell performance[9]. In recent years, many studies have found that lncRNA can exert its biological function as competing endogenous RNA (ceRNA). CeRNAs, also known as miRNA "molecular sponges," have revealed a novel re-

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gulatory mechanism that long non-coding RNAs "absorb" miRNAs and affect miRNA downstream functions.

Growth arrest specific5 (GAS5) is a lncRNA which acts as tumor suppressor[12]. Studies have shown that GAS5 is lowly expressed in various tumor tissues, suggesting its role as tumor suppressor genes. Besides, overexpression of GAS5 can inhibit tumor growth, invasion and metastasis. Meanwhile, overexpression of GAS5 can induce cell apoptosis and increase the sensitivity of cells to chemotherapy drugs[13-15]. Scholars have also confirmed that high expression of GAS5 can lead to hypoxia/ischemic brain injury and apoptosis of primary hippocampal neurons[16]. However, the regulation mechanism of GAS5 in cerebral infarction remains to be studied. The primary purpose of this study was to investigate whether growth arrest specific5 (GAS5) could regulate the expression of DCDC2 through competitive binding to miR-365a-3p, thus leading to increased neuronal death in cerebral cortex.

Materials and Methods

Establishment of Rat MCAO Model

Animal experimental procedures were approved by the Second Affiliated Hospital of Dalian Medical University Ethics Committee. The mouse middle cerebral artery occlusion (MCAO) model was prepared according to a previous study[17]. The specific operations were as follows: after anesthesia, mice were bluntly dissected to the anterior cervical muscle. The common carotid artery (CCA) was isolated. Afterward the external carotid artery (ECA) was separated sequentially.

A line thread (head diameter 0. 23 mm, trunk diameter 0. 18 mm) was inserted from the external carotid artery hole and then through the common carotid artery to the internal carotid artery, then up to the middle cerebral artery (depth about 12 mm). After the thrombus was blocked for 1 h, blood flow from the common carotid artery to the internal carotid artery was restored. Intraoperative dressings were kept warm. Mice were postoperatively placed in cages with clean litter for free access to water and food. The digital table method was used to randomly divide the mice into the sham group and the MCAO model group. The sham-operation group was performed with the same surgical procedure except for thread placement.

Neuronal Culture and Neuronal Oxygen-Glucose Deprivation (OGD) Ischemia Model

Primary cultured cerebral cortical neurons were isolated from mouse pups within 24 hours of birth. The suckling mice were placed in PBS solution, then the whole brain was decapitated to separate neonatal cerebral cortex while the meninges and blood vessels were dissected. Tissues were digested with trypsin for 15 minutes and digested with Dulbecco's Modified Eagle Medium (DMEM) containing 10% horse serum and 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). After filtered with 200 mesh stainless steel cell strain, cells were planted on D-polylysine (0.1 g/L) pretreated Petri dishes. The cells were cultured in normoxic environment (5% CO_2, 95% O_2) in a 37°C humidity incubator for 4 to 6 hours and then cultured with neurobasal medium containing 2% B27 and 2 mmol/L L-Glutamine. The medium was replaced every 3 days. After maturation, cells were cultured in glucose-free DMEM medium and placed in a hypoxic incubator with mixed gas (5% CO_2, 5% O_2, and 90% N2). After 5 hours, the sugarless medium was changed back into the neurobasal medium. Cells were continuously cultured in a normoxic incubator for 24 hours.

Neurological Function Score

Animals were trained for 3 consecutive days prior to surgery, and scores were taken as the baseline one day before injury. Then neurological function evaluations of each mouse were performed at 1, 3, 7, 14, 21, and 28 days after reperfusion of MCAO respectively according to the modified neurological score[18]. Neurological defect was graded on a scale of 0-15 (0=no neurobehavioral abnormalities; 15=neuron death).

Calculation of the Volume of Cerebral Infarction by TTC Staining

After neurobehavioral and motor function tests, mice were sacrificed and the brains were rapidly decapitated. Brain slices of approximately 1.5 mm in thickness were stained with 2,3,5-triphenyl tetrazolium chloride (TCC) (Sigma-Aldrich, St. Louis, MO, USA). After TCC staining, the brain slices were immersed in 4% neutral paraformaldehyde for preservation. After photographing, the brain infarct contours were outlined using Ulead Photo Express 2.0 image analysis software. Injury measurement software was used to measure cerebral ischemic area and the infarct volume was calculated for each brain.

RNA Isolation and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) using an RNA extraction kit. A reverse transcription reaction was performed using the PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan). The miRNA quantitative PCR procedure was performed according to the miScript SYBR Green PCR Kit instructions. The sequences of the primers are listed below: GAS5 (F: 5'-CTTGCCTGGACCAGCTTAAT-3', R: 5'-CAAGCCGACTCTCCATACCT-3'), miR-365a-3p (F: 5'-ACACTCCAGCTGGGTCCGAGCCTGGGTCTC-3', R: 5'-TGGTGTCGTGGAGTCG-3'), GAPDH (F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'), DCDC2 (F: 5'-TGGTGTATCGAAATGGGGATGC-3', R: 5'-GCTGATGTCACCTCATAAAGCA -3') .

Infection and Transfection of Cells

Recombinant lentiviral virus and control vectors were constructed by GenePharma (Shanghai, China). The neurons were digested and seeded in a 24-well plate (5 x 10^5 cells per well). Cells were infected by lentivirus in a multiplicity of infection (MOI) value of 40. Polybrene agent was added to enhance the infection rate in the ratio of 1:200. After 24 hours, the virus-containing medium was removed and replaced with fresh culture medium. For transfection, cells were transfected with miR-365a-3p mimics and inhibitor as well as their respective controls according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Dual Luciferase Reporter Gene Assay

The 3'UTR sequences of GAS5 and DCDC2 were downloaded from the NCBI website to construct GAS5 and DCDC2 wild-type sequences, GAS5 WT 3' UTR and DCDC2 WT 3' UTR as well as their mutant sequences. The cells were then plated in 96-well plates. 50 pmol/L mir-365a-3p mimics or negative controls were co-transfected with the constructed GAS5 and DCDC2 wild-type or mutant plasmids (80 ng). After 48 hours of transfection, the dual luciferase reporter gene assay system was used to detect fluorescence intensity.

TUNEL Assay

48 hours after transfection cells were seeded to detect neuronal apoptosis. TUNEL staining was performed strictly in accordance with the procedures shown in the Kekele Apoptosis Detection Kit (Dojindo, Kumamoto, Japan). Those cells with brownish yellow granules under the light microscope were recognized as TUNEL-positive apoptotic cells.

Western Blot

Total proteins were extracted by radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China). Proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) while the immunoblots were incubated overnight at 4°C with the primary antibodies. After washed in Tris-buffered saline and Tween 20 (TBST) (Beyotime, Shanghai, China) for 3 times, the immunoblots were incubated with horseradish peroxidase (HRP) -labeled antibodies for 2 h at room temperature. Protein bands were determined by imaging analysis system with enhanced chemiluminescence (ECL) imaging (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. Measurement data were analyzed by *t*-test. Data were expressed as mean±standard deviation. The difference was statistically significant at $p<0.05$.

Results

GAS5 is Highly Expressed in Cerebral Infarction

We detected the expression level of GAS5 in cerebral cortical neurons of mice with MCAO cerebral infarction and control mice by qRT-PCR. The results of qRT-PCR showed that GAS5 was highly expressed in cerebral cortical neurons in MCAO cerebral infarction model (Figure 1A). Subsequently, we found that GAS5 was also highly expressed in the oxygen-glucose deprivation (OGD) model of primary neurons cultured *in vitro* (Figure 1B). Next, MCAO cerebral infarction mouse was injected with LV-shGAS5 and LV-GAS5 to knockdown or increase the expression of GAS5 by tail vein injection. Then the infarct volume of mice was calculated by TTC staining. The results showed that the infarct size decreased in the LV-shGAS5 group while the infarct size of LV-GAS5 increased (Figure 1C). Subsequently, the neurological function of each group of mice was evaluated by the foot fault test. The results

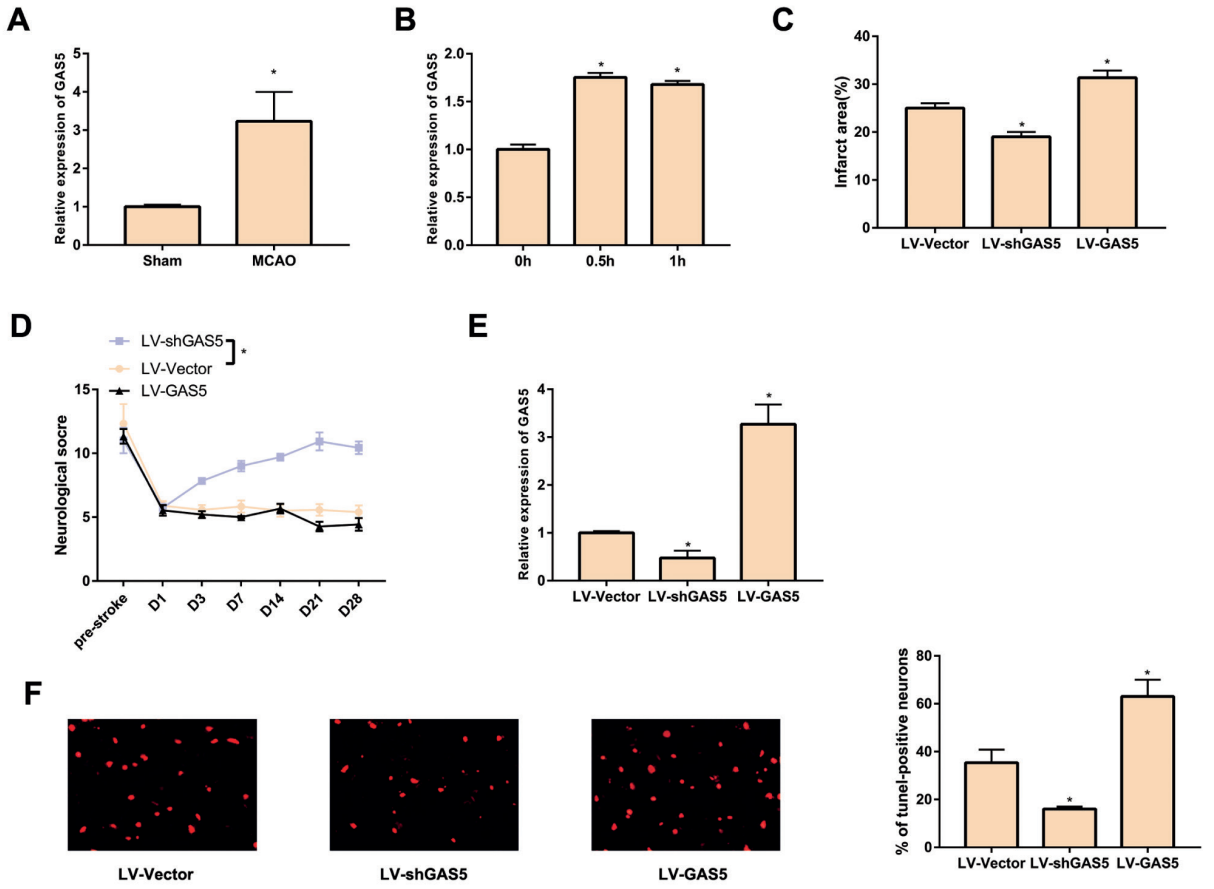

Figure 1. GAS5 is highly expressed in cerebral infarction brain. GAS5 is highly expressed in in cerebral cortical neurons of MCAO cerebral infarction mice model. GAS5 is highly expressed in the oxygen-glucose deprivation (OGD) model of primary cultured neurons *in vitro*. GAS5 expression in MCAO mice model by tail vein injection of LV-shGAS5 and LV-GAS5. Foot injury test evaluation of nerve function of mice was shown. GAS5 expression in primary neuronal cells transfected with LV-shGAS5 and LV-GAS5. Tunel assay in primary neuronal cells transfected with LV-Vector, LV-shGAS5 and LV-GAS5

showed that the LV-shGAS5 group scored higher than the LV-Vector group, while the LV-GAS5 group and the LV-Vector group had no significant difference (Figure 1D). Besides, the primary neurons cells were infected with LV-shGAS5 and LV-GAS5 respectively. It was found that LV-GAS5 infection significantly increased the expression of GAS5, while LV-shGAS5 infection significantly inhibited the expression of GAS5 (Figure 1E). Further analysis by Tunel assay confirmed the effect of GAS5 on neuronal apoptosis. As shown in Figure 1F, the apoptotic cells in the LV-shGAS5 group decreased compared with that in the LV-Vector group. Meanwhile, the apoptotic cells in the LV-GAS5 group increased (Figure 1F). These results demonstrated that GAS5 is highly expressed in cerebral infarction brain and that GAS5 is related to cell apoptosis.

GAS5 Regulates miR-365a-3p Expression

By bioinformatics prediction of miRNAs that could bind GAS5, we found that miR-365a-3p had the highest binding score. Reporter gene assay showed that after transfection of miR-365a-3p cells, the luciferase activity in the GAS5-WT 3'UTR group was decreased, while no difference was observed in the GAS5-MUT 3'UTR luciferase activity (Figure 2A). These results indicated that GAS5 could bind with miR-365a-3p. Then, we predicted the target gene of miR-365a-3p DCDC2 and performed functional analysis. After transfection of miR-365a-3p cells, the luciferase activity of the DCDC2-WT 3'UTR group was decreased, while the DCDC2-MUT 3'UTR luciferase activity was not significantly different (Figure 2B). These results indicated that DCDC2 may be the target gene of miR-365a-3p. Subse-

quently, we detected the expression of miR-365a in primary neuronal infected with LV-shGAS5 or LV-GAS5. We found that GAS5 knockdown elevated the expression of miR-365a-3p and GAS5 overexpression decreased the expression of miR-365a-3p (Figure 2C), which suggested that GAS5 could negatively regulate miR-365a-3p expression. In addition, the expression of DCDC2 was detected in primary neuronal cells transfected with miR-365a-3p mimics or miR-365a-3p inhibitor. It was found that the expression of DCDC2 in miR-365a-3p mimics group was decreased and that in miR-365a-3p inhibitor group was increased (Figure 2D), which indicated that DCDC2 is the downstream target gene of miR-365a-3p.

DCDC2 Promotes Neuronal Apoptosis

We detected the mRNA expression of DCDC2 in primary neuronal cells transfected with LV-shGAS5 or LV-GAS5 as well as their controls. Our results showed that the expression of DCDC2 in LV-shGAS5 group was decreased compared with LV-Vector group, while DCDC2 expression was increased in LV-GAS5 group (Figure 3A). The same result was proved by Western blot (Figure 3B). These results showed that GAS5 could positively regulate the expression of DCDC2. In addition, we detected the expression level of DCDC2 in cerebral cortical neurons of mice with MCAO cerebral infarction and control mice by quantitative real-time

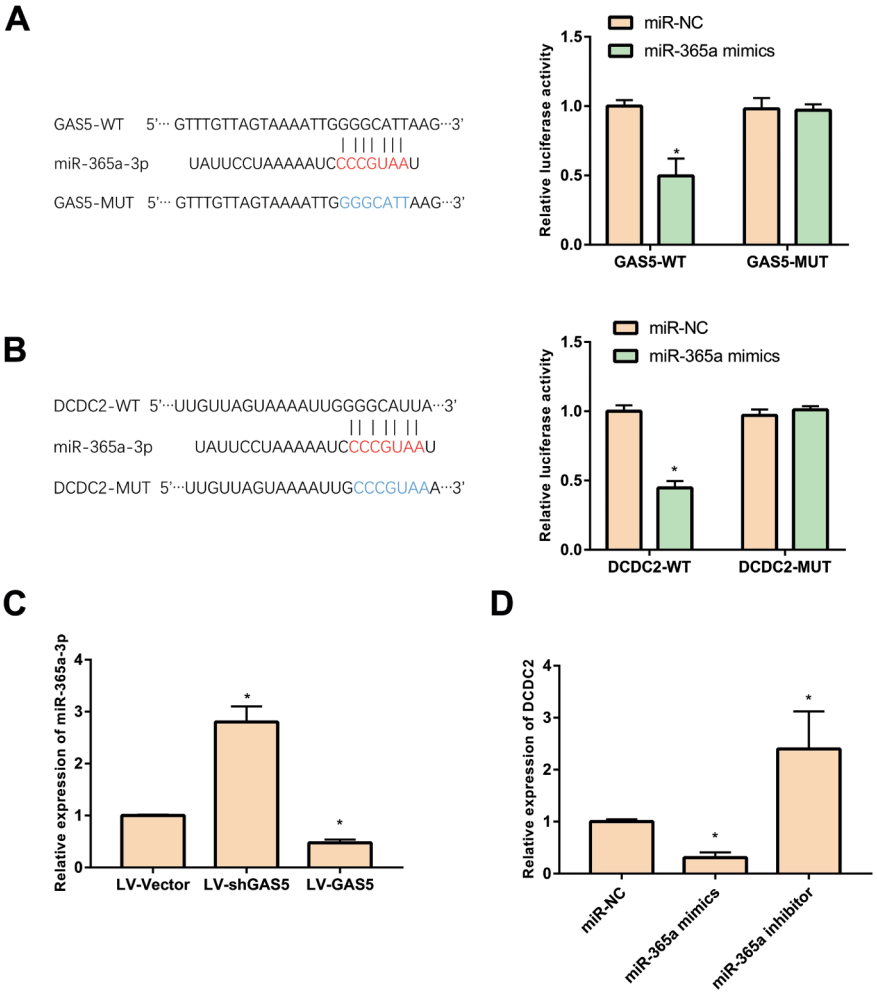

Figure 2. GAS5 regulates miR-365a-3p expression. Luciferase assay of cells co-transfected with GAS5-WT or GAS5-MUT reporter plasmids and miR-NC or miR-365a-3p mimics. Luciferase assay of cells co-transfected with DCDC2-WT or DCDC2-MUT reporter plasmids and miR-NC or miR-365a-3p mimics. miR-365a-3p expression after primary neuronal cells transfected with LV-Vector, LV-shGAS5, and LV-GAS5. DCDC2 expression was detected in primary neuronal cells after transfection with miR-NC, miR-365a-3p mimics, and miR-365a-3p inhibitor.

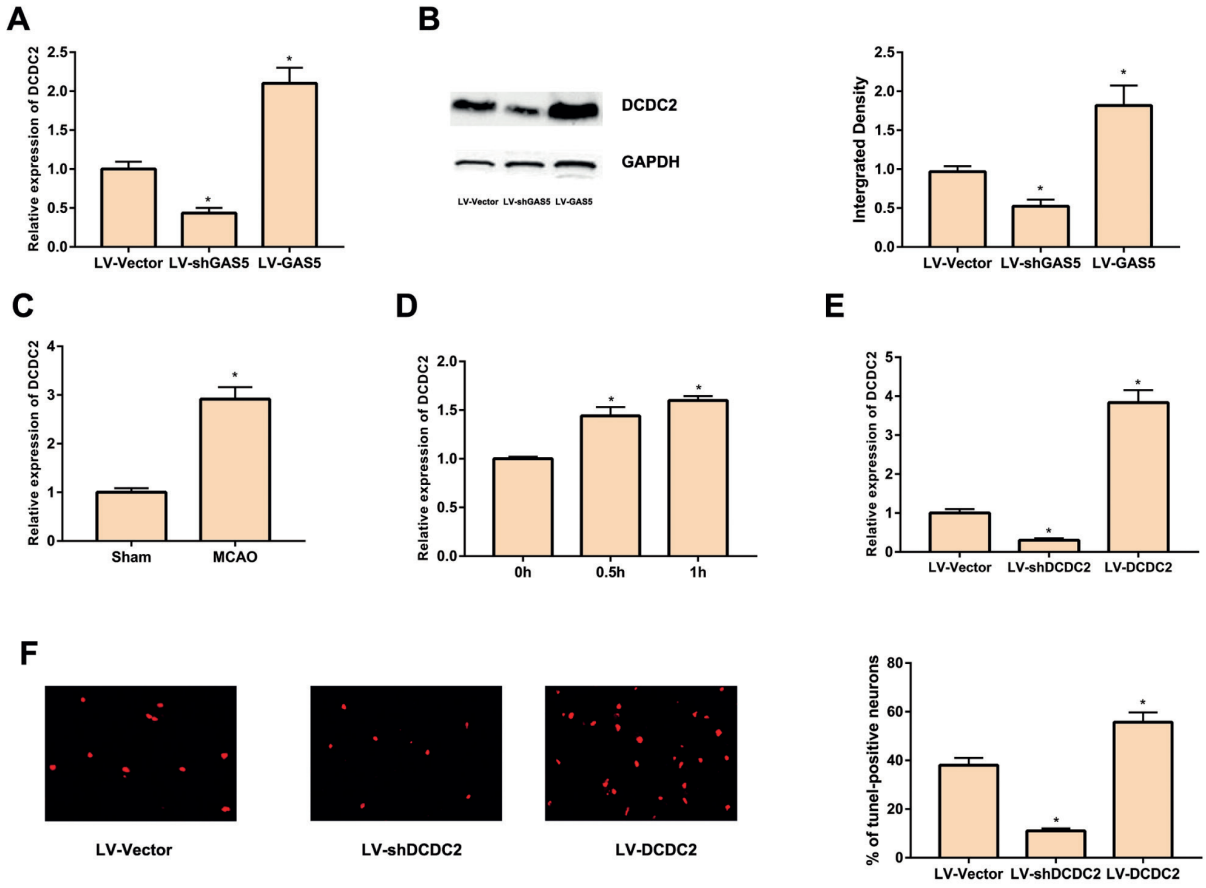

Figure 3. DCDC2 promotes neuronal apoptosis. DCDC2 expression was detected by qPCR in primary neuronal cells transfected with LV-Vector, LV-shGAS5, and LV-GAS5. DCDC2 protein expression was detected by Western blot in primary neuronal cells transfected with LV-Vector, LV-shGAS5, and LV-GAS5. DCDC2 was highly expressed in cerebral cortical neurons in a mouse model of MCAO with cerebral infarction. High expression of DCDC2 in oxygen-glucose deprivation (OGD) model of primary cultured neurons *in vitro*. DCDC2 expression was detected in primary neuronal cells transfected with LV-Vector, LV-shDCDC2, and LV-DCDC2. Tunel assay was performed to detect the cell apoptosis after transfection ofLV-shDCDC2 and LV-DCDC2.

PCR. The results showed that GAS5 was highly expressed in cerebral cortical neurons of mice models with MCAO cerebral infarction (Figure 3C). Subsequently, we found that DCDC2 was also highly expressed in the OGD model of primary neuronal cells cultured *in vitro* (Figure 3D). Then, the primary neuronal cells were transfected with LV-shDCDC2 or LV-DCDC2 to detect the expression of GAS5. Results showed that LV-shDCDC2 significantly inhibited the expression of DCDC2, while LV-DCDC2 significantly increased the expression of GAS5 (Figure 3E). Meanwhile, we examined the effect of DCDC2 on neuronal apoptosis by Tunel assay, which showed that the number of apoptotic cells in the LV-shDCDC2 group was decreased compared with that of the LV-Vector group, while the apoptotic cells in the LV-DCDC2 group were increased (Figure 3F). These above findings revealed that DCDC2 could promote neuronal apoptosis

GAS5 Regulates the Expression of Bcl-2 and Bax

Next, we investigated the effect of GAS5 on apoptosis-related proteins Bcl2 and Bax by LV-shGAS5 and LV-GAS5 infection of primary neurons cells. The results showed that LV-shGAS5 significantly inhibited the expression of pro-apoptosis protein Bax and promoted the expression of Bcl-2, while LV-GAS5 infection promoted the expression of Bax and inhibited Bcl2 expression (Figure 4), which suggested that GAS5 could regulate the expression of Bcl-2 and Bax.

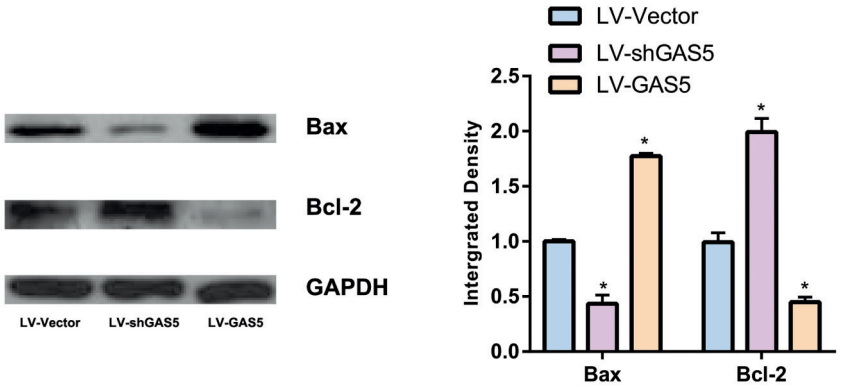

Figure 4. GAS5 promotes neuronal apoptosis. LV-Vector, LV-shGAS5 and LV-GAS5 were transfected into primary neurons to detect the expression of pro-apoptotic protein Bax and apoptosis-inhibiting protein Bcl-2

Discussion

Acute cerebral infarction usually occurs in middle-aged and elderly people. It is characterized by high incidence of morbidity, disability and mortality. Cerebral infarction causes brain damage due to ischemia and hypoxia. Long-term neurological impairment may lead to permanent deficits in brain function[19-20]. In addition, due to lack of adequate nutritional support, peripheral infarct dark neurons may eventually turn into apoptosis[21]. Therefore, how to save neurons and inhibit apoptosis affects the recovery of neurological function after cerebral infarction.

GAS5 is located on human chromosome 1q25.1 which consists of 4983 bp. Studies have found that the expression of GAS5 is decreased in many malignant tumors. Studies have also shown that overexpression of GAS5 in many cell lines could lead to tumor cell growth arrest through inducing apoptosis and regulating cell cycle. In recent years, many studies have found that lncRNAs can also interact with miRNAs and participate in the regulation of the expression of target genes, resulting in cell proliferation and withering. For example, in prostate cancer, lncRNA PTENP1 has been shown to absorb miR-19 and miR-20a, which can release their inhibition of PTEN and lead to the up-regulation of the well-known tumor suppressor gene PTEN. PTEN could inhibit the PI3K signaling pathway and inhibits cell growth in turn[22]. In addition, lncRNA HOTAIR targets miR-331-3p in the form of ceRNA in gastric cancer, thereby regulating the expression of the famous oncogene HER2[23]. In this study, GAS5 expression was significantly increased in cerebral cortical neurons of MCAO cerebral infarction mice model and primary neuronal cell OGD model cultured *in vitro*. High expression of GAS5 could promote neuronal death after cerebral infarction. The dual luciferase reporter gene results showed that GAS5 could target and bind to miR-365a-3p, thus regulating its expression, alleviating the degradation of DCDC2 by miR-365a-3p.

Apoptosis inhibitory protein Bcl-2 and apoptosis-promoting factor Bax are important members of apoptotic molecules family, which has been widely confirmed in regulating apoptosis of brain cells. The role of Bcl-2 and Bax has been widely confirmed and is one of the last common pathways of apoptosis[24,25]. DCDC2 is located at the 6p22.2 site, which was proved to be a strong candidate for dyslexia. Its expression product is a bicorticoid cytoplasmic protein that can promote interaction of neurons with cell membrane microtubules. Besides, it has been shown to promote the migration of neurons to the ventricle during embryonic development and the movement of neurons to the cerebral cortex during maturation. In this study, we found that DCDC2 expression was significantly increased in cerebral cortical neurons of mice models of MCAO and in primary neuronal cell OGD models. Overexpression of DCDC2 in primary neuronal cells promoted cell death. In addition, overexpression and knockdown of GAS5 showed that GAS5 could promote the apoptosis of primary neurons by inhibiting Bcl-2 and promoting Bax, thus participating in the development of cerebral infarction.

Conclusions

We showed that GAS5 levels are markedly increased in cerebral cortical neurons of MCAO mice models and OGD model. In addition, we

found that GAS5 could promote nerve cell death through the GAS5/miR-365a-3p/DCDC2 regulatory network.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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