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MicroRNA miR-505-5p Promotes Oxygen-Glucose Deprivation/Reoxygenation-Induced Neuronal Injury via Negative Regulation of CREG1 in Cultured Neuron-Like Cells

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Cerebral ischemia/reperfusion (I/R) injury is associated with various cardiovascular and cerebrovascular diseases with high disability, morbidity, and mortality rates. MicroRNAs (miRNAs) are related to the pathogenesis of the above diseases. MiR-505-5p, a kind of such miRNAs, was found to be highly expressed in cerebral I/R injuries, but the mechanism of action of this miRNA in cerebral I/R injury was unclear. In our study, up-regulation of miR-505-5p was detected in cultured oxygen-glucose deprivation/reoxygenation (OGD/R)-subjected PC12 and HEC293 cells. Silencing of miR-505-5p in OGD/R-affected neuron-like cells not only decreased the OGD/R-injury (according to cell viability, SOD increase, and LDH and MDA decreases), but also reduced apoptosis (decreasing the cleaved caspase-3 and PARP protein levels). Interestingly, expression of *CREG1* (Cellular Repressor of E1A-stimulated Genes 1) was low in OGD/R-subjected neurons; it was verified as a possible target gene of miR-505-5p. *CREG1* knockdown can reverse the effect of miR-505-5p silencing in OGD/R-induced neural injury. Taken together, the data obtained provide a new target for cerebral I/R injury treatment.

Keywords: oxygen-glucose deprivation/reoxygenation, PC12 and HEC293 cells, neuronal injury, microRNAs, miR-505-5p, *CREG1*.

INTRODUCTION

Cerebral ischemia/reperfusion (I/R)-related injury, as one of the most common types of stroke, is associated with various cardiovascular and cerebrovascular diseases [1]; the respective events (oxidative stress, neuronal apoptosis, and neurotoxicity) can lead to severe damage of the brain [2]. High disability, morbidity, and mortality rates are consequences of cerebral I/R injury [3, 4]. Recently, thrombolytic therapy is most frequently used to prevent such injuries [5]. However, the strategies for cerebral I/R injury treatment are not sufficiently effective. Oxidative stress has been the major cause of brain damage after cerebral I/R injury, resulting in a shortage of oxygen and glucose supply [6, 7]. Therefore, it is necessary to significantly improve our knowledge of oxygen-glucose deprivation/reoxygenation (OGD/R) induced by cerebral I/R injury.

MicroRNAs (miRNAs) containing approximately 22 nucleotides are small non-coding RNAs, which can negatively regulate mRNA translation or promote target RNA degradation [8, 9]. Abnormal miRNA expression has been reported to be related to the development of cardiovascular and cerebrovascular diseases [10]. For example, miR-125b inhibition was found to alleviate cerebral I/R injury through directly targeting CK2α/NADPH oxidase signaling [11]. Furthermore, previous studies showed that miR-193 can cause toxic aldehyde accumulation and tyrosine hydroxylase dysfunction via targeting *ALDH2* under I/R conditions [12]. Moreover, modulation of expression of miR-544 was reported to inhibit an inflammatory response and cell apoptosis through targeting *IRAK4* after cerebral I/R events [13]. Interestingly, a high miR-505-5p expression level was detected in cerebral I/R injury by GSE82146 analysis. On the one hand, Chung et al. [14] discovered that miR-

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505-5p enhanced cell death by targeting *PFKFB4* in glioblastoma cells. On the other hand, Ma et al. [15] proved that miR-505-5p decreased cell proliferation by regulating *FZD4* in cervical carcinoma. However, the function of miR-505-5p in cerebral I/R injury is still mostly unclear.

In our study, we examined the decrease of miR-505-5p in cultured neuron-like cells (PC12 and HEK293) subjected to OGD/R-like conditions. MiR-505-5p knockdown ameliorated OGD/R-induced neuronal injury and apoptosis. The *cellular repressor of E1A-stimulated genes* 1 (*CREG1*) was verified to be a target of miR-505-5p. We concluded that modulation of miR-505-5p can regulate OGD/R-induced neuronal injury through targeting the *CREG1*. This provides us with a new therapeutic strategy for OGD/R-induced neuronal injury.

METHODS

Cell Cultures and Transfection. PC12 cells and HEK293 cells were harvested from the Institute of Basic Medical Sciences of the Chinese Academy of Sciences. PC12 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml streptomycin, and 100 U/ml penicillin at 37°C in a 5% CO_2 incubator. HEK293 cells were grown in DMEM supplemented with 10% FBS. PC12 cells were then transfected with NC mimics, miR-505-5p mimics, and an NC inhibitor, or a miR-505-5p inhibitor via a Lipofectamine 2000 reagent (Invitrogen, USA).

Real-Time PCR Assay. Total RNA was extracted using TRIzol reagent. The cDNA was reverse-transcribed via a reverse transcription kit (Applied Biosystems, USA). Subsequently, PCR reactions were used to detect the miR-505-5p or *CREG1* levels by a Taqman Human MicroRNA Assays Kit (Applied Biosystems, USA). The relative mRNA level was analyzed with a $2^{-\Delta\Delta Cq}$ method; U6 or *GAPDH* was used as the loading control. In this study, the primers were as follows (Table 1):

Western Blotting. The control or OGD/R neurons were extracted via 1% RIPA lysis buffer. Protein quantitation was performed with a BCA kit (BioTeke, China). Proteins were then separated through 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, the PVDF membranes were incubated with primary antibodies, namely CREG1 (1:200, Santa Cruz Biotechnology, USA), cleaved caspase-3 (1:1000, Cell Signaling Technology, USA), cleaved PARP (1:1000, Santa Cruz Biotechnology, USA), and β-actin (1:10000, Santa Cruz Biotechnology, USA), overnight at 4°C. After washing, the PVDF membranes were incubated with the secondary antibody (1:5000, Santa Cruz Biotechnology, USA) at 37°C for over 1 h. Finally, the bands were captured and quantified.

Cell Viability Assay. The transfected cells (10^4 cells/well) were then seeded into 96-well plates and grown in a CO_2 incubator at 37°C. Subsequently, the cells from each well were supplemented with 10 µl CCK-8 solution for 4 h. The absorbance values were detected at 450 nm.

LDH Assay. Samples of the supernatant (50 µl) were taken from transfected cells and transferred into 96-well plates. The reaction mixture (combination of a matrix buffer and cell supernatant) were incubated at 37°C for 30 min. Subsequently, 50 µl of stop solution was added to the 96-well plate. The OD value was tested at 450 nm to estimate lactate dehydrogenase (LDH) activity.

MDA and SOD Assay. Proteins from the transfected cells were extracted. The protein content was quantified with a BCA protein assay kit. Then, the malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were examined by the respective assay kits.

T a b l e 1. Primers used.

Gene	Sequence (5'-3')
miR-505-5p	RT primer GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGAC ACATCAA
	Forward Primer GCGCGGGAGCCAGGAAG
	Reverse primer GTGCAGGGTCCGAGGT
CREG1	Forward Primer TGGATATTGCAAAGCATTCG
	Reverse primer TCTGGTGTCACGATTTTTGG
U6	Forward primer CTCGCTTCGGCAGCACATATAC
	Reverse primer AATATGGAACGCTTCACGAATTTG
GAPDH	Forward primer TGCACCACCAACTGCTTAGC
	Reverse primer GGCATGGACTGTGGTCATGAG

Apoptosis Assay. The cells were harvested and resuspended with 200 µl binding buffer. Subsequently, the cell suspension was supplemented with 5 µl propidium iodide (PI) and 5 µl Annexin-V fluorescein isothiocyanate (FITC). After incubation for 20 min in the dark, 150 µl binding buffer was added to each tube. Finally, cell apoptosis was detected via a flow cytometer.

Luciferase Report Assay. The wild type of the *CREG1 3'-UTR* sequence (*CREG1-WT*), containing a miR-505-5p binding site, and the mutant type of the *CREG1 3'-UTR* sequence (*CREG1-MUT*), avoiding miR-505-5p binding, were cloned into a pGL3 promoter vector (Genscript, China). Then the HEK293 cells were co-transfected with *CREG1-WT, CREG1-MUT*, miR-505-5p mimics, or NC mimics for 48 h. Finally, the relative luciferase activities were assayed.

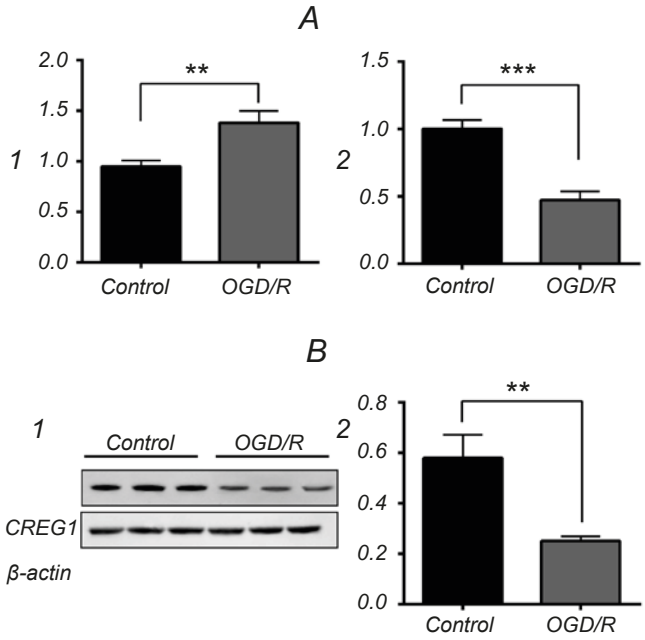
Statistics. The numerical data are presented as means ± s.d. Statistical analysis was conducted via GraphPad Prism 5. The Student's *t*-test was used for comparisons of two different groups. In addition, one-way or two-way ANOVA was used for comparison among multiple groups. In this study, differences with $P < 0.05$ were considered statistically significant. All results were repeated at least three times.

RESULTS

Up-Regulation of miR-505-5p Is Detected in OGD/R-Subjected Neurons. To examine the miR-505-5p expression level in OGD/R affected neurons, an OGD/R model was established. The real-time PCR revealed that the expression of miR-505-5p was increased and that of *CREG1* decreased in OGD/R-subjected neuron-like cells (Fig. 1A). Furthermore, the decrease of the CREG1 protein level was found using Western blotting (Fig. 1B). These findings suggest that the miR-505-5p amount can be reduced in OGD/R-subjected neurons.

MiR-505-5p Silencing Alleviates the OGD/R-Induced Neural Injury. To explore the function of miR-505-5p in the control of OGD/R-induced neural injury, miR-505-5p was successfully knocked down in PC12 cells with OGD/R treatment (Fig. 2A). The CCK-8 assay determined that OGD/R treatment led to the decrease in cell viability, whereas miR-505-5p silencing elevated the latter index (Fig. 2B). Furthermore, OGD/R treatment can enhance the LDH and MDA levels and inhibit SOD. However, miR-505-5p silencing reversed changes in the OGD/R-induced LDH, MDA, and SOD levels (Fig. 2C). These findings suggest that miR-505-5p silencing can alleviate the OGD/R-induced neural injury.

MiR-505-5p Silencing Inhibits OGD/R-Induced Neuron Apoptosis. We further analyzed the effect of miR-505-5p on OGD/R-related apoptosis of the tested neuron-like cells. Flow cytometry assay showed that the intensity of cell apoptosis was higher in OGD/R-treated PC12 cells than that in the control. However, miR-505-5p silencing caused the decrease of OGD/R-induced neuronal apoptosis (Fig. 3A). Moreover, OGD/R treatment promoted the cleaved caspase-3 and cleaved PARP protein levels, whereas miR-505-5p silencing suppressed these indices (Fig. 3B). The data reveal that miR-505-5p silencing can moderate OGD/R-induced neuron apoptosis.



F i g. 1. Up-regulation of miR-505-5p induced by oxygen-glucose deprivation/reoxygenation (OGD/R) in cultured neuron-like cells decreases the CREG1 protein level. A) Relative expressions of miR-505-5p (1) and CREG1 mRNA (2) in control and OGD/R-subjected cells; the respective levels under control conditions are taken as 1.0. B) Results of Western blotting (1) and estimation of expression of CREG1 protein (arbitrary units) (2). **, $P < 0.01$, and ***, $P < 0.001$ in intergroup comparisons.

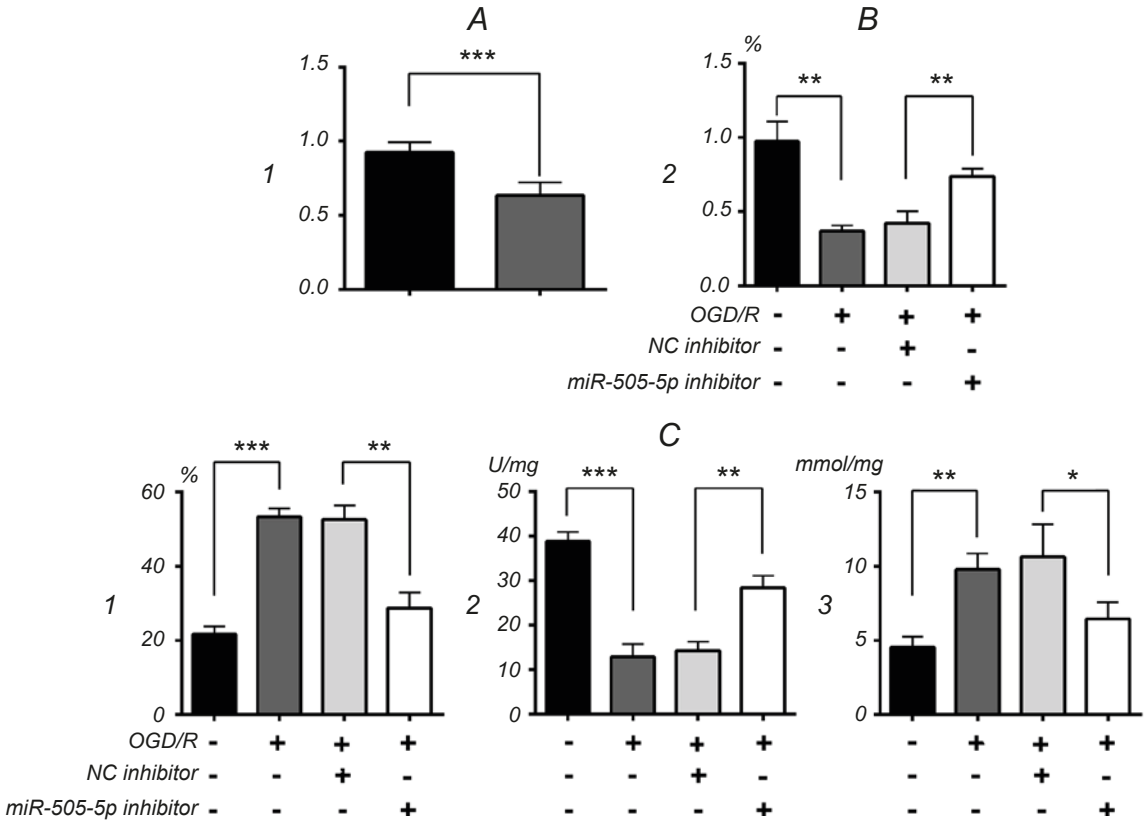
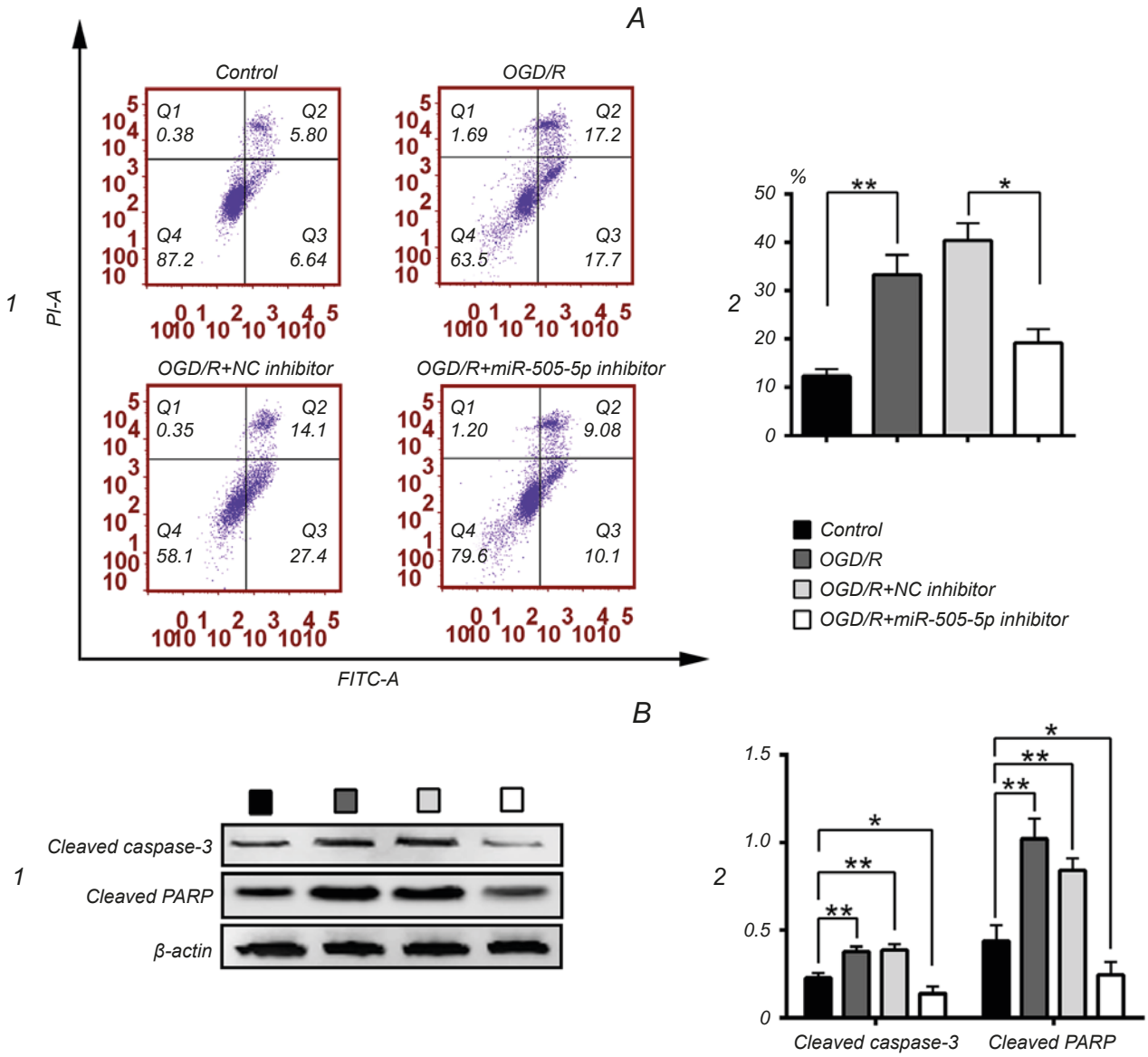


Fig. 2. MiR-505-5p silencing alleviates the OGD/R-induced neural injury. A) CREG1 mRNA overexpression level under conditions of applications of NC and miR-505-5p inhibitors. B) Cell viability (1) measured under conditions of isolated and combined applications of the injuring factors (shown below the columns). C) Effects of the mentioned factors on the LDH (%, 1), SOD (U/mg, 2), and MDA (mmol/mg) levels (3) were analyzed via LDH, SOD and MDA assays, respectively. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$; Designations of conditions in C are similar to those in B.

***CREG1* Is Verified as a Target Gene of miR-505-5p.** To clarify the mechanism underlying the role of miR-505-5p in OGD/R-induced neuron apoptosis, we predicted that miR-505-5p can bind with *CREG1* (Fig. 4A). The miR-505-5p overexpression was examined (Fig. 4B). The relative luciferase activity was dramatically inhibited in HEK293 cells co-transfected with miR-505-5p and *CREG1 WT*. Nevertheless, there was no effect on relative luciferase activity in HEK293 cells co-transfected with miR-505-5p and *CREG1 MUT* (Fig. 4C). Moreover, Western blotting demonstrated that miR-505-5p overexpression decreased the CREG1 protein level, whereas miR-505-5p knockdown enhanced this index in OGD/R-affected neurons (Fig. 4D). These findings show that the *CREG1* is a target of miR-505-5p.

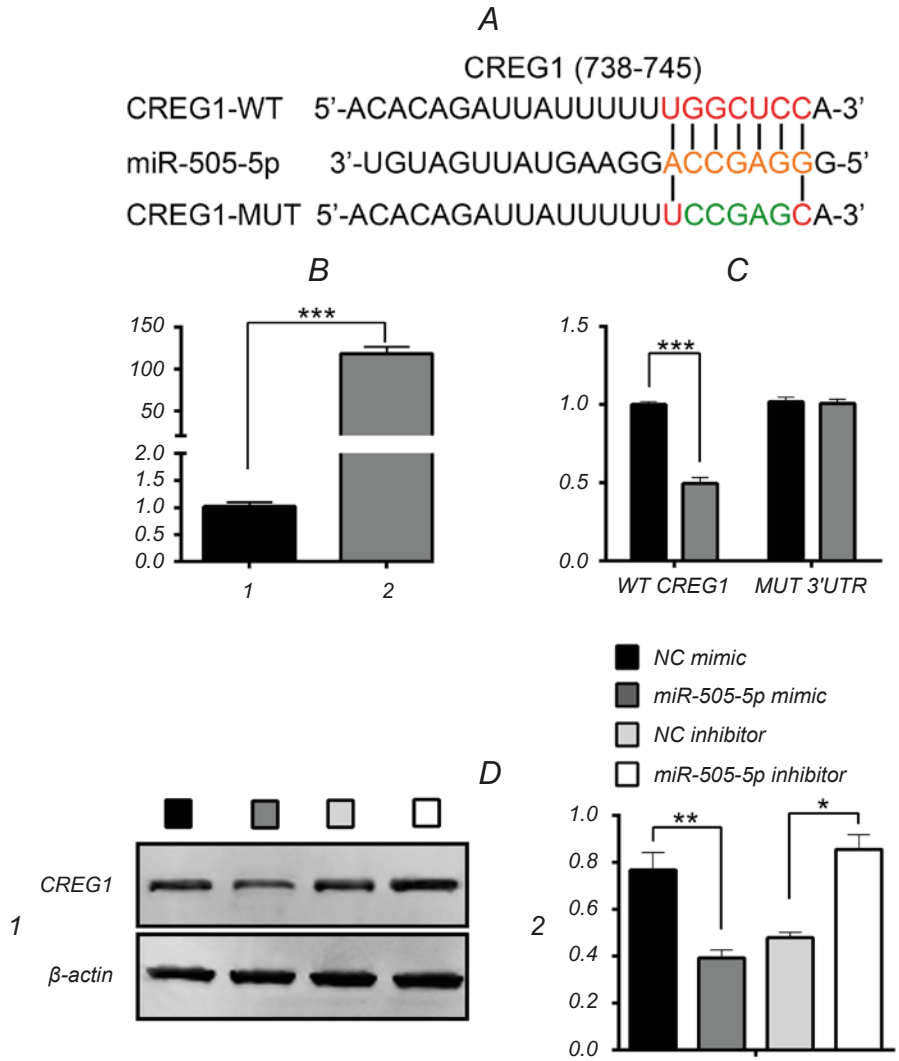
MiR-505-5p Silencing Elevates the Level of OGD/R-Induced Neuron Apoptosis by Inhibition of *CREG1*. To show how miR-505-5p manifested its role in OGD/R-affected neurons by the influence on the *CREG1*, this gene was successfully knocked down (Fig. 5A). As was shown above, miR-505-5p silencing increases cell viability, whereas *CREG1* knockdown can rescue it (Fig. 5B). Similarly, *CREG1* knockdown reversed the effect of miR-505-5p silencing on the LDH, SOD, and MDA levels (Fig. 5C). Furthermore, miR-505-5p silencing moderated cell apoptosis, whereas both miR-505-5p silencing and *CREG1* knockdown intensified this process (Fig. 5D). Western blotting revealed that miR-505-5p silencing enhanced CREG1 expression, as well as reduced the cleaved caspase-3 and cleaved PARP protein levels. However, in the presence of *CREG1* knockdown, the protein levels of CREG1, cleaved caspase-3, and cleaved PARP were rescued (Fig. 5E). These findings indicate that miR-505-5p silencing can increase OGD/R-induced neuron apoptosis by inhibition of *CREG1* expression.



F i g. 3. Inhibition of OGD/R-induced apoptosis of cultured neuron-like cells by silencing of miR-505-5p. A) Detection of the intensity of cell apoptosis: results of flow cytometry (1), and mean rates of apoptosis, % (2), in the control, after OGD/R, after OGD/R+NC inhibitor, and OGD/R+miR-505-5p inhibitor. B) Cleaved caspase-3 and cleaved PARP protein levels measured via Western blotting: results of Western blotting (1), and relative protein levels of cleaved caspase-3 and cleaved PARP (2), left and right groups of columns, respectively; designations of the columns are similar to those in A2. Other designations are similar to those in Figs. 1 and 2.

DISCUSSION

In our study, the high miR-505-5p expression was detected in cultured OGD/R-subjected neuron-like cells. Furthermore, miR-505-5p silencing was successfully preformed in PC12 cells. MiR-505-5p down-regulation caused increases in cell viability and SOD activity, as well as decreases in cell apoptosis and LDH and MDA levels. Interestingly, *CREG1* proved to be a target of miR-505-5p. A low *CREG1* level was measured in PC12 cells with miR-505-5p mimics, and a high *CREG1* level was induced by the miR-505-5p inhibitor. Notably, the effect of miR-505-5p silencing on the cell viability, LDH, SOD, and MDA levels, and cell apoptosis can be reversed by *CREG1* knockdown. Our findings indicate that miR-505-5p can demonstrate its function on OGD/R-induced neuronal injury by down-regulation of the *CREG1*.

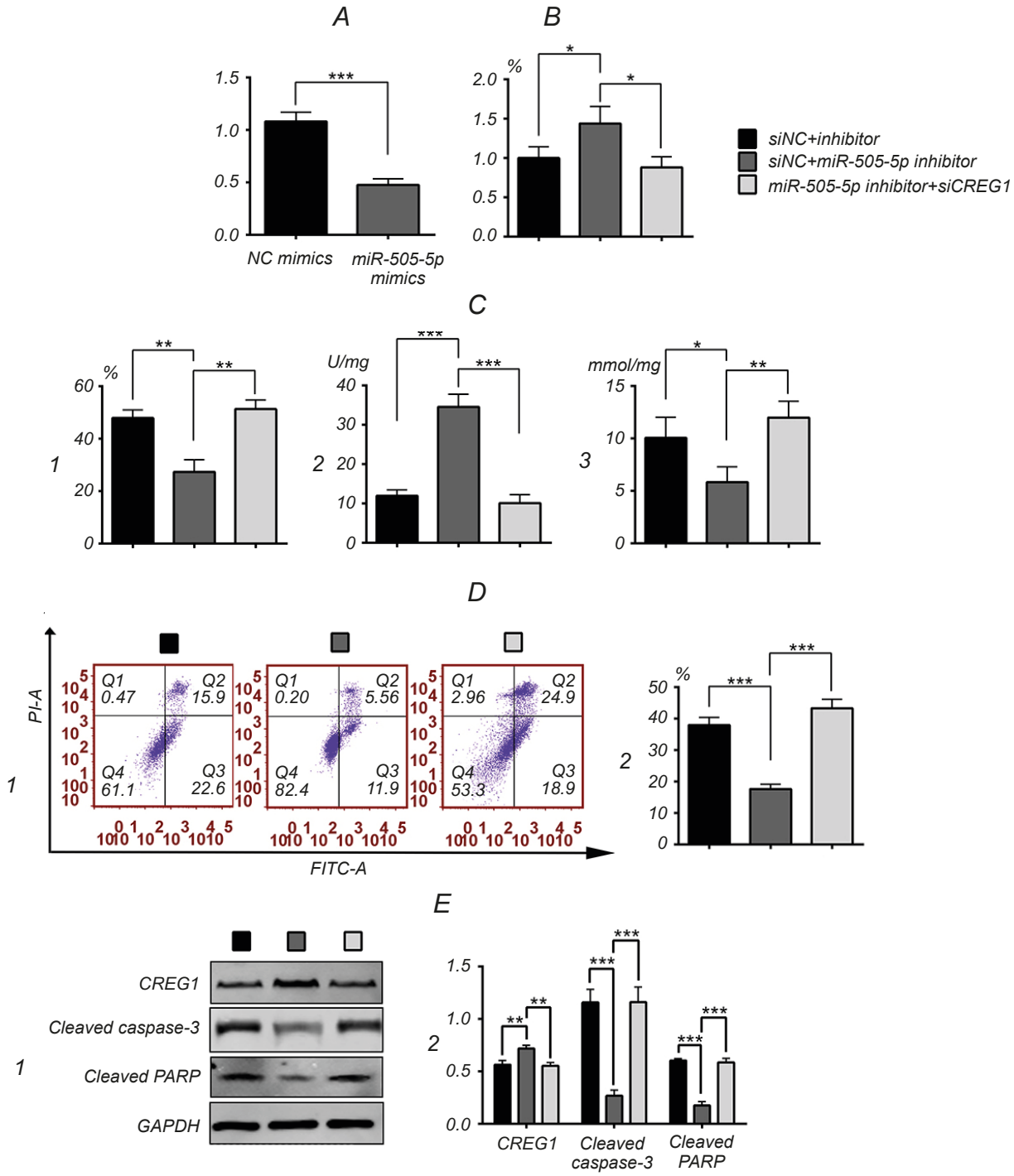


F i g. 4. Verification of the *CREG1* gene as a target of miR-505-5p. A) Prediction of the binding site for miR-505-5p. B) Mean level of overexpression of miR-505-5p measured using real-time PCR: at application of NC mimics (1), and at that of miR-505-5p mimic (2). C) Relative luciferase activity in *WT CREG1* and MUT 3 UTR at the action of the same agents as in B. D) Western blotting demonstrating the CREG1 protein level: results of Western blotting (1), and relative mean CREG1 protein levels (2). Other designations are similar to those in Figs. 1–3.

Previously, Li et al. [16] demonstrated that miR-144-3p can suppress cell viability as well as enhance the intensities of oxidative stress and cell apoptosis in OGD/R-affected neurons. However, its silencing can reduce OGD/R-induced neuronal injury. In addition, Wang et al. [17] demonstrated that miR-186-5p can be inhibited in a OGD/R model, and it can promote neuron apoptosis. Furthermore, Liang et al. [11] revealed that OGD/R treatment decreased manifestations of cell apoptosis and the levels of LDH and caspase-3 proteins in PC12 cells with miR-125b inhibition. In our study, we found that up-regulated miR-505-5p in OGD/R-subjected neurons exerts significant effects, which is consistent with other miRNAs-related phenomena in previous studies. Additionally, miR-505-5p silencing was shown to improve cell viability and the SOD state, to suppress cell apoptosis, and to decrease the LDH and MDA levels in OGD/R-affected neurons. These findings suggest that miR-505-5p silencing is able to considerably protect neurons against OGD/R-induced injury.

In further investigations of the effect of miR-505-5p on OGD/R-induced neuronal injury, miR-505-5p can be considered a target for the *CREG1* gene. Furthermore, the action of miR-505-5p mimics led to decreased *CREG1* expression, whereas the miR-505-5p inhibitor enhanced the

CREG1 level. Hence, the mechanism of interaction between miR-505-5p and *CREG1* was explored in the OGD/R cell model. The *CREG1* was found to demonstrate a low expression in OGD/R-subjected neurons in this study. As was shown in previous studies, during spontaneous immortalization of Li–



F i g. 5. Elevation of OGD/R-induced apoptosis by inhibition of *CREG1* due to silencing of miR-505-5p. A) Results of estimation of CREG1 expression with real-time PCR; B) estimation of cell viability using CCK-8 assay; C) estimation of the LDH (1), SOD (2), and MDA (3) levels using the respective assays; D) detection of cell apoptosis using flow cytometry; E) measurements of the levels of CREG1, cleaved caspase-3, and cleaved PARP proteins using Western blotting: (1) results of Western blotting, (2) relative mean CREG1, cleaved caspase-3, and cleaved PARP protein levels. Designations are similar to those in Figs. 3 and 4.

Fraumeni syndrome fibroblasts, the *CREG1* should be suppressed during immortalization and improved in senescence [18]. Importantly, accumulating evidence revealed that miRNAs can be involved in the progression of some diseases via targeting the *CREG1*. For example, miR-31 was reported to affect phenotypic modulation of human vascular smooth muscle cells through targeting the *CREG1* [19]. MiR-505-5p was shown to target the *3'-UTR* of S100 calcium-binding protein A4 and to mediate epithelial mesenchymal migration and invasion [20]. This microRNA was convincingly proved to bind with the *CREG1*. Besides, *CREG1* knockdown reversed the effect of miR-505-5p silencing on OGD/R-induced injury. All these data imply that miR-505-5p can play a key role in OGD/R-induced neuronal injury through negatively regulating the *CREG1*.

Therefore, we demonstrated that miR-505-5p can enhance OGD/R-induced neuronal injury through negative regulation of the *CREG1* gene. This will provide novel targets for the therapy of cerebral I/R injury. Although the effect of miR-505-5p on OGD/R-induced neuronal injury was explored, other binding sites of miR-505-5p may be found. Therefore, to clarify the mechanism of miR-505-5p involved in OGD/R-induced neuronal injury, additional experiments should be conducted in the future.

The experiments were carried out on cultured cells, and, thus, confirmation of the correspondence to ethical standards for experiments on animals is not necessary.

The authors, Y. Gao, G. Nan, and L. Chi, declare the absence of any conflict in commercial or financial relations, relationships with organizations or persons that in any way could be related to the study, and also in interrelations of the co-authors.

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