LncRNA FGD5-AS1 acts as a competing endogenous RNA for miRNA-223 to lessen oxygen-glucose deprivation and simulated reperfusion (OGD/R)-induced neurons injury

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Abstract

The purpose of this study was to evaluate whether FGD5-AS1 participates in oxygen-glucose deprivation and simulated reperfusion (OGD/R)-induced neurons injury and the detailed mechanism. An OGD/R model was established using the primary cortical neuron isolated from the brains of Sprague-Dawley rats. qRT-PCR and western blot were performed to detect the RNA and protein expression levels, respectively. Cell counting kit 8 (CCK8) and flow cytometry assays were used to evaluate the proliferation and apoptosis of neurons. The luciferase reporter assay was used to verify the interaction between lncRNA FGD5-AS1 and miRNA-223. We found that the expression of FGD5-AS1 is decreased in neurons suffering from OGD/R. Up-regulation of FGD5-AS1 could recover proliferation and inhibit apoptosis of OGD/R-injured neurons. In addition, the interaction between FGD5-AS1 and miRNA-223 were verified. The expression of miRNA-223 was negatively correlated with the level of FGD5-AS1. In turn, the expression of insulin-like growth factor-1 receptor (IGFIR, a target gene of miR-223) was positively associated with the level of FGD5-AS1. Simultaneously down-regulating miR-223 and over-expressing FGD5-AS1 as well as IGF1R exhibited an additional effect of extenuating OGD/R damage i.e. increasing neuron proliferation and reducing neuron apoptosis. In conclusion, our findings indicated that FGD5-AS1 may protect the neuron against OGD/R injury via acting as a ceRNA for miR-223 to mediate IGF1R expression, which contributes to a deeper understanding of ischemic stroke and provide a promising therapeutic target for this disease.

Key words: OGD/R, ischemic stroke, ceRNA, proliferation, apoptosis.

Introduction

Stroke is one of the main causes of death and disability in the world [1], with ischemic stroke as the most common type of all strokes [6]. During ischemic stroke, the reduction of oxygen and glucose supply results in neuronal injury [7]. Rapid blood flow restoration is an important approach for its treatment. However, it usually exacerbates ischemic brain tissue injury after reperfusion [14,19]. Although the study on the treatment and prevention of ischemic stroke has attracted increasing attention, its cellular mechanism is still indefinite due to its complexity. A better understanding of the pathological mechanism of stroke will be helpful to develop better strategies for its diagnosis and treatment.

In recent years, disordered long non-coding RNAs (lncRNAs, over 200 nucleotides) have been illustrated to take a vital regulatory part in post-transcriptional gene expression in pathological aspects of ischaemic stroke [11,17]. Many research have revealed

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that various RNA transcripts having the microRNA (miRNA)-binding sites could modulate each other via competing for shared miRNAs, therefore acting as competing endogenous RNAs (ceRNAs) [20,21]. LncRNAs have recently been reported to involve in ceRNA mechanisms by competing with mRNAs (the targets of miRNA) for miRNA molecules and then modulate miRNA-involved target suppression [21]. Multiple lncRNAs have been observed in ceRNA crosstalk in ischemic stroke. For instance, lncRNA GAS5 was recently identified as a ceRNA for miR-137 to modulate Notch1 in middle cerebral artery occlusion-injured brain [3]. LncRNA SNHG1 was reported to modulate cerebrovascular pathologies as a ceRNA for miR-18a, thereby modulating the de-repression of its endogenous target hypoxia-inducible factor 1α (HIF-1α) [22].

In a previous study, it was identified that miR-223 was over-expressed in neurons suffering from oxygen-glucose deprivation and simulated reperfusion (OGD/R) and it suppressed the growth of OGD/R-injured neurons by repressing insulin-like growth factor-1 receptor (IGF1R) expression [5]. IGF1R is known as a transmembrane receptor tyrosine kinase which is implicated in the regulation of IGF bioactivity [13]. Interactions between cells utilizing the IGF-1/IGF1R signalling cascades play a key role in the survival of neuronal cells against stress and injury [12,15].

Here, we want to explore the lncRNA that is involved in OGD/R-induced neuron injury by competing with IGF1R for miR-223. Through bioinformatics prediction, we identified that lncRNA FGD5 antisense RNA 1 (FGD5-AS1) was a potential lncRNA that could interact with miR-223. FGD5-AS1 was found to target miR-125a-3p as well as miR-142-3p in the ceRNA network in periodontitis [10]. In addition, it has been suggested as a prognostic biomarker and therapeutic target in clear cell kidney carcinoma [24]. However, the function of FGD5-AS1 in neurons suffering from OGD/R, and whether it could interact with miR-223 remains unrevealed. Thus, in the present study, we have evaluated whether FGD5-AS1 participates in OGD/R-induced neurons injury and if yes, whether it did so through competing with IGF1R for miR-223.

Material and methods

Primary cortical neuron isolation and OGD/R model construction

Primary cortical neurons were isolated from the brains of embryonic day 18 (E18) Sprague-Dawley rats. At first, cerebral cortices were isolated and digested by trypsin. Then cells were placed on the plates with poly-L-lysine coated culture cluster (Corning Incorporated, USA) and cultured in neurobasal medium (Thermo Fisher Scientific) supplemented with 2% B27 NeuroMix, 10 mM cytosine-β-D-arabinofuranoside, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C with 5% CO_2.

The OGD/R model was established as per our previous description [5]. In brief, the primary cortical neurons were cultured in glucose-free Earle's balanced salt solution (BSS) in an anaerobic incubator with 95% N_2 and 5% CO_2 at 37°C. After 2 h of OGD treatment, the medium was changed to a normal neurobasal medium and recovered under a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 12 h. The neurons in the sham group received the same treatment without OGD exposure.

Transfection

pcDNA3.1, pcDNA3.1-lncRNA FGD5-AS1, pcDNA3.1-IGF1R, miR-223 agomir/antagomir and their corresponding NC were all bought from YouBio (Changsha, China). Primary cortical neurons were transfected with pcDNA3.1, pcDNA3.1-lncRNA FGD5-AS1, pcDNA3.1-IGF1R, miR-223 agomir/antagomir or their corresponding NC, or cotransfected with pcDNA3.1-lncRNA FGD5-AS1 and miR-223 antagomir or pcDNA3.1-lncRNA FGD5-AS1, miR-223 antagomir and pcDNA3.1-IGF1R using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell viability assay

The viability of primary cortical neurons was detected using a cell counting kit-8 (CCK8, Dojindo, Tokyo, Japan) according to the manufacturer's description. In brief, cell suspensions were prepared and seeded into 96-well plates with 1,000 cells/well. Then the neurons were cultured under standard conditions and cell viability was tested every 24 h. When testing, 10 μl of CCK8 reagent was added to each well followed by being incubated for 1.5 h at 37°C. Then the optical density (OD) at 450 nm was detected using a microplate reader. The viability curve was plotted using Graph Pad Prism 5.

Cell apoptosis analysis

The neurons were collected and washed by precooled PBS. Then the neurons were re-suspended in

binding buffer to a concentration of 1-5 × 10^6/ml. Next, 100 µl of cell suspension was added into a 5 ml tube followed by adding 5 µl of Annexin V/FITC and 5 µl of Propidium Iodide (PI) into the tube. After being incubated for 15 min in the dark, 400 µl of 1 × Annexin V binding buffer were added into the tube. Cell apoptosis was detected using flow cytometry (BD, USA).

qRT-PCR

Total RNA of primary cortical neurons was extracted using TRIzol (Invitrogen). PrimeScript RT Reagent Kit (Takara, Japan) or MiScript Reverse Transcription kit (Qiagen) was used for reverse transcription as appropriate. SYBR Premix Ex Taq II (TaKaRa, Japan) or Mi-Script SYBR-Green PCR kit (Qiagen) was used to perform qPCR on 7900HT real-time PCR system to determine the expression level of FGD5-AS1, IGF1R and miR-223, as appropriate. GAPDH or U6 was used for normalization. The primers used for qPCR were as follows:
- lncRNA FGD5-AS1 F: 5'-AACAGTGCCTATGTGGACGG-3'
- lncRNA FGD5-AS1 R: 5'-CCCATCACAGAGGTCCACAC-3'
- miR-223 F: 5'-CGTGTATTTGACAAGCTGAGTTG-3'
- miR-223 R: 5'-AACGCTTCACGAATTTGCGT-3'
- IGF1R F: 5'-GTGTGGATCGCGATTTCTGC-3'
- IGF1R R: 5'-GTACATGCTCTGGGTGCTGT-3'
- U6 F: 5'-CTCGCTTCGGCAGCACA-3'
- U6 R: 5'-AACGCTTCACGAATTTGCGT-3'
- GAPDH F: 5'-ACACCCACTCCTCCACCTTT-3'
- GAPDH R: 5'-TTACTCCTTGGAGGCCATGT-3'.

The relative expression level of FGD5-AS1, IGF1R and miR-223 was calculated using a 2-$^{\Delta\Delta Ct}$ method.

Luciferase reporter assay

The binding sites between FGD5-AS1 and miR-223 were predicted using TargetScan (http://www.targetscan.org). The wild type (WT) and mutant type (Mut) 3'UTR sequences of FGD5-AS1 were synthesized and ligated into the pmiR-RB-REPORT^{TM} vector. Then the cells were co-transfected with pmiR-WT or pmiR-Mut and miR223-agomir negative control (NC) or miR223-agomir using Lipofectamine200 (Invitrogen). After 48 h transfection, a Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) was used to test the activities of Firefly and Renilla luciferase consecutively.

Western blot

Western blot was performed as described previously [5]. The protein bands were detected using an enhanced chemiluminescence detection kit (ECL, Pierce Biotechnology Inc.). The density of the bands was analysed utilizing Quantity One software. Tubulin was used as an intrinsic quality control.

Statistical analysis

Statistical analysis of the experimental data was performed using SPSS22.0 and GraphPad Prism 6.0 software. All data were presented as Mean ± standard deviation (SD). Student's t-test was used for single comparison and one way analysis of variance (ANOVA) was used for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

Results

The expression of FGD5-AS1 was down-regulated in neurons suffering from OGD/R

In our study, an OGD/R model was constructed using primary cortical neurons isolated from Sprague-Dawley rats. Then we detected the expression changes of FGD5-AS1 in neurons subjected to OGD/R by qRT-PCR and found that the expression level of FGD5-AS1 was significantly decreased in neurons injured by OGD/R compared with that in the sham group (Fig. 1A, $p < 0.01$). These results suggested the involvement of FGD5-AS1 in OGD/R-induced injury.

Overexpression of FGD5-AS1 in neurons mitigates OGD/R-induced injury

In order to explore the biological function of FGD5-AS1 in OGD/R-induced injury, we overexpressed it by transfecting pcDNA3.1-lncRNA FGD5-AS1 into neurons. We can easily observe that the level of FGD5-AS1 in neurons transfected with pcDNA3.1-lncRNA FGD5-AS1 significantly increased about 3.51 folds of that in the Vehicle group (neurons transfected with pcDNA3.1, $p < 0.01$, Fig. 1B). Then we detected the effect of FGD5-AS1 up-regulation on the proliferation of neurons by CCK8 assay. The results exhibited that the proliferative ability of neurons in the OGD/R + vehicle group was significantly suppressed compared with the sham group. While overexpression of FGD5-AS1 significantly recovered the proliferation ability compared to the OGD/R + vehicle group ($p < 0.01$, Fig. 1C). Flow cytometry was then used to investigate the effect of FGD5-AS1 on neurons apop-
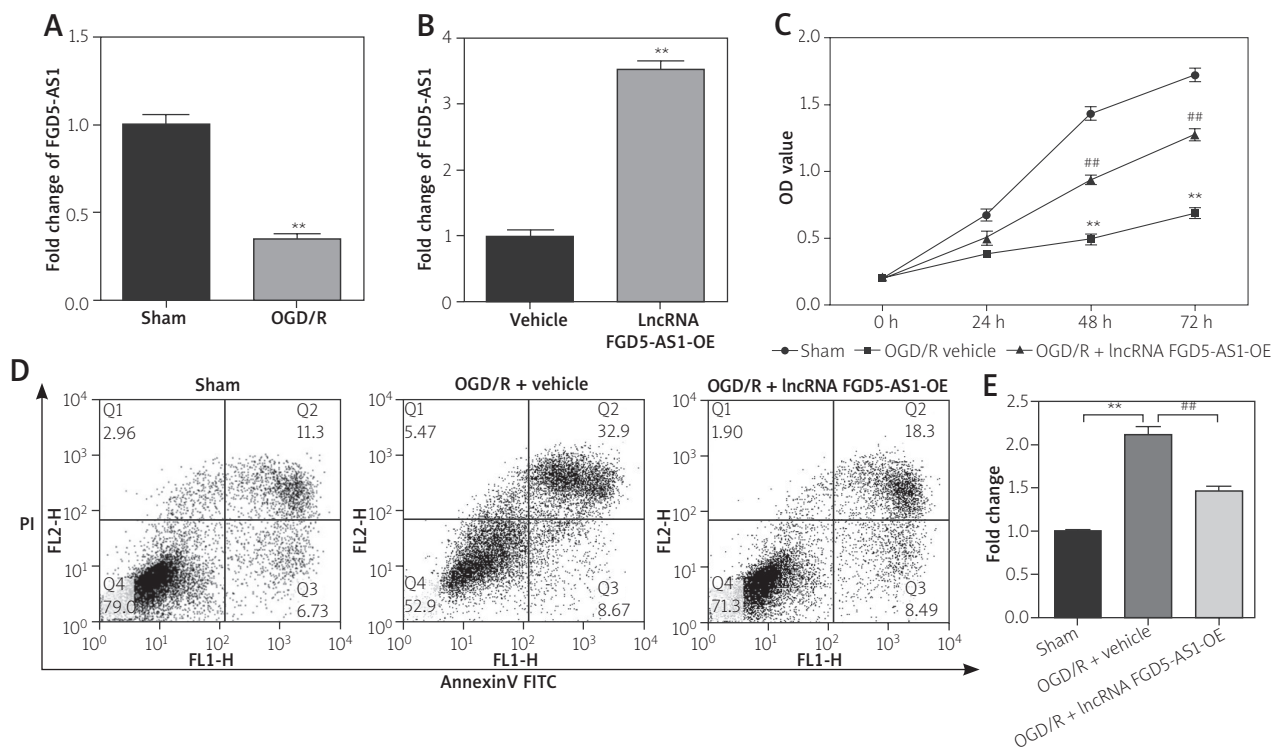
Fig. 1. Overexpression of FGD5-AS1 extenuates OGD/R-induced neuron injury. **A**) FGD5-AS1 expression is decreased in OGD/R-injured neurons. **B**) The expression of FGD5-AS1 was significantly up-regulated in neurons transfected with pcDNA-FGD5-AS1. **C**) By CCK8 assay, we found that overexpression of FGD5-AS1 could recover the proliferation ability of OGD/R-injured neurons. **D**) Cell apoptosis analysis revealed that overexpression of FGD5-AS1 reduced the apoptosis rate of OGD/R-injured neurons. **E**) The apoptosis rate of neurons. **p < 0.01 vs. the sham or vehicle group. ##p < 0.01 vs. the OGD/R + vehicle (vector) group. OE – overexpression.

tosis. We found that the apoptotic rate in the OGD/R + vehicle group was notably higher than that in the sham group. However, when we up-regulated FGD5-AS1 in the neurons, the apoptotic rate of neurons damaged by OGD/R was significantly decreased compared to the OGD/R + vehicle group ($p < 0.01$, Fig. 1D, E). Based on the above results, we speculated that overexpression of FGD5-AS1 in neurons could extenuate OGD/R-induced injury.

FGD5-AS1 could interact with miR-223

Through bioinformatics prediction, we found that FGD5-AS1 could interact with miR-223. The possible binding sites between these 2 molecules were predicted by Targetscan and presented in Figure 2A. Luciferase reporter assay illustrated that the luciferase activity of FGD5-AS1-WT was significantly decreased in the miR-223 agomir group compared with the miR-223 agomir NC group ($p < 0.01$, Fig. 2B).

While there was almost no difference of FGD5-AS1-Mut luciferase activity between the miR-223 agomir group and miR-223 agomir NC group. These results indicated that FGD5-AS1 could interact with miR-223.

FGD5-AS1 participates in the regulation of OGD/R-induced neurons injury via regulating miR-223/IGF1R axis

In our previous study, we demonstrated that IGF1R is a target of miR-223 and the expression of IGF1R is negatively regulated by miR223. Here, we want to explore how FGD5-AS1 affects the expression of miR-223/IGF1R and whether together with regulation of FGD5-AS1/miR-223/IGF1R could synergistically extenuate OGD/R injury. Our results showed that the expression of miR223 was up-regulated in neurons injured by OGD/R and overexpression of FGD5-AS1 decreased the level of miR-223 in OGD/R-injured neurons. In OGD/R neurons trans-

fected with pcDNA-FGD5-AS1 and miR223 antagomir, the level of miR-223 was further decreased compared to the OGD/R + FGD5-AS1 overexpression (OE) group ($p < 0.01$, Fig. 3A). On the contrary, the expression of IGF1R was down-regulated in neurons injured by OGD/R and overexpression of FGD5-AS1 increased both the mRNA and protein levels of IGF1R in OGD/R-injured neurons. Up-regulation of FGD5-AS1 and down-regulation of miR-223 together, further up-regulated the level of IGF1R. In OGD/R neurons transfected with pcDNA-FGD5-AS1, miR223 antagomir and pcDNA- IGF1R, the level of IGF1R increased to more than 2 folds of the sham group ($p < 0.01$, Fig. 3B-D).

Then we investigated the effect of FGD5-AS1/miR-223/IGF1R on the proliferation and apoptosis of neurons subjected to OGD/R. Compared with the OGD/R+FGD5-AS1-OE group, the proliferation of neurons in the OGD/R + FGD5-AS1-OE + miR-223 antagomir group was significantly increased. In the OGD/R + FGD5-AS1-OE + miR-223 antagomir + IGF1R-OE group, the proliferation was notably higher than that in the OGD/R + FGD5-AS1-OE + miR-223 antagomir group and even higher than that in the sham group ($p < 0.01$, Fig. 4A). The flow cytometry analysis showed that the apoptotic rate in the OGD/R + FGD5-AS1-OE + miR-223 antagomir group was notably decreased compared to the OGD/R + FGD5-AS1-OE group. And in the OGD/R + FGD5-AS1-OE + miR-223 antagomir + IGF1R-OE group, the apoptotic rate was further decreased compared to the OGD/R + FGD5-AS1-OE + miR-223 antagomir group ($p < 0.01$, Fig. 4B, C). Collectively, these data hinted that down-regulation of miR-223 and up-regulation of FGD5-AS1 and IGF1R together, could notably extenuate OGD/R injury in neurons.

Discussion

LncRNAs are considered to be promising therapeutic targets for ischemic stroke, owing to their abnormal expression and various functions in ischemic stroke [4,18,23]. Recent research indicates that lncRNAs take part in ceRNA crosstalk by competing with the mRNAs for their shared miRNAs, which play critical roles in the occurrence and progression of human disease [21]. In our previous study, the involvement of miR-223/IGF1R in OGD/R-induced neuron injury has been proved. MiR-223 was illustrated to exhibit an inhibitory effect on the prolif-

A

FGD5-AS1 WT 5′CCUGACCUUUCGCCAACUGACU3′

miR-223-3p 3′ACCCCAUAAACUGUUUGACUGU

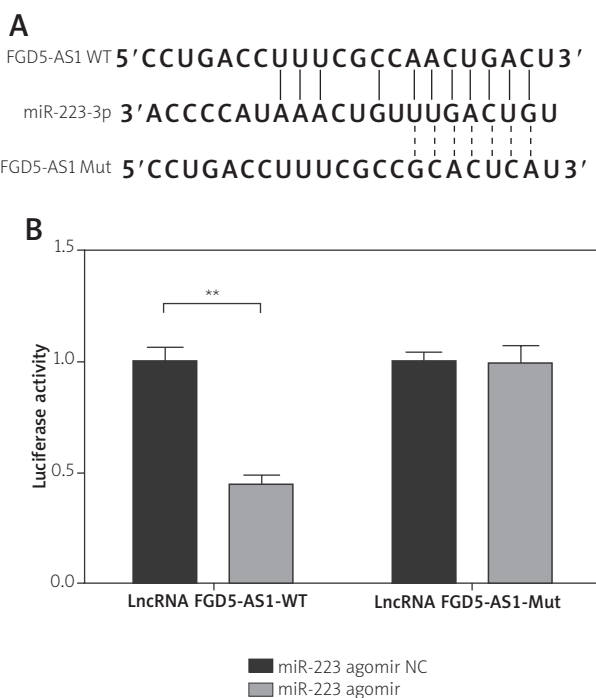FGD5-AS1 Mut 5′CCUGACCUUUCGCCGCACUCAU3′

B

Fig. 2. FGD5-AS1 is a target of miR-223. **A**) The putative miR-223 binding sequence of the wild type (WT) and mutation (Mut) sequence of FGD5-AS1. **B**) FGD5-AS1-WT and FGD5-AS1-Mut were co-transfected with miR-223 agomir or miR-223 agomir NC into neurons. Luciferase activity was measured. **$p < 0.01$. NC – negative control

eration of OGD/R-injured neurons via retarding IGF1R expression [5]. Here, lncRNA FGD5-AS1 was forecasted and verified as a ceRNA for miR-223. The expression of FGD5-AS1 was reduced significantly in neurons subjected to OGD/R and overexpression of FGD5-AS1 could notably weaken neurons injury caused by OGD/R.

FGD5 belongs to the Rho guanine nucleotide exchange factor (Rho GEF) family [8,16] and has been demonstrated to regulate proangiogenic action of VEGF in vascular endothelial cells [8]. Rho GEFs could activate Rho GTPases which modulate cytoskeleton and complicated in various cellular processes including gene expression, cell cycle progression as well as cell motility [16]. Many Rho GEFs have been reported to be associated with the development of ischemic stroke or other cardiovascular disease, such as ARHGEF10 [9], Arhgef1 [2] etc. FGD5-AS1 (Gene ID: 100505641), which affiliated
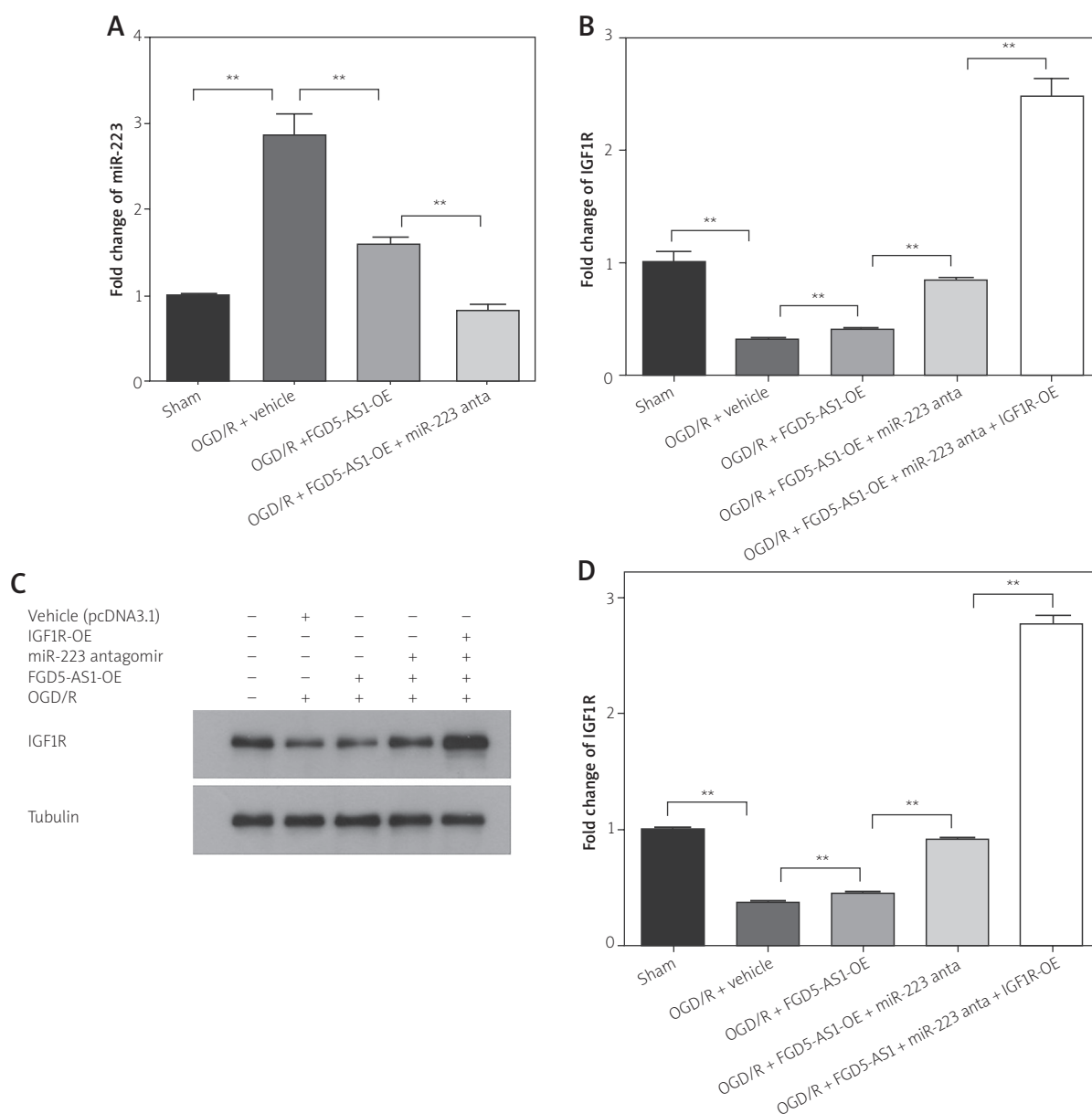
Fig. 3. The expression of miR-223 and IGF1R in neurons. **A**) The level of miR-223 in OGD/R-injured neurons transfected with pcDNA-FGD5-AS1 or pcDNA-FGD5-AS1 and miR-223 antagomir was detected by qRT-PCR. **B**) The mRNA expression level of IGF1R in OGD/R-injured neurons transfected with pcDNA-FGD5-AS1 or pcDNA-FGD5-AS1 and miR-223 antagomir or pcDNA-FGD5-AS1, miR-223 antagomir and pcDNA-IGF1R was detected by qRT-PCR. **C**) The protein expression of IGF1R in OGD/R-injured neurons was detected by western blot. **D**) Quantification of the protein expression level of IGF1R. ****$p < 0.01$. OE – overexpression, miR-223 anta – miR-223 antagomir.

with the non-coding RNA class, is an antisense RNA of FGD5. To date, few reports regarding the function of FGD5-AS1 have been available. In the human gene database (https://www.genecards.org/), the association between FGD5-AS1 and neuroblasto-

ma has been indicated. The biological function of FGD5-AS1 in stroke remains unclear. In our present study, FGD5-AS1 is predicted and demonstrated as a ceRNA for miR-223, which has been reported to involve in OGD/R-induced neurons injury by regulat-
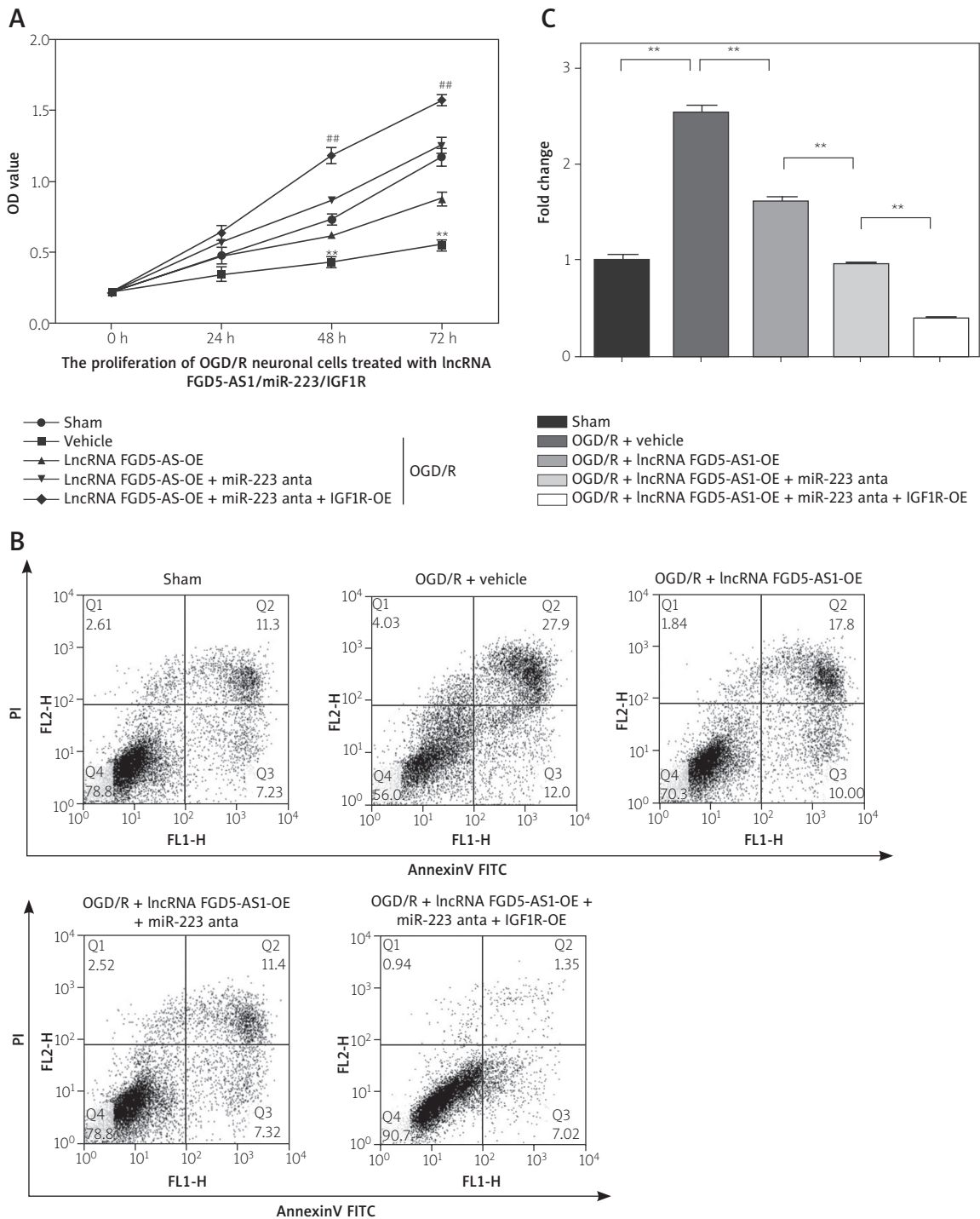
Fig. 4. Simultaneous down-regulation of miR-223 and overexpression of FGD5-AS1 as well as IGF1R, exhibited an additive/additional effect of extenuating OGD/R damage. **A**) The effect of simultaneous down-regulation of miR-223 and overexpression of FGD5-AS1 as well as IGF1R on the proliferation of OGD/R-injured neurons. **p < 0.01 vs. sham group. ##p < 0.01 vs. OGD/R + lncRNA FGD5-AS-OE + miR-223 anta group. **B**) The effect of simultaneous down-regulation of miR-223 and overexpression of FGD5-AS1 as well as IGF1R on the apoptosis of OGD/R-injured neurons. **C**) The apoptosis rate of neurons. **p < 0.01. OE – overexpression, miR-223 anta – miR-223 antagomir.

ing IGF1R [5]. The expression of miR-223 was negatively regulated by FGD5-AS1 as we found that overexpression of FGD5-AS1 significantly decreases the level of miR-223. On the contrary, the level of IGF1R was positively associated with FGD5-AS1 (Fig. 3). These experimental data indicated that FGD5-AS1 is competing with IGF1R for miR-223 in neurons.

To further illustrate that FGD5-AS1 exhibits its protective role in OGD/R-induced injury by modulating miR-223/IGF1R axis, we changed the levels of FGD5-AS1/miR-223/IGF1R together in OGD/R-injured neurons. The results revealed that simultaneous down-regulation of miR-223 and overexpression of FGD5-AS1 as well as IGF1R, exhibited an additional effect of extenuating OGD/R damage. These findings suggested that FGD5-AS1 protected the neuron against OGD/R injury via acting as a ceRNA for miR-223 to mediate IGF1R expression.

In conclusion, our study demonstrated for the first time that FGD5-AS1 played a protective role in OGD/R-induced neuron injury, which is partly via competitively binding with miR-223 to regulate IGF1R expression. This study deepened our understanding on the pathogenesis of ischaemic stroke, as well as provided a novel potential biomarker for stroke therapy.

Disclosure

The authors report no conflict of interest.

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