

# miR-30a-5p inhibits hypoxia/reoxygenation-induced oxidative stress and apoptosis in HK-2 renal tubular epithelial cells by targeting glutamate dehydrogenase 1 (GLUD1)

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**Abstract.** MicroRNAs (miRNAs) are reported to be involved in renal hypoxia/reoxygenation (H/R) damage. To investigate this further, human kidney (HK-2) cells were cultured, subjected to H/R and the function of miR-30a-5p and glutamate dehydrogenase 1 (GLUD1) was evaluated. The results showed that, miR-30a-5p was downregulated and GLUD1 was upregulated in HK-2 cells exposed to H/R. The relationship between miR-30a-5p and GLUD1 was determined using dual luciferase assays. Primary HK-2 cells were cultured in H/R and transfected with negative control 1 (NC1), negative control 2 (NC2), mimic, inhibitor or GLUD1 siRNA plasmids. Reactive oxygen species (ROS) generation, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, and the rate of apoptosis in HK-2 cells were assessed. The results showed that, miR-30a-5p mimic reduced the production of ROS in HK-2 cells treated with H/R, but increased the activity of SOD, CAT and GPx. In addition, miR-30a-5p mimic significantly decreased H/R-mediated apoptosis, decreased the expression of bax and activity of caspase-3 and enhanced the expression of bcl-2. However, miR-30a-5p inhibitor showed the opposite effect with regard to the degree of oxidative damage and apoptosis in H/R-induced HK-2 cells. Silencing GLUD1 rescued the influence of miR-30a-5p inhibitor on oxidative injury and apoptosis in HK-2 cells stimulated with H/R. These results demonstrated that under H/R conditions, miR-30a-5p can reduce oxidative stress *in vitro* by targeting *GLUD1*, which may be a novel therapeutic target for liver failure and worth further study.

## Introduction

Acute kidney injury (AKI) is currently the main cause of high mortality in patients with acute disease (1). In recent years, the molecular and pathological mechanisms of AKI has been elucidated; however, most studies were focused on signaling pathways such as the PI3K/Akt (2), Nrf2/HO-1 (3), and TLR4/NF- $\kappa$ B (4) pathways. MicroRNAs (miRNAs) have been reported to play a vital regulatory role in many physiological pathways, including multiple acute tissue injury, for example the miR-30b-3p pathway, which is involved in acute lung injury (5) and the miR-30a-5p pathway, which can alleviate inflammatory responses and oxidative stress stimulated by spinal cord injury by targeting the Neurod 1/MAPK/ERK axis (6). In addition, miR-30a-5p is involved in the process of inflammation and apoptosis in PC-12 cells mediated by lipopolysaccharide (7). Nevertheless, the regulatory mechanism of miR-30a-5p in the physiological process of renal I/R injury at the gene level has not been fully investigated.

The glutamate dehydrogenase 1 (*GLUD1*) gene encodes glutamate dehydrogenase (GDH), which is mainly located in the mitochondria of the liver, myocardium and renal cells (8). It is well known that GDH acts as a critical enzyme for catalyzing the entry of glutamine into the tricarboxylic acid cycle (9) and reducing NAD(P) to NAD(P)H, which can be used in bio-synthetic pathways (10). As an important antioxidant and maintainer of intracellular calcium homeostasis (11), Ginsenoside Rg1 (Rg1) was demonstrated to alleviate hypoxia/reoxygenation (H/R)-induced cell damage and mitochondrial dysfunction by regulating GDH imbalance (12). More importantly, the bioinformatics prediction website TargetScan (<http://www.targetscan.org>) demonstrated that the 3'-UTR region of *GLUD1* can specifically bind to miR-30a-5p. Therefore, we hypothesized that miR-30a-5p participated in the H/R injury by targeting the *GLUD1* gene.

In the present study, human renal tubule epithelial cells (HK-2) were used to construct a H/R cell model *in vitro*, to research the molecular mechanism of miR-30a-5p on oxidative stress and apoptosis from the perspective of the *GLUD1* gene.

## Materials and methods

**HK-2 cell culture.** HK-2 cells were provided by the American Type Culture Collection (Rockville). RPMI-1640 medium (HyClone) was used to culture cells in a thermostatic incubator

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(Thermo Scientific) with 5% CO<sub>2</sub>, at 37°C. Fetal bovine serum (10%, Sigma), penicillin (100 IU/ml), streptomycin (100 µg/ml), and human recombinant epidermal growth factor (10 ng/ml, Gibco) were added to the RPMI-1640 medium. At 80% confluency, 0.25% trypsin was used for digestion and cells were passaged at a ratio of 1:3.

**Establishment of the model of hypoxia/reoxygenation (H/R).** HK-2 cells were incubated in a 96-well plate at a density of 5x10<sup>4</sup> cells/well. After 24 h culture, for the H/R group, HK-2 cells were subjected to hypoxic treatment (containing 1% O<sub>2</sub> and 5% CO<sub>2</sub>, with 94% N<sub>2</sub>, temperature was maintained at 37°C) for 12 h in serum-free medium without glucose. Subsequently, the cells were switched to a humidified cell incubator (BB 15; Thermo Electron LED GmbH) containing 5% CO<sub>2</sub> and 95% O<sub>2</sub> at 37°C for 2 h for reoxygenation with normal medium. For the control group, HK-2 cells were cultured in a humidified cell incubator containing 5% CO<sub>2</sub> at 37°C for 14 h.

**HK-2 cell groups and transfections.** HK-2 cells has been assigned to 10 groups and treated as follows: Experiment 1: i) control group (cells without any treatment); ii) H/R group (cells exposed to H/R); experiment 2: iii) H/R + negative control 1 (NC1) group (cells treated with mimic NC before H/R was induced); iv) H/R + mimic group (cells treated with miR-30a-5p mimic before H/R was induced); v) H/R + NC2 group (cells treated with inhibitor NC before H/R was induced); vi) H/R + inhibitor group (cells treated with miR-30a-5p inhibitor before H/R was induced); experiment 3: vii) H/R + NC2 + si-control group (cells treated with inhibitor NC and siRNA NC to GLUD1 before H/R was induced); viii) H/R + inhibitor + si control group (cells treated with miR-30a-5p inhibitor and siRNA NC to GLUD1 before H/R was induced); ix) H/R + NC2 + si-GLUD1 (cells treated with inhibitor NC and siRNA to GLUD1 before H/R was induced); and x) H/R + inhibitor + si-GLUD1 (cells treated with miR-30a-5p inhibitor and siRNA to GLUD1 before H/R was induced).

Mimic NC, inhibitor NC, miR-30a-5p mimic, miR-30a-5p inhibitor, siRNA NC and siRNA GLUD1 were provided by GenePharma Co., Ltd. Cell transfection was conducted following the instructions in the Lipofectamine 2000 transfection kit (Thermo Fisher Scientific, Ltd.). Forty-eight hours after transfection, HK-2 cells were subjected to H/R treatment as mentioned above.

**Dual luciferase reporter analysis.** Bioinformatics analysis TargetScan (<http://www.targetscan.org>) was used in the present study to predict the binding sites of GLUD1 and miR-30a-5p. A wild-type fragment that contained the binding site for miR-30a-5p was designed to target the 3'-untranslated region (3'-UTR) of the *GLUD1* gene and synthesized by Genechem Corp. The fragment was designed to have a restriction endonuclease site *Hind*III and *Spe*I. The target fragment was inserted into the pMIR-REPORT™ Luciferase vector plasmid (Ambion) using T4 DNA/RNA ligase and labelled as pMIR-GLUD1-wild-type (WT). The binding site of miR-30a-5p was mutated in the WT plasmid to produce the pMIR-GLUD1-mutant-type (MUT) plasmid. HK-2 cells were inoculated in 12-well plates with a density of 2x10<sup>5</sup> cells/well,

and co-transfected with recombinant plasmids (WT and MUT) and miR-30a-5p mimic for 48 h according to the groups described above. Cells were collected as described earlier. A luciferase kit (Promega) was used to determine firefly luciferase (A) and *Renilla* luciferase (B), respectively. The internal reference was represented by firefly luciferase, meaning that the fluorescence activity (C) was evaluated by the ratio of B:A.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** After total RNA was extracted from the HK-2 cells in each group using TRIzol® reagent (Promega Corp.) as per the manufacturer's protocol, it was used to synthesize cDNA using a reverse transcription kit and following the manufacturer's instructions (Takara). *GLUD1* was detected by RT-qPCR using a SYBR-Green qPCR Super Mix (Invitrogen). For miR-30a-5p, a Taqman MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to synthesize cDNA and a Taqman Universal Master Mix II (Roche Applied Science) was used to perform RT-qPCR in accordance with the manufacturer's protocol. The primer sequences are presented in Table I. U6 and β-actin were used as the internal reference for miRNA-30a-5p and *GLUD1*, respectively, the 2<sup>-ΔΔC<sub>q</sub></sup> method (13) was utilized to calculate the relative expression level of the target gene. Experiments were independently repeated three times.

**Western blot analysis.** RIPA lysis buffer (Beyotime Biotechnology) was used to extract total protein from cells. Cells were incubated on ice for 30 min, centrifuged at 12,000 x g for 10 min at 4°C, then total protein concentration was measured in the supernatants using a bicinchoninic acid kit (Pierce). SDS loading buffer was mixed with the protein samples and boiled at 100°C for 10 min at 50 µg/well. Proteins were resolved on a 10% SDS-PAGE gel, and then transferred to polyvinylidene fluoride (PVDF) membranes. After 1 h blocking in 5% bovine serum albumin at 25°C, the membrane was incubated with primary antibodies (all supplied by Abcam) against GLUD1 (ab166618, 1:1,000), cleaved-caspase-3 (ab32042, 1:500), caspase-3 (ab13847, 1:500), Bax (ab32503, 1:1,000), Bcl-2 (ab32124, 1:1,000) and GAPDH (ab8245, 1:1,000) overnight at 4°C. Next, the membrane was rinsed using Tris-buffered saline.

The membrane was washed in Tween-20 (TBST) four times for 5 min each and treated with secondary antibodies (1:2,000; Santa Cruz Biotechnology) for 1 h. The signals were determined using an electrogenerated chemiluminescence reagent (GE Healthcare Life Sciences) after washing in TBST three times for 5 min. ImageJ software (NIH, Bethesda) was used to detect intensity of the bands and, with GAPDH as the internal reference, the expression of these proteins was quantified.

**Determination of intracellular ROS concentration.** The fluorescent probe of 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma) was used to monitor the intracellular reactive oxygen species (ROS) concentration. After the HK-2 cells reached a density of 1x10<sup>4</sup> cells/ml, the culture medium was removed, and the cells were washed three times with D-Hank's solution. HK-2 cells were then cultured with 40 µM DCFH-DA for 30 min at 37°C. After incubation,

Table I. Primer sequences for RT-qPCR.

Primer sequences	Forward (5'→3')	Reverse (5'→3')
<i>miR-30a-5p</i>	TGTAACATCCTCGAC TGGAAG	TGCGTGTCTGGAGTC
<i>U6</i>	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
<i>GLUD1</i>	TATCCGGTACAGCACTGACG	GCTCCATGGTGAATCTTCGT
<i>β-actin</i>	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC

the cells were washed again with D-Hank's solution three times. To identify the nucleus, the cells were then stained with Hoechst 33342 (Beyotime Institute of Biotechnology) at room temperature for 5 min, then washed again with D-Hank's solution three times and 1 ml of fresh culture medium was added for resuspension. A fluorescence microplate reader (Tecan) was used to evaluate the fluorescence intensity, with excitation wavelengths at 485 nm and emission wavelengths at 535 nm. Experiments were performed in triplicate.

**Measurement of SOD, CAT, GPx, GST and GR activities.** To quantify enzyme activity, HK-2 cells were collected after 48 h culture, cells were lysed on ice with 100  $\mu$ l of RIPA buffer for 20 min and the supernatant was collected after centrifugation at 6,000  $\times$  g for 30 min at 4°C. Commercial kits (JianCheng Bioengineering Institute) were used to quantify the activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (GPx) in accordance with the manufacturer's instructions. The activity of glutathione S-transferase (GST) enzyme in the cells was measured according to the method of Habig *et al* (14) and the activity of glutathione-reductase (GR) was detected based on the method reported by Aviram *et al* (15). These experiments were repeated in triplicate.

**Flow cytometry.** Flow cytometry was employed to measure cell apoptosis. HK-2 cells were digested with 0.25% trypsin after 48 h in culture and centrifuged at 1,000  $\times$  g for 5 min at room temperature. The cell pellet was resuspended in phosphate-buffered saline (PBS) solution and the cell density was adjusted to 12 $\times$ 10<sup>5</sup> cells/ml. Cell suspension (100  $\mu$ l) was removed and stained with AnnexinV-FITC/PI staining kit (Carlsbad) according to the manufacturer's instructions. BD FACSCalibur flow cytometer (BD Biosciences) was used to analyze the stained cells and calculate the number of apoptotic cells in each group. All experiments were repeated three times.

**Statistical analysis.** Data were analyzed using SPSS 21.0 statistical software (SPSS Inc.), and conducted three times to calculate the mean value and standard deviation. Results are presented as mean  $\pm$  standard deviation (SD) and the Student's t-test was used to calculate the difference between two groups, while one-way ANOVA and Turkey's post-hoc test were utilized to analyze the differences between multiple groups. In cases of non-normal distribution, Mann-Whitney and Kruskal Wallis tests were performed. P<0.05 was considered statistically significant.

## Results

**Decreased expression of miR-30a-5p inversely correlated with GLUD1 protein expression in HK-2 cells with or without H/R stimulation.** Expression of miR-30a-5p and *GLUD1* was examined in HK-2 cells from each group. H/R-stimulated HK-2 cells were decreased in miR-30a-5p expression when compared with cells with no treatment (P<0.05; Fig. 1A). Furthermore, the H/R group had a significant increase in *GLUD1* expression compared with the control group (P<0.05; Fig. 1B). This result suggested that miR-30-5p and the *GLUD1* gene have vital effects on renal injury. To investigate whether miR-30a-5p and *GLUD1* were involved in the process of H/R injury, over-expression and inhibition of miR-30a-5p as well as silencing of *GLUD1* gene were performed in HK-2 cells. RT-qPCR determination showed that in the cells with or without H/R stimulation, mimics transfected with miR-30a-5p markedly promote the expression of miR-30a-5p and inhibited the expression of *GLUD1*, while inhibitors transfected with miR-30a-5p notably reduced the expression of miR-30a-5p and increased the expression of *GLUD1* (all P<0.05). In addition, silencing *GLUD1* by introducing *GLUD1* siRNA distinctly suppressed the expression of *GLUD1* in HK-2 cells (P<0.05; Fig. 1C-D).

**GLUD1 is a target gene of miR-30a-5p.** Bioinformatics prediction analysis (<http://www.targetscan.org>) demonstrated that the 3'-UTR region of *GLUD1* can specifically bind to miR-30a-5p (Fig. 2A). Dual luciferase reporter analysis was conducted to confirm this hypothesis, and revealed that compared with HK-2 cells in the NC1 + WT group, the mimic + WT group showed a sharp decline in luciferase activity (P<0.05), whereas cells in the mimic + MUT and NC1 + MUT had no changes in luciferase activity (Fig. 2B; all P>0.05).

Furthermore, we determined the protein levels of *GLUD1* in HK-2 cells following either up- or downregulation of miR-30a-5p. RT-qPCR results showed that the mimic group increased the level of miR-30a-5p, and the inhibitor group decreased the level of miR-30a-5p compared with the NC1 and NC2 groups, respectively (Fig. 2C). In addition, cells transfected with mimic were associated with a decrease in *GLUD1* protein expression and increased *GLUD1* protein expression in the inhibitor group, as determined by western blot analysis (Fig. 2D). These results suggested that *GLUD1* could function as the target gene for miR-30a-5p.

**miR-30a-5p inhibits oxidative stress in H/R-stimulated HK-2 cells.** In order to investigate the influence of miR-30a-5p in oxidative damage in HK-2 cells that undergo H/R, we investigated intracellular ROS generation and the activities of SOD,

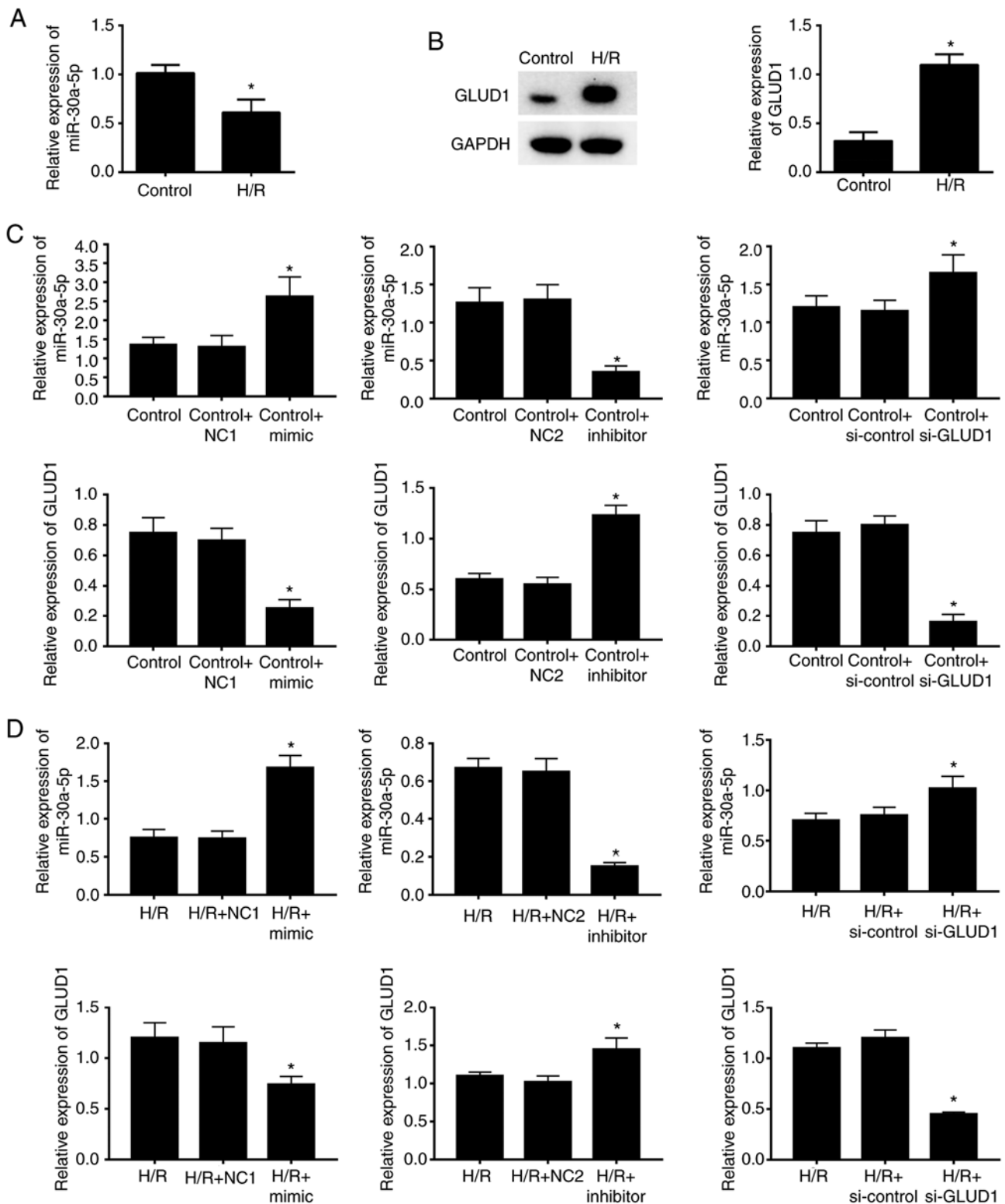


Figure 1. Expression of miR-30a-5p and GLUD1 in hypoxia/reoxygenation-treated HK-2 renal tubular epithelial cells. (A) Expression of miR-30a-5p detected by RT-qPCR. (B) Expression of miR-30a-5p detected by western blot analysis. (C) miR-30a-5p and GLUD1 expression transfected with miR-30a-5p mimic, inhibitor and NC and siRNA GLUD1 and NC in HK-2 renal tubular epithelial cells without hypoxia/reoxygenation. (D) miR-30a-5p and GLUD1 expression transfected with miR-30a-5p mimic, inhibitor and NC and siRNA GLUD1 and NC in HK-2 renal tubular epithelial cells after hypoxia/reoxygenation. \* $P < 0.05$  vs. control.

CAT, GPx, GST and GR in each group. As shown in Fig. 3, Hoechst 33342 was used to identify the nucleus, which appears blue and intracellular ROS generation appears green. Intracellular ROS generation significantly increased in the

H/R group with decreased activities of SOD, CAT, GPx, GST and GR when compared with the normal control group (all  $P < 0.05$ ). Moreover, the levels of the oxidative stress indicators in the H/R group have no significant difference compared with

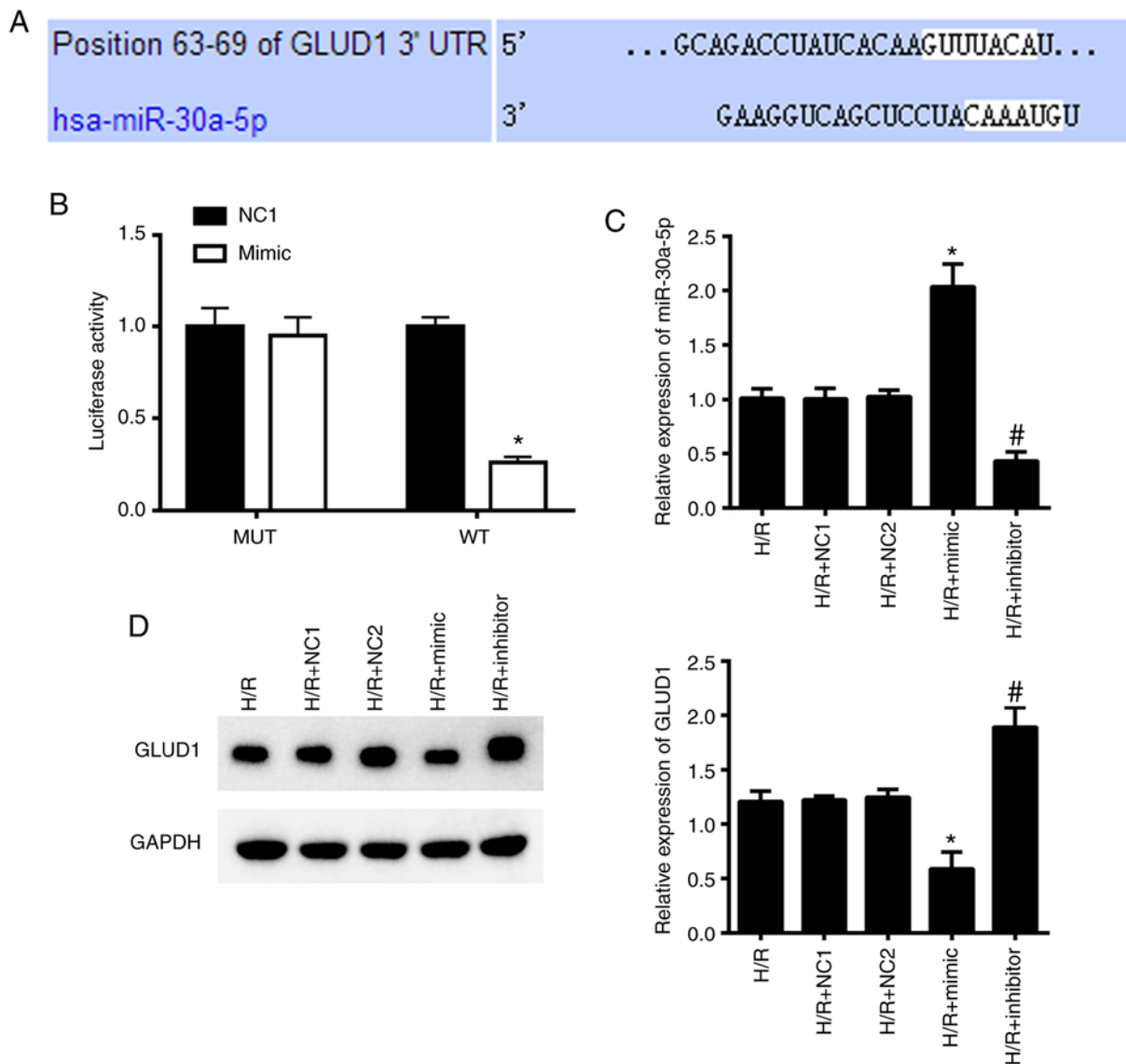


Figure 2. miR-30a-5p directly targets GLUD1. (A) Predicted binding site between miR-30a-5p and GLUD1 using TargetScan software. (B) GLUD1 is a target of miR-30a-5p, as shown by dual-luciferase reporter assay. (C) Expression of miR-30a-5p detected by RT-qPCR. (D) Expression of miR-30a-5p detected by western blot analysis. \* $P < 0.05$  vs. H/R + NC1, # $P < 0.05$  vs. H/R + NC2.

the NC groups (all  $P > 0.05$ ). Compared with the NC group, the H/R + mimic group showed a decrease in intracellular ROS generation and significantly elevated levels for SOD, CAT, GPx, GST and GR. By contrast, the H/R + inhibitor group had the completely opposite result (all  $P < 0.05$ ). This result demonstrated the negative action of miR-30a-5p in oxidative stress in H/R-stimulated HK-2 cells.

*miR-30a-5p repressed H/R-induced apoptosis in HK-2 cells.* Flow cytometry was utilized to evaluate apoptosis in HK-2 cells in each group (Fig. 4A). There was a significant increase in cell apoptosis in the H/R group compared with the control group ( $P < 0.05$ ), but there was no difference between the NC groups and H/R (all  $P > 0.05$ ). In comparison to the NC groups, H/R + mimic showed a downregulation in apoptosis whereas the H/R + inhibitor group had upregulation in apoptosis (all  $P < 0.05$ ).

Western blot analysis revealed a significant increase in Bax/Bcl-2 and cleaved caspase-3/caspase-3 expression in the

H/R and NC groups (all  $P < 0.05$ ). Furthermore, Bax/Bcl-2 and cleaved caspase-3/caspase-3 expression were significantly decreased in the H/R + mimic group compared to the NC group (all  $P < 0.05$ ). Nevertheless, the trend of Bax/Bcl-2 and cleaved caspase-3/caspase-3 were evidently contrary in the H/R + inhibitor group (all  $P < 0.05$ ; Fig. 4B). Notably, miR-30a-5p suppressed cell apoptosis in H/R-stimulated HK-2 cells.

*Inhibition of GLUD1 rescues the influence of miR-30a-5p inhibitor in oxidative damage stimulated by H/R.* To assess whether miR-30a-5p contributed to oxidative stress in HK-2 cells exposed to H/R by regulating GLUD1, we suppressed the expression of GLUD1 in HK-2 cells along with inhibition of miR-30a-5p. We found that expression of GLUD1 protein was notably reduced when *GLUD1* gene was downregulated with the siRNA ( $P < 0.05$ ; Fig. 5A). Furthermore, when compared with the H/R + NC2 + si-control group, intracellular ROS generation was significantly increased in the H/R + inhibitor + si-control group with decreased activities of GST, SOD, CAT, GPx and

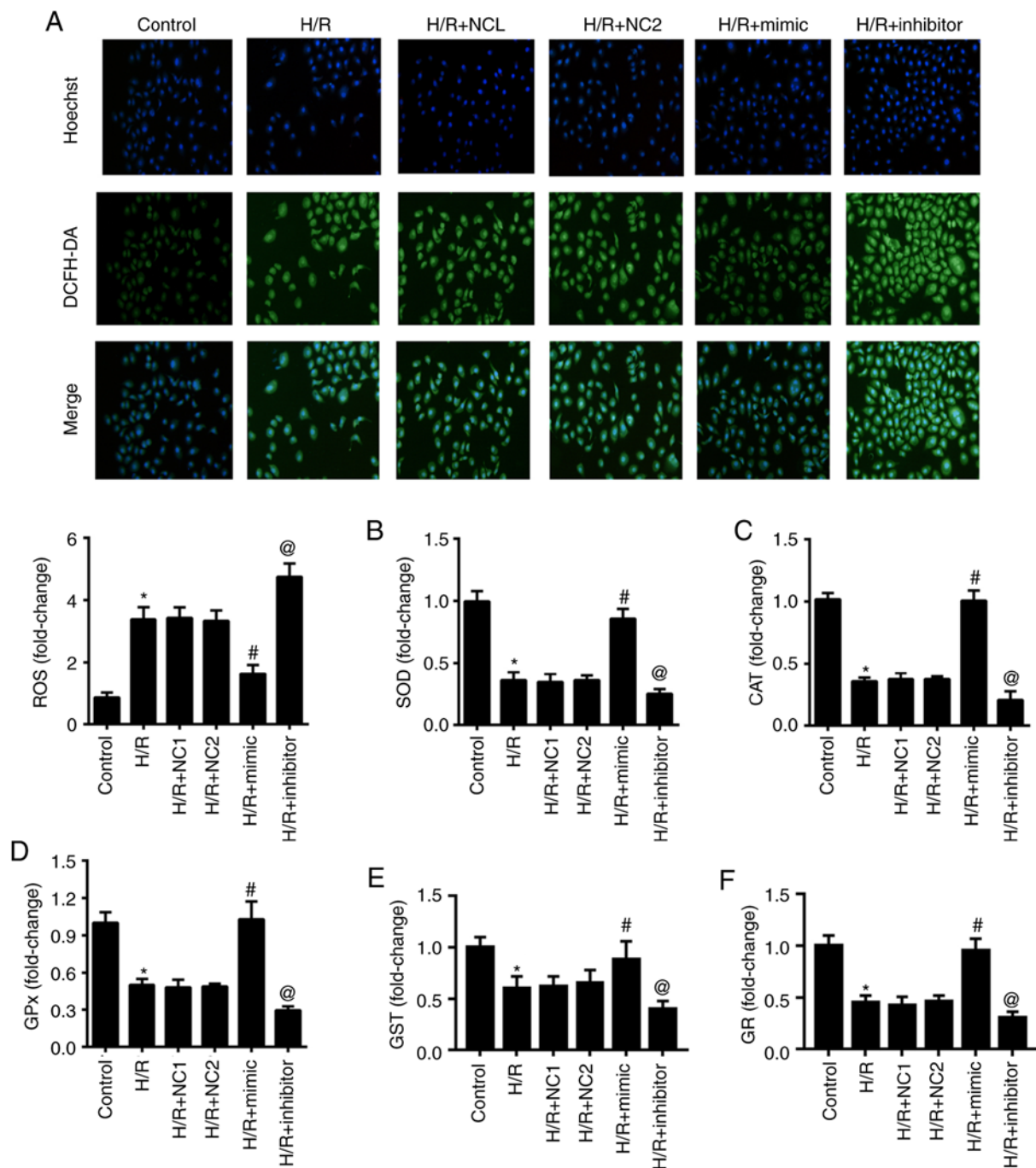


Figure 3. miR-30a-5p inhibits hypoxia/reoxygenation-induced oxidative stress in HK-2 renal tubular epithelial cells. (A) The production of ROS in HK-2 cells induced by H/R. (B) The activity of SOD in HK-2 cells induced by H/R. (C) The activity of CAT in HK-2 cells induced by H/R. (D) The activity of GPx in HK-2 cells induced by H/R. \* $P < 0.05$  vs. control, # $P < 0.05$  vs. H/R + NC1, @ $P < 0.05$  vs. H/R + NC2.

GR. By contrast, reduced generation of intracellular ROS and GST as well as increased activities of SOD, CAT, GPx and GR were observed in the H/R + NC2 + si-GLUD1 group ( $P < 0.05$ ). Simultaneously, interference of GLUD1 expression abolished miR-30a-5p inhibitor effects on H/R-induced oxidative stress in the H/R + inhibitor + si-GLUD1 group ( $P < 0.05$ ; Fig. 5B-G). Thus, inhibition of GLUD1 rescues the impact of miR-30a-5p inhibitor on oxidative stress in HK-2 cells.

*Inhibition of GLUD1 rescues the role of miR-30a-5p inhibitor in apoptosis in HK-2 cells.* As shown in Fig. 6A, when compared with the H/R + NC2 + si-control group, the rate of apoptosis in

HK-2 cells was increased with inhibition of miR-30a-5p in the H/R + inhibitor + si-control group and reduced by the suppression of GLUD1 in the H/R + NC2 + si-GLUD1 group (all  $P < 0.05$ ). Moreover, the downregulated expression of GLUD1 mitigated the function of miR-30a-5p inhibitor on apoptosis in HK-2 cells in the H/R + inhibitor + si-GLUD1 group (all  $P < 0.05$ ).

Consistent with annexin V/PI assay, the ratio of Bax/Bcl-2 and the ratio of cleaved-caspase-3/caspase-3 were markedly increased in the H/R + inhibitor + si-control group when compared with the H/R + NC2 + si-control group. However, the ratio of Bax/Bcl-2 and the ratio of cleaved-caspase-3/caspase-3 were reversed in the H/R + NC2 + si-GLUD1 group (Fig. 6B).

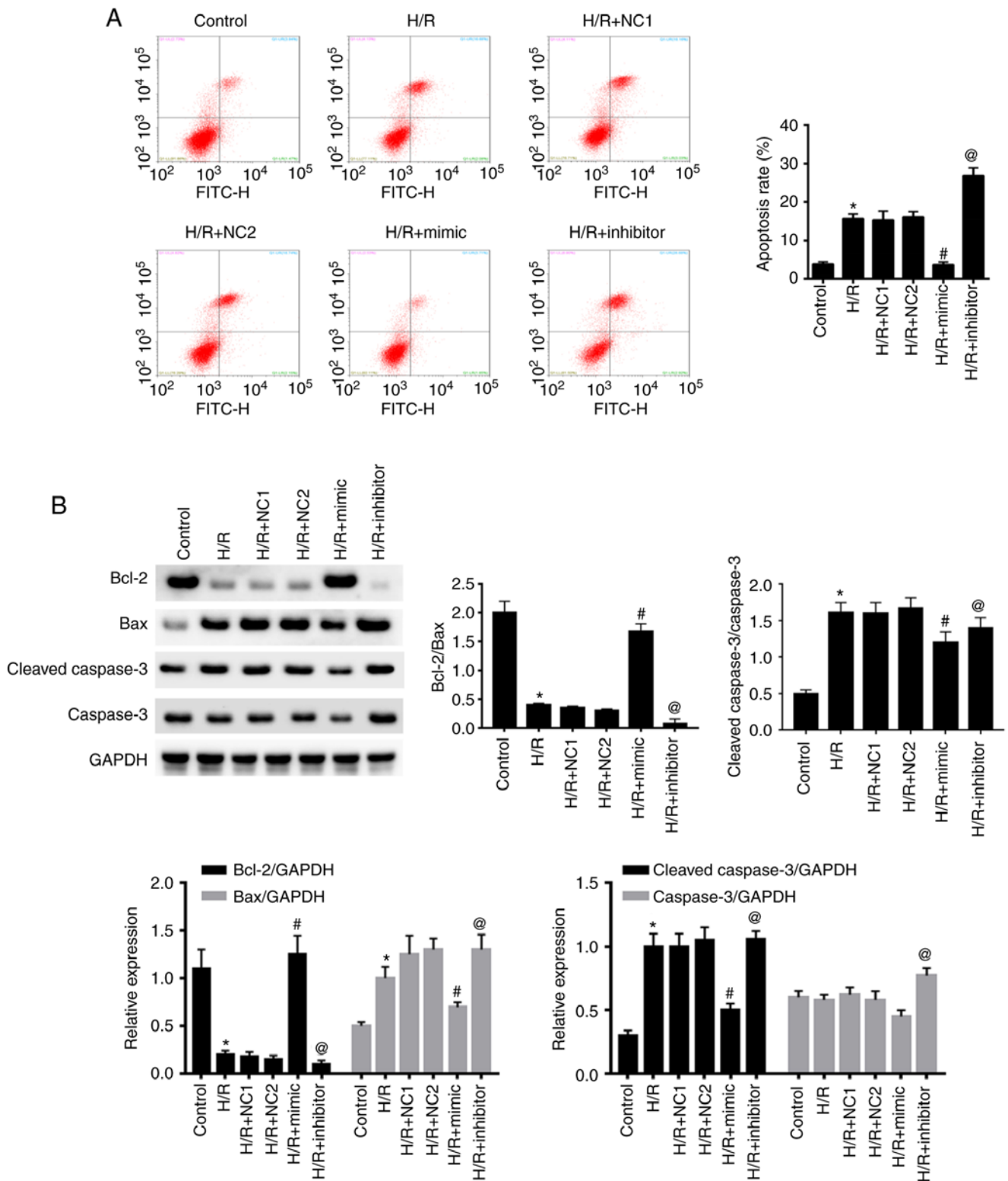


Figure 4. miR-30a-5p inhibits hypoxia/reoxygenation-induced apoptosis in HK-2 renal tubular epithelial cells. (A) Flow cytometry to detect apoptosis induced by H/R. (B) Western blot to detect the expression of proteins related to apoptosis induced by H/R. \*P<0.05 vs. control, #P<0.05 vs. H/R + NC1, @P<0.05 vs. H/R + NC2.

A reduced ratio of Bax/Bcl-2 and cleave-caspase-3/caspase-3 was observed in the H/R + inhibitor + si-GLUD1 group compared with the H/R + inhibitor + si-control group (all P<0.05; Fig. 6C-E). These results indicated that silencing the *GLUD1* gene significantly attenuated the influence of miR-30a-5p inhibitor on apoptosis in HK-2 cells.

### Discussion

An important effect associated with oxidative damage is that the renal tubular epithelial cells are the first involved in acute renal injury (16,17). Emerging evidence indicates that miR-30a-5p acts as a suppressor for cancer, regulating the



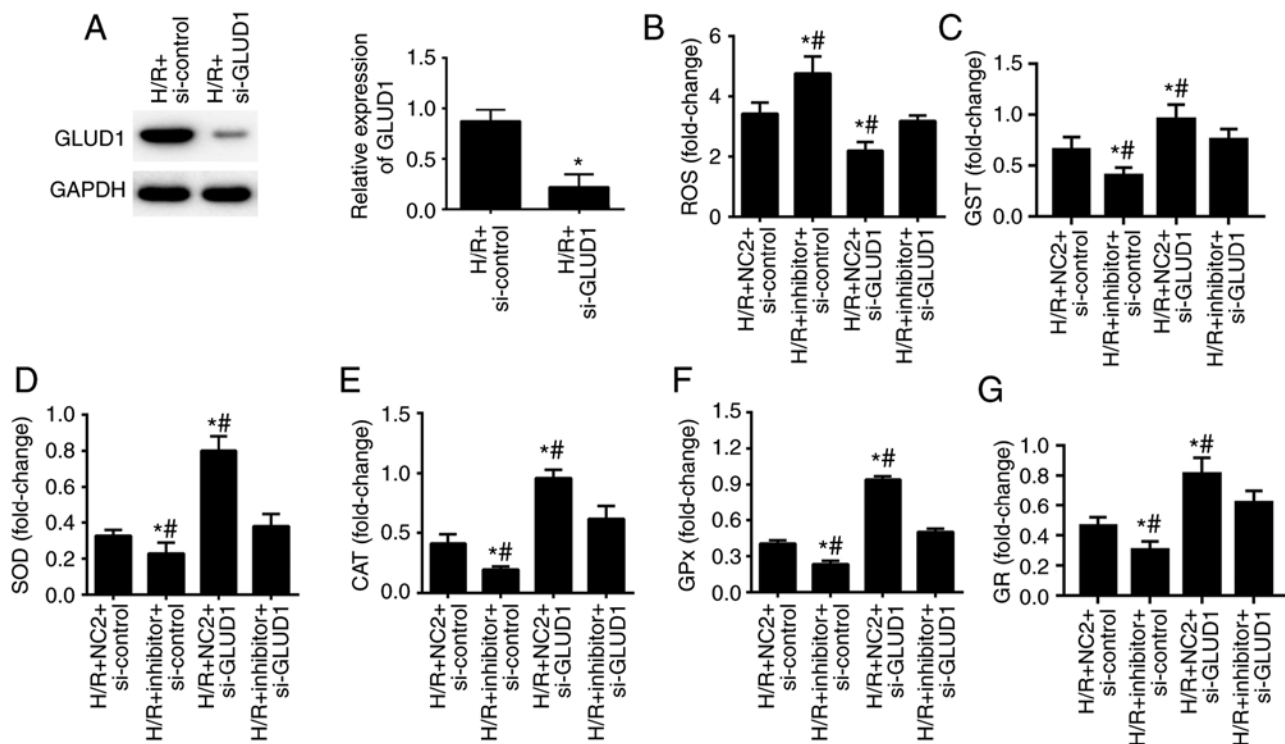


Figure 5. Inhibition of GLUD1 rescues the effect of miR-30a-5p inhibitor on oxidative stress in HK-2 renal tubular epithelial cells. (A) The protein expression of GLUD1 in HK-2 cells detected by western blot analysis. (B) The production of ROS in HK-2 cells induced by H/R. (C) The activity of SOD in HK-2 cells induced by H/R. (D) The activity of CAT in HK-2 cells induced by H/R. (E) The activity of GPx in HK-2 cells induced by H/R. \* $P < 0.05$  vs. H/R + NC2 + si-control, # $P < 0.05$  vs. H/R + inhibitor + si-GLUD1.

growth, migration, and invasion of various tumors, including maintaining renal function during acute renal injury (18-20). This study aimed to explore the miR-30a-5p effect on HK-2 renal tubular epithelial cells in renal injury.

miR-30a-5p binding to the 3'-UTR region of the *GLUD1* gene was confirmed by dual-luciferase reporter assay in the present study. GDH, encoded by *GLUD1*, is an important mitochondrial enzyme that is associated with amino acid metabolism and the tricarboxylic acid cycle (21,22). It has been reported that GDH is mainly distributed in the mitochondria of liver cells and only increases significantly when liver cells are damaged (23,24). Thus, overexpression of the *GLUD1* gene was suspected to be involved in the course of renal injury. Our results demonstrated that HK-2 cells under H/R exposure reduced the level of miR-30a-5p, which upregulated the expression of its target gene, *GLUD1*. This is similar to previous studies (23,24). Results of the present study laid the foundation for studying the mechanism of miR-30a-5p affecting renal H/R damage on a molecular basis.

Oxidative stress is directly related to the occurrence and development of I/R injury in myocardial cells and can be regarded as an important treatment target for I/R injury (25). In addition to the marked increase in ROS production and GST, which affects cellular mitochondrial damage and renal dysfunction (26,27), decreased activity of the intracellular antioxidants SOD, CAT, GPx and GR, were observed leading to oxidative stress (28,29). To further investigate the effect of the downregulation of *GLUD1* by miR-30a-5p on H/R injury, the status of oxidative stress in HK-2 cells was analyzed. In the model of H/R injury, HK-2 cells transfected

with miR-30a-5p mimic and *GLUD1* siRNA enhanced the production of ROS along with reduced activities of SOD, CAT, GPx, GST and GR, whereas cells in the miR-30a-5p inhibitor group aggravated the oxidative stress induced by H/R. Consistent with the present study, Fu *et al* (30) reported that miR-30a-5p relieved oxidative stress in spinal cord by regulating Neurod 1 and the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway. Zhou *et al* (5) reported that miR-30b-5p exerts a critical role in acute lung injury in children. More importantly, we also found that inhibition of *GLUD1* attenuated the effect of miR-30a-5p inhibitor on oxidative stress in HK-2 cells. This suggests that miR-30a-5p influences oxidative stress in the processes of renal injury by negative regulation of *GLUD1*.

As a consequence of oxidative stress, apoptosis is significantly involved in the pathogenesis of renal injury and by reducing cell apoptosis can effectively ameliorate renal injury (31,32). Mitochondria are not only the site of oxidative metabolism and energy transformation, but also have the function of regulating programmed cell death. Oxidative stress directly leads to mitochondrial damage, which causes the initiation of the mitochondrial apoptosis program. Bax/Bcl-2 as well as cleaved-caspase-3/caspase-3 are critical markers of apoptosis. The former promotes apoptosis, while the latter inhibits apoptosis and they regulate apoptosis by controlling the permeability of mitochondrial membranes (33). In addition, the *Bcl-2* gene can regulate the activity of cleaved-caspase-3, which eventually leads to apoptotic cascade (34,35). Our research showed that, when the level of miR-30a-5p was upregulated



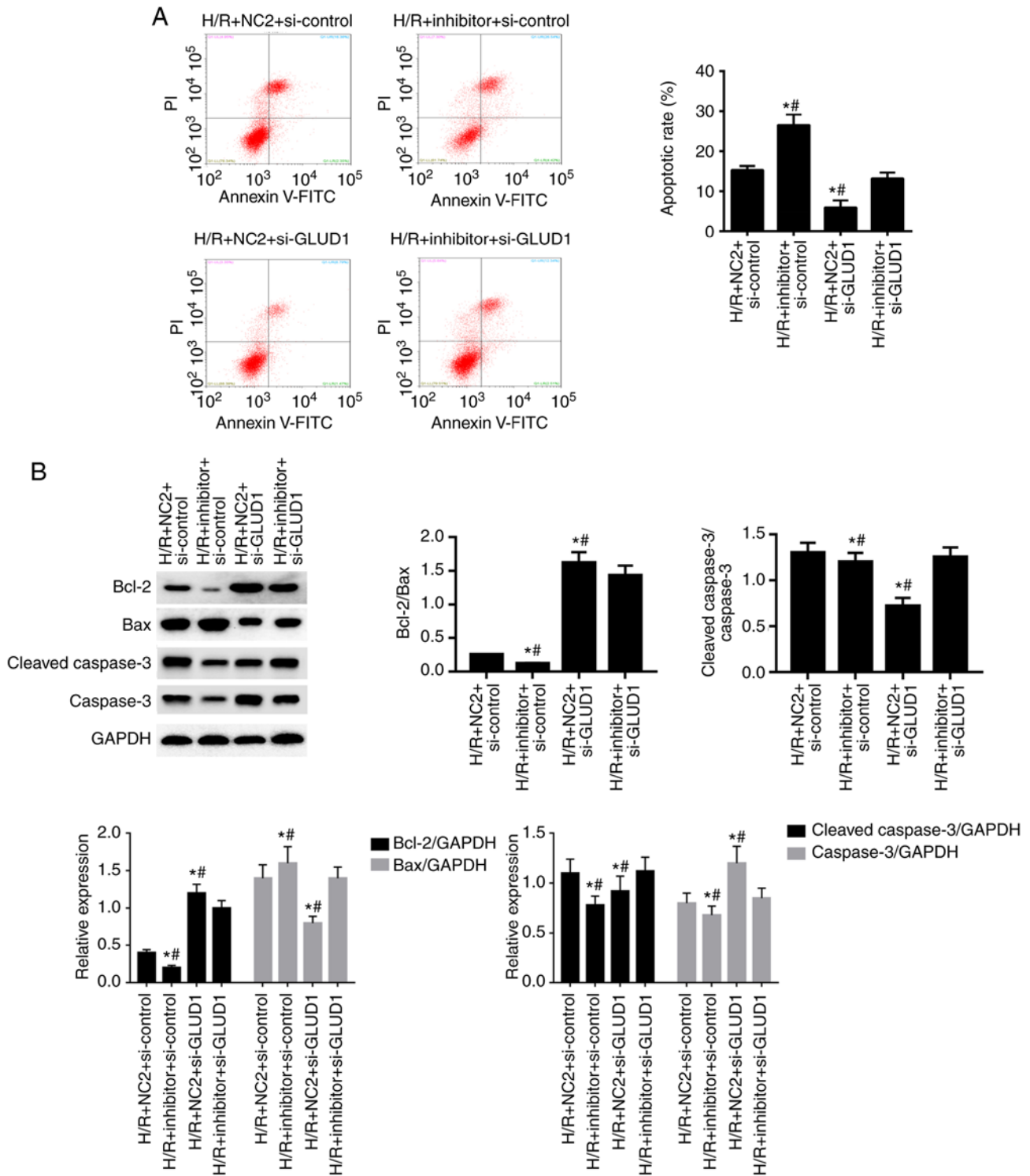


Figure 6. Inhibition of GLUD1 rescues the effect of miR-30a-5p inhibitor on apoptosis in HK-2 renal tubular epithelial cells. (A) Flow cytometry to detect apoptosis induced by H/R. (B) Western blot to detect the expression of proteins related to apoptosis induced by H/R. \*P<0.05 vs. H/R + NC2 + si-control, #P<0.05 vs. H/R + inhibitor + si-GLUD1.

or the level of GLUD1 was suppressed, the cell apoptosis rate stimulated by H/R was reduced. The opposite results occurred after miR-30a-5p expression was inhibited. Western blot results revealed that cells containing miR-30a-5p mimics and GLUD1 siRNA have a significant reduction in the ratio of Bax/Bcl-2 and the ratio of cleaved-caspase-3/caspase-3. The results were the opposite in HK-2 cells when treated with miR-30a-5p inhibitor. Similar to our result, Chien *et al* (35) found that

after renal ischemia reperfusion in rats, the rate of apoptosis in renal cells increased with time as well as the increasing ratio of Bax/Bcl-2 and the ratio of cleaved-caspase-3/caspase 3. Furthermore, reducing the expression of GLUD1 weakened the impact of miR-30a-5p inhibitor on apoptosis in HK-2 cells treated with H/R. Thus, miR-30a-5p affects apoptosis induced by renal injury by targeting the *GLUD1* gene. However, there remain several limitations to our study. For example, lack of

antioxidant enzyme activity detection in H/R cells as well as the detection of convincing indicators of cell apoptosis, which may complement and refine the results in the current manuscript. More importantly, whether the downstream signaling pathway of miR-30a-5p plays a role in H/R injury was not thoroughly investigated, and may be explored in a further study.

In conclusion, the findings of our study indicate that, during H/R miR-30-5p helps to avoid oxidative stress and prevent apoptosis by negatively regulating GLUD1. Therefore, miR-30-5p mimic is potentially therapeutic for renal injury and worth further study.

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### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

YH conceived and designed this study. XL, DC and TZ performed the experiments and collected data. ZY and RX analyzed and interpreted the experimental data. YH and XL wrote the manuscript. YH reviewed and revised the paper. All authors approved the version to be published.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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