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ORIGINAL RESEARCH

Circular RNA circNRIP1 Sponges microRNA-138-5p to Maintain Hypoxia-Induced Resistance to 5-Fluorouracil Through HIF-1α-Dependent Glucose Metabolism in Gastric Carcinoma

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Background: Hypoxia-induced chemoresistance is recognized as a major obstacle to the successful treatment of gastric cancer (GC). Circular RNAs (circRNAs) have been proposed to implicate in resistance to chemotherapeutic drugs. However, whether circNRIP1 is involved in the development of hypoxia-induced 5-fluorouracil (5-FU) resistance remains largely unknown.

Methods: Gene expression was evaluated using quantitative real-time polymerase chain reaction and Western blot. The impact of circNRIP1 on hypoxia-induced resistance to 5-FU was investigated by determining glucose consumption, lactate production and glucose-6-phosphate (G6P) levels. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide assay was performed to assess cell survival.

Results: circNRIP1 was upregulated in GC cells. Hypoxia induced the upregulation of circNRIP1 and reduced the sensitivity of GC cells to 5-FU, as evidenced by the increase in multidrug resistance 1 gene, P-glycoprotein, hypoxia-inducible factor-1α (HIF-1α) and G6P levels, glucose consumption, lactate production, as well as cell survival. Silencing of circNRIP1 enhanced the sensitivity of GC cells to 5-FU under a hypoxic condition. microRNA (miR)-138-5p was confirmed as a downstream target gene of circNRIP1, and upregulation of miR-138-5p could reverse the effect of circNRIP1 on hypoxia-induced 5-FU resistance. Additionally, HIF-1α was a target gene of miR-138-5p. More significantly, the effect of circNRIP1 on hypoxia-induced 5-FU resistance was markedly blocked by 2-DG treatment.

Conclusion: circNRIP1 functioned as a miR-138-5p sponge to enhance hypoxia-induced resistance to 5-FU through modulation of HIF-1α-dependent glycolysis, which provides a novel potential approach to overcome hypoxia-induced 5-FU resistance in GC.

Keywords: 5-fluorouracil, gastric carcinoma, circular RNA circNRIP1, miR-138-5p, multidrug resistance 1 gene, P-glycoprotein, hypoxia-inducible factor-1α, glucose-6-phosphate

Introduction

Gastric cancer (GC) is one of the most prevalent and aggressive malignancies in the alimentary tract, which is till the third leading cause of cancer-related mortalities throughout the world.[1] The incidence of GC is on the rise, approximately 1000 000 new cases are diagnosed annually in the world.[2] The lack of characteristic manifestations makes GC arduous to diagnose in its early stage, which is the main

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reason leading to poor prognosis.[3] 5-fluorouracil (5-FU) is currently a first-line agent for the clinical treatment of GC, which affects the de novo synthesis of DNA by multiple mechanisms, such as incorporation into RNA or DNA, and suppression of thymidine synthase, resulting in cell death.[4] Despite their overall efficacy, GC cells often develop resistance to 5-FU. Thus, novel strategies to overcome the resistance of GC cells to 5-FU are urgently needed.

Chemoresistance is the chief cause of clinical chemotherapy failure. The mechanism of chemoresistance is complex, in which tumor microenvironment potentially acts as a principal factor.[5] Oxygen deprivation (hypoxia) is a hallmark of solid tumors, which is caused by the consumption of oxygen by rapidly proliferating cells that exceeds the oxygen supply.[6] Hypoxia is able to promote tumorigenesis, the epithelial-to-mesenchymal transition (EMT), tumor metastasis, as well as affect tumor cell responsiveness to anti-tumor agents.[7] In the last years, hypoxia-induced chemoresistance has been recognized as a major obstacle to the successful treatment of GC.[8] Hence, further exploration of hypoxia-induced chemoresistance is of significance for GC therapy.

Recently, multiple lines of evidence increasingly linked dysregulation of noncoding RNAs (ncRNAs), such as microRNAs (miRNAs) and circular RNAs (circRNAs), to a wide range of biological processes.[9] Notably, miRNAs have been extensively investigated in human cancers, where they potentially participate in the process of human cancers by complementary binding to the 3′untranslated regions (3′UTR) of their target genes.[10] In comparison, the involvement of circRNAs in the process of human cancers is much less certain. circRNAs, a novel class of RNAs with a covalently closed-loop structure, are much more resistant to degradation by exonuclease.[11,12] The regulatory function of circRNAs in the tumorigenesis of GC has been documented: circPSMC3 as a competitive endogenous RNA (ceRNA) restrained the proliferation and metastasis of GC cells by sponging miR-296-5p.[13–15] Also, hsa_circ_0067582 and hsa_circ_0005758 have been reported to be underexpressed in GC tissues and associated with GC patients' stages, suggesting their potential role for GC diagnosis.[16] Additionally, circ-RanGAP1 acted as a sponge for miR-877-3p to upregulate the expression of vascular endothelial growth factor and then facilitate the migration and invasion of GC cells.[17] Besides, hsa_circ_0000467 was identified as a novel independent diagnostic and prognostic factor for GC, and silencing of hsa_circ_0000467 was able to inhibit the proliferation and metastasis and promote the apoptosis of GC cells.[18] circNRIP1 (circ_0004771), a novel identified circRNA, is transcribed from the nuclear receptor interaction protein (NRIP) gene.[19] circNRIP1 has been reported to be upregulated in GC and acted as a promoter of GC progression. For instance, Zhang et al argued that circNRIP1 functioned as a miR-149-5p sponge to promote the proliferation, migration, and invasion of MKN-45 and BGC-823 cells through regulation of the AKT1/mTOR signaling pathway.[20] More recent studies by Xie's group demonstrated that silencing of circNRIP1 restrained the proliferation and induced the apoptosis of MCF-7 and MDA-MB-231 cells by regulating the miR-653/Zinc finger E-box binding homeobox 2 axis.[21] Nevertheless, its precise role in hypoxia-induced chemoresistance in GC has never been studied.

Herein, we investigated the contribution of circNRIP1 to hypoxia-induced chemoresistance in GC, and its downstream regulatory mechanism was also explored. The results manifested that circNRIP1 functioned as a miR-138-5p sponge to maintain hypoxia-induced resistance to 5-FU through hypoxia-inducible factor-1α (HIF-1α)-dependent glucose metabolism in GC, which provides a potential therapeutic therapy for combating hypoxia-induced resistance in GC.

Materials and Methods

Cell Culture and Treatment

Human normal gastric cell line GES-1 and GC cell lines (SGC-7901, AGS, MKN-45, MGC-803, HGC-27, and BGC-823) were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All the cells were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), and grown in a 5% CO_2 incubator at 37°C. For hypoxia experiments, SGC-7901 and BGC-823 were cultured at 37°C in a multigas incubator with 5% CO_2, 94% N_2, and 1% O_2.

miR-138-5p, miR-ctrl, small interfering RNA targeting NRIP1 (si-NRIP1), lentivirus for overexpressing NRIP1 (Lv-NRIP1), and their negative control (Ctrl) were purchased from Gene Pharma (Shanghai, China). These constructs were transfected into SGC-7901 and BGC-823 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in keeping with the manufacturer's instructions. Cells were infected with Lv-NRIP1 or Lv-Ctrl at a multiplicity of infection of 20. After incubation for 24 h, supernatant from these wells were replaced with

complete culture medium and cultured for another 24 h for subsequent analyses.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cells using Trizol reagent (Invitrogen) following the product manual. cDNA was synthesized using oligo-dT and superscript II (Life Technologies, Grand Island, NY, USA) and tested for circNRIP1, multidrug resistance 1 gene (MDR1) and β-actin expression using the Platinum SYBR Green qPCR SuperMix-UDG with Rox (Life Technologies). miR-138-5p expression was determined using TaqMan® assay (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. The primers used for qRT-PCR analysis were listed as follows: circNRIP1: forward primer 5′-TCCGGATGACATCAGAGCTAC-3′ and reverse primer 5′-TCAAGTGTGCATCTTCTGGCT-3′; MDR1: forward primer 5′-GCTGTCAAGGAAGCCAA TGCCT-3′ and reverse primer 5′-TGCAATGGCGATC CTCTGCTTC-3′; β-actin: forward primer 5′-TTGTTA CAGGAAGTCCCTTGCC-3′ and reverse primer 5′-ATGCTATCACCTCCCCTGTGTG-3′; miR-138-5p: forward primer 5′-GCTTAAGGCACGCGG-3′ and reverse primer 5′-GTGCAGGGTCCGAGG-3′; U6: forward primer 5′-CTCGCTTCGGCAGCACA-3′ and reverse primer 5′-AACGCTTCACGAATTTGCGT-3′. Gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method using β-actin and U6 as endogenous controls.

Western Blot

Cell lysates were prepared by scraping SGC-7901 and BGC-823 cells with lysis buffer, and then electrophoresed on a 14% sodium dodecyl sulfate-polyacrylamide gel. After gel electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane. Following this, the membranes were blocked with 5% skim milk, probed with primary antibodies against HIF-1α (Abcam, Cambridge, MA, USA) and P-glycoprotein (p-gp; Abcam), and then immunoblotted with horseradish peroxidase-conjugated secondary antibody (Abcam). The proteins were visualized by enhanced chemiluminescence (Solarbio, Beijing, China) following the product manual.

Measurement of Glucose Uptake and Lactate

SGC-7901 and BGC-823 cells were collected, homogenized and centrifuged after treatment. The supernatant of SGC-7901 and BGC-823 cells was collected and tested for the amount of glucose and lactate using a glucose/lactate assay kit (Jiancheng Bioengineering Institute, Nanjing, China), as directed by the manufacturer's instructions.

Measurement of Glucose-6-Phosphate (G6P) Levels

The level of G6P in cells was evaluated using a G6P Assay Kit with WST-8 (Beyotime, Shanghai, China) per manufacturer's instructions.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolim Bromide (MTT) Assay

Cell survival was evaluated using the MTT assay (Beyotime), according to the manufacturer's instructions. SGC-7901 and BGC-823 cells were collected and seeded in a 96-well plate overnight after transfection. Thereafter, SGC-7901 and BGC-823 cells were incubated with increasing doses (0, 1, 2.5, 5, 10 and 20 μM) of 5-FU under a hypoxic condition. After 48 h of incubation, 10 μL of MTT solution was added into each well. After 4 h of incubation, SGC-7901 and BGC-823 cells were treated with 100 μL of formazan solution for 3 h and assayed for the absorbance of each well at 570 nm using a microplate reader.

Luciferase Reporter Assay

The circNRIP1 or 3′UTR of HIF-1α sequence containing the miR-138-5p-binding sites was amplified and subcloned into pGL3 luciferase reporter (Promega, Madison, WI, USA) to generate the HIF-1α-wt and circNRIP1-wt constructs. Meanwhile, mutations were introduced into the miR-138-5p-binding sites in HIF-1α or circNRIP1 sequence and then inserted into pGL3 luciferase reporter to creat the HIF-1α-mut and circNRIP1-mut constructs. These reporters were transfected into SGC-7901 and BGC-823 cells together with miR-138-5p or miR-ctrl. After 48 h, transfected cells were collected, lysed and analyzed using Dual-Luciferase reporter assay system from Promega, according to the instruction of the manufacturer.

Statistical Analysis

Data were given as the mean ± standard error of the mean. Statistical tests were done by one-way analysis of variance or student's *t* test using SPSS 20.0 (IBM, SPSS, Chicago,

IL, USA) software. A probability value of $P<0.05$ was considered to be the limit of statistical significance.

Results

circNRIP1 Was Upregulated in GC Cells Under Hypoxia

To evaluate the role of circNRIP1 in GC, we examined the expression level of circNRIP1 using qRT-PCR. The results showed that circNRIP1 expression was upregulated in GC cell lines (SGC-7901, AGS, MKN-45, MGC-803, HGC-27, and BGC-823) relative to the normal gastric cell line GES-1 (Figure 1A). To explore the effect of hypoxia on the expression of circNRIP1 in GC cells, GC cells were exposed to hypoxia or normoxia and then tested for circNRIP1 expression. Compared to the normoxia group, hypoxia treatment caused a marked elevation of circNRIP1 expression level in GC cell lines (SGC-7901, AGS, MKN-45, and BGC-823) (Figure 1B–E).

Hypoxia Treatment-Induced Resistance to 5-FU in GC Cells

As shown in Figure 2A and B, hypoxia treatment increased the mRNA levels of MDR1 and the protein levels of p-gp in SGC-7901 and BGC-823 cells. Moreover, hypoxia treatment led to an elevation of HIF-α levels in SGC-7901 and BGC-823 cells (Figure 2C). In addition, marked increases of glucose consumption and lactate production were discovered in SGC-7901 and BGC-823 cells cultured under hypoxia (Figure 2D and E). Likewise, hypoxia administration led to an obvious increase of G6P levels in SGC-7901 and BGC-823 cells (Figure 2F). In parallel, the cytotoxic effect of 5-FU was assessed in SGC-7901 and BGC-823 cells under a normoxic or hypoxic condition using MTT assay. The results showed that 5-FU dose-dependently reduced the cell survival rate of SGC-7901 and BGC-823 cells under a normoxic condition. However, hypoxia treatment obviously blocked the cytotoxic effect of 5-FU on survival of SGC-7901 and BGC-823 cells (Figure 2G).

Knockdown of circNRIP1 Inhibited Hypoxia-Induced 5-FU Resistance in GC Cells

To evaluate the contribution of circNRIP1 to hypoxia-induced 5-FU resistance in GC cells, SGC-7901 and BGC-823 cells were transfected with si-ctrl or si-NRIP1 and then incubated under a hypoxic condition. As expected, the expression of circNRIP1 was reduced in BGC-823 cells transfected with si-NRIP1 under a normoxic or hypoxic

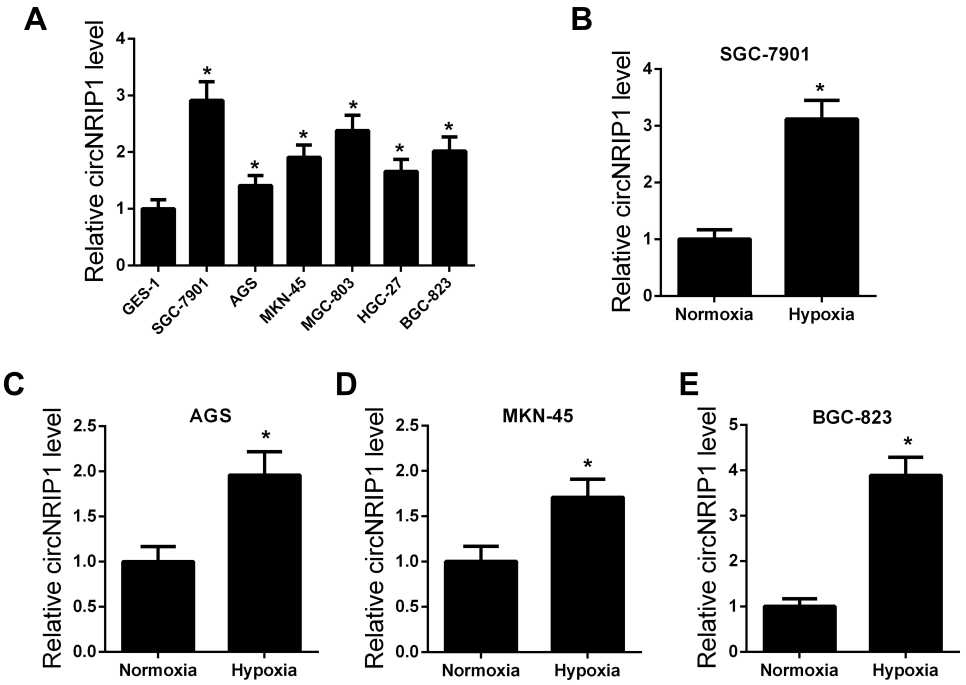

Figure 1 circNRIP1 was upregulated in GC cells under hypoxia. (**A**) qRT-PCR analysis of circNRIP1 expression showing increased expression of circNRIP1 in GC cell lines (SGC-7901, AGS, MKN-45, MGC-803, HGC-27, and BGC-823). (**B**–**E**) GC cell lines (SGC-7901, AGS, MKN-45, and BGC-823) were exposed to hypoxia and tested for circNRIP1 expression using qRT-PCR. *P< 0.05.

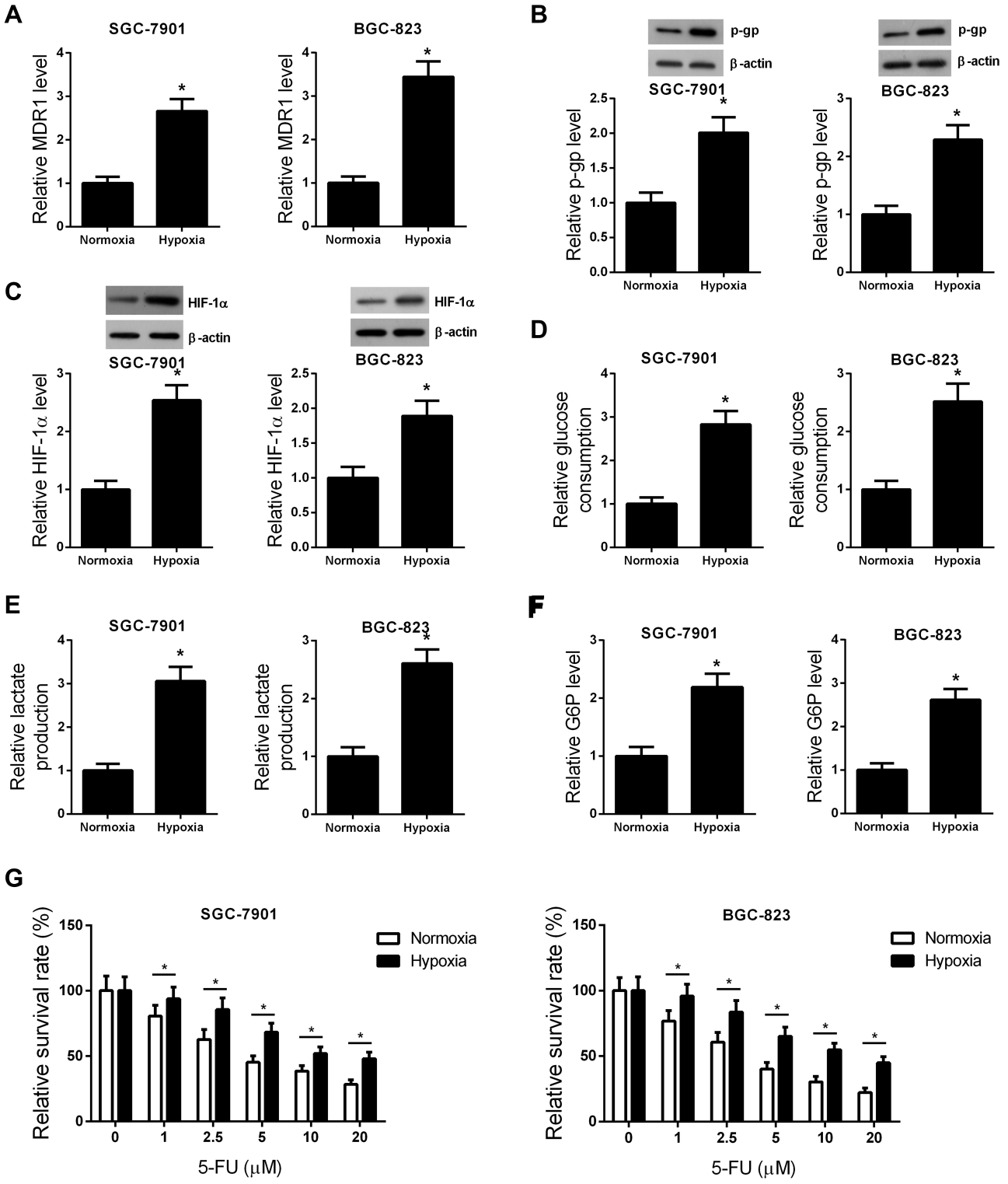

Figure 2 Hypoxia treatment-induced resistance to 5-FU in GC cells. (**A**) qRT-PCR analysis of MDR1 expression in SGC-7901 and BGC-823 cells under a normoxic or hypoxic condition. (**B**) Western blot analysis of p-gp expression in SGC-7901 and BGC-823 cells under a normoxic or hypoxic condition. (**C**) Western blot analysis of HIF-α expression in SGC-7901 and BGC-823 cells under a normoxic or hypoxic condition. The glucose consumption (**D**) and lactate production (**E**) were measured in SGC-7901 and BGC-823 cells under a normoxic or hypoxic condition. (**F**) qRT-PCR analysis of G6P levels in SGC-7901 and BGC-823 cells under a normoxic or hypoxic condition. (**G**) MTT assay was applied to detect cell survival after SGC-7901 and BGC-823 cells treated with increasing doses (0, 1, 2.5, 5, 10 and 20 μM) of 5-FU under a normoxic or hypoxic condition. *$P < 0.05$.

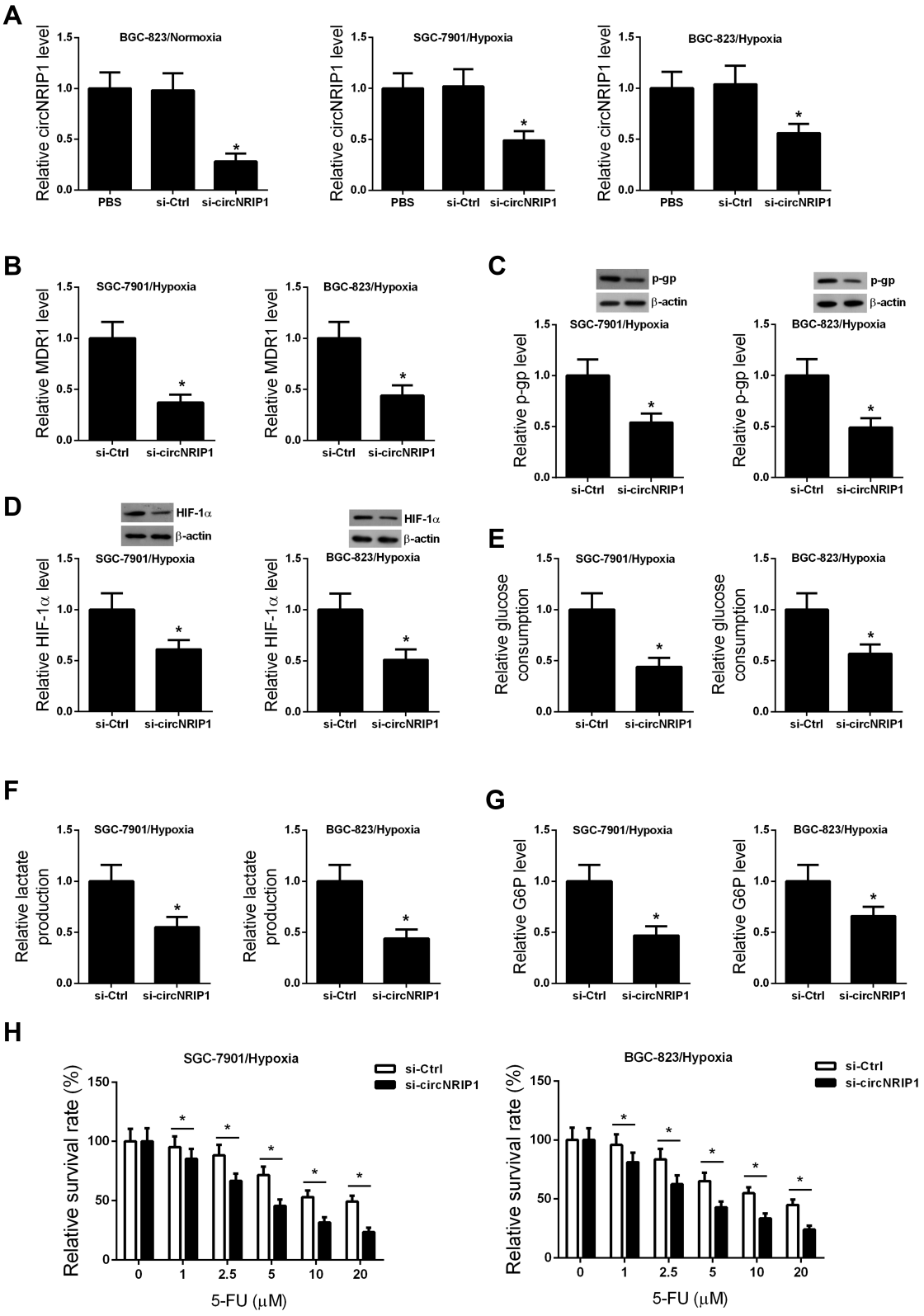

Figure 3 Knockdown of circNRIP1 inhibited hypoxia-induced 5-FU resistance in GC cells. (**A**) SGC-7901 and BGC-823 cells transfected with si-ctrl or si-NRIP1 were incubated under hypoxic condition for 24 h, followed by qRT-PCR analysis of circNRIP1 gene. (**B**) qRT-PCR analysis of MDR1 expression in SGC-7901 and BGC-823 cells transfected with si-ctrl or si-NRIP1 under a hypoxic condition. (**C** and **D**) Western blot analysis of p-gp and HIF-1α levels in SGC-7901 and BGC-823 cells transfected with si-ctrl or si-NRIP1 under a hypoxic condition. (**E**) The glucose consumption and (**F**) lactate production were measured in SGC-7901 and BGC-823 cells transfected with si-ctrl or si-NRIP1 under a hypoxic condition. (**G**) qRT-PCR analysis of G6P levels in SGC-7901 and BGC-823 cells transfected with si-ctrl or si-NRIP1 under a hypoxic condition. (**H**) SGC-7901 and BGC-823 cells transfected with si-ctrl or si-NRIP1 were grown under a hypoxic condition, followed by incubation with different doses (0, 1, 2.5, 5, 10 and 20 μM) of 5-FU. Cell survival was measured by MTT assay. *$P < 0.05$.

condition, as well as in SGC-7901 cells transfected with si-NRIP1 under a hypoxic condition (Figure 3A). Moreover, SGC-7901 and BGC-823 cells transfected with si-NRIP1 showed a decrease in MDR1 mRNA levels and p-gp protein levels under a hypoxic condition (Figure 3B and C). A clear reduction in HIF-1α protein levels was also observed in SGC-7901 and BGC-823 cells transfected with si-NRIP1 under a hypoxic condition (Figure 3D). Furthermore, knockdown of circNRIP1 suppressed glucose consumption and lactate production, as well as G6P levels in SGC-7901 and BGC-823 cells under a hypoxic condition (Figure 3E–G). Compared with the si-ctrl group, silencing of circNRIP1 increased the sensitivity of SGC-7901 and BGC-823 cells to 5-FU under a hypoxic condition, as evidenced by a decrease in cell survival (Figure 3H).

circNRIP1 Functioned as a miR-138-5p Sponge in GC Cells

To investigate the mechanisms by which circNRIP1 regulates hypoxia-induced 5-FU resistance in GC cells, we explored the miRNAs downstream of circNRIP1 using starbase website analysis. circNRIP1 sequence was predicted to harbor a putative miR-138-5p-binding site (Figure 4A). The expression level of miR-138-5p was markedly reduced in SGC-7901 and BGC-823 cells exposed to hypoxia compared with that in cells exposed to normoxia (Figure 4B).

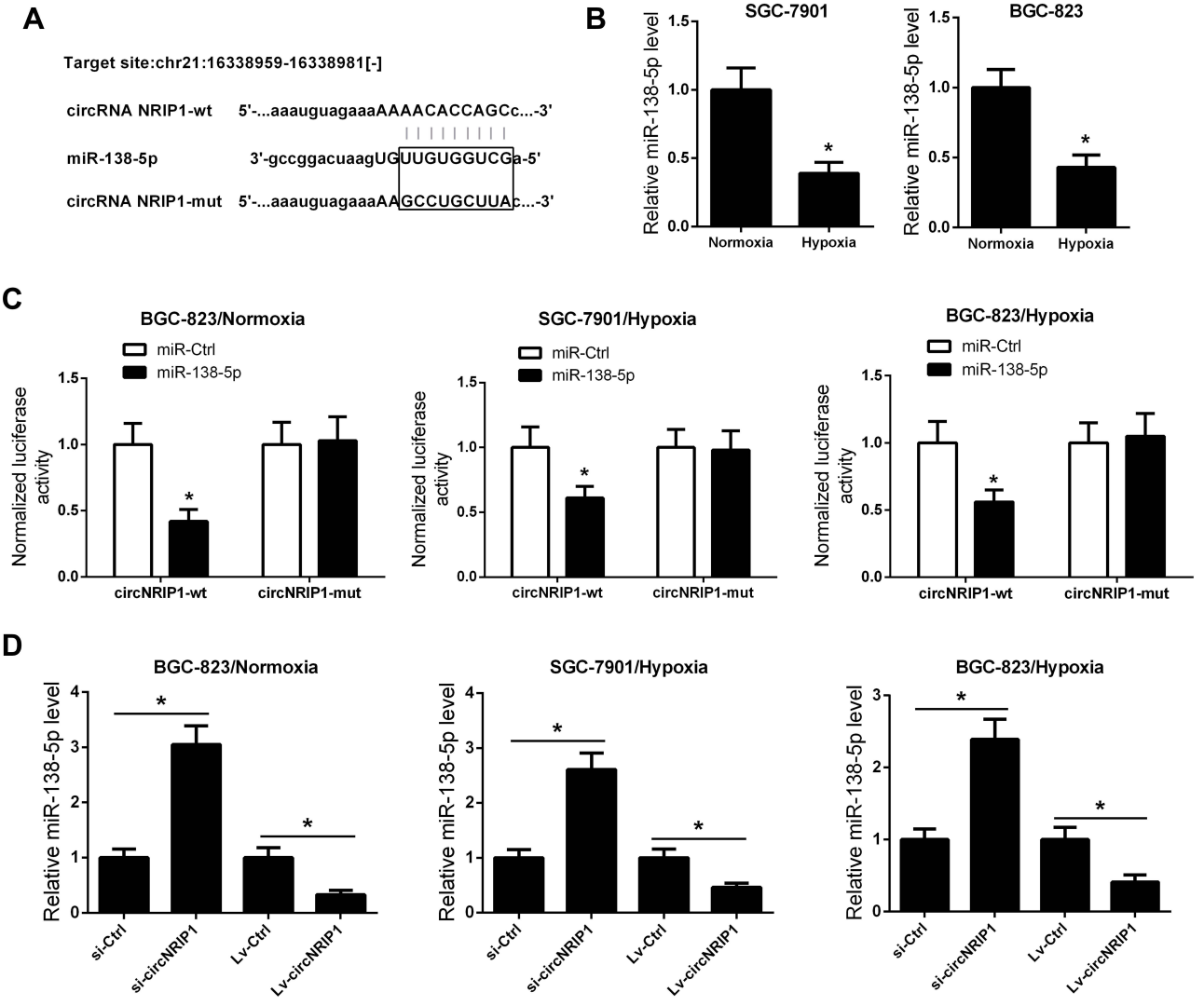

Figure 4 circNRIP1 functioned as a miR-138-5p sponge in GC cells. (**A**) A putative miR-138-5p-binding site exists in the sequence of circNRIP1 and eleven point mutations were generated in the binding site. (**B**) qRT-PCR analysis of miR-138-5p expression showed reduced expression of miR-138-5p in SGC-7901 and BGC-823 cells exposed to hypoxia. (**C**) Luciferase reporter assay was performed in BGC-823 cells cultured under normoxia, as well as in SGC-7901 and BGC-823 cells exposed to hypoxia. (**D**) SGC-7901 and BGC-823 cells transfected with si-NRIP1, Lv-NRIP1 or paired controls were incubated under a normoxic or hypoxic condition, and tested for miR-138-5p expression using qRT-PCR. *$P < 0.05$.

Luciferase reporter assay showed that upregulation of miR-138-5p suppressed the luciferase activity of reporters containing NRIP1-wt in BGC-823 cells under a normoxic condition, and such suppression was absent with mutations in the miR-138-5p-binding site. Similar results were discovered in SGC-7901 and BGC-823 cells exposed to hypoxia (Figure 4C). In addition, silencing of circNRIP1 markedly increased the expression levels of miR-138-5p in BGC-823 cells cultured under normoxia, as well as in SGC-7901 and BGC-823 cells exposed to hypoxia, while upregulation of circNRIP1 represented the opposite effects (Figure 4D).

circNRIP1 Regulated Hypoxia-Induced 5-FU Resistance in GC Cells by Sponging miR-138-5p

To identify if circNRIP1 regulates hypoxia-induced 5-FU resistance in GC cells by sponging miR-138-5p, SGC-7901 and BGC-823 cells were co-transfected with miR-138-5p or miR-ctrl and Lv-NRIP1 or Lv-ctrl, and then incubated under a hypoxic condition. The expression level of miR-138-5p was remarkably increased in SGC-7901 and BGC-823 cells transfected with miR-138-5p under a hypoxic condition, which was blocked by upregulation of circNRIP1 (Figure 5A). Upregulation of miR-138-5p reduced the mRNA levels of MDR1 and the protein levels of p-gp in SGC-7901 and BGC-823 cells exposed to hypoxia, and these effects were obviously abrogated following Lv-NRIP1 transfection (Figure 5B and C). Additionally, upregulation of miR-138-5p restrained the consumption of glucose and the production of lactate, as well as reduced the levels of G6P in SGC-7901 and BGC-823 cells exposed to hypoxia. However, the reduced glucose consumption, lactate production and G6P level induced by miR-138-5p were greatly overturned by upregulation of circNRIP1 (Figure 5D–G).

circNRIP1 Regulated HIF-1α Expression by Targeting miR-138-5p

It was found that the 3′UTR region of HIF-1α contains a miR-138-5p-binding site by Targetscan analysis (Figure 6A). To confirm the interaction between miR-138-5p and HIF-1α, luciferase reporter assay was performed in SGC-7901 and BGC-823 cells exposed to hypoxia. The results showed that upregulation of miR-138-5p dramatically reduced the luciferase activity of reporters containing HIF-1α-wt, which was greatly overturned after upregulation of circNRIP1. While the luciferase activity of reporters containing HIF-1α-mut was unaltered following miR-138-5p, miR-ctrl, Lv-NRIP1 or Lv-ctrl transfection (Figure 6B). Upregulation of miR-138-5p decreased the protein level of HIF-1α in SGC-7901 cells exposed to hypoxia, but this decrease was blocked following Lv-NRIP1 transfection (Figure 6C). Meanwhile, similar results were obtained in BGC-823 cells exposed to hypoxia (Figure 6D).

2-DG Reversed circNRIP1-Mediated Increase of 5-FU Resistance and HIF-1α Expression in GC Cells

To further investigate whether circNRIP1 regulates hypoxia-induced 5-FU resistance in GC cells through HIF-1α-dependent glycolysis, BGC-823 cells were transfected with Lv-NRIP1 or Lv-ctrl, followed by treatment with 2-DG under a hypoxic condition. As displayed in Figure 7A and B, transfection of Lv-NRIP1 markedly elevated the mRNA level of MDR1 and the protein level of p-gp in BGC-823 cells exposed to hypoxia, while cotreatment with Lv-NRIP1 and 2-DG strikingly recuperated MDR1 mRNA level and p-gp protein level. In addition, glucose consumption and lactate production were conspicuously increased following Lv-NRIP1 transfection in BGC-823 cells exposed to hypoxia, which was markedly conversed by 2-DG treatment (Figure 7C and D). Simultaneously, the levels of G6P were substantially increased in BGC-823 cells transfected with Lv-NRIP1 under a hypoxic condition, however, circNRIP1-mediated increase of G6P levels was markedly abolished by 2-DG treatment (Figure 7E). Furthermore, a marked elevation of HIF-1α was discovered in BGC-823 cells transfected with Lv-NRIP1 under a hypoxic condition, and this elevation was reversed by 2-DG treatment (Figure 7F). Besides, upregulation of circNRIP1 reduced the sensitivity of BGC-823 cells to 5-FU accompanied by the increased cell survival, and this action was marked overturned by 2-DG administration (Figure 7G).

Discussion

It is well documented that hypoxia-induced 5-FU resistance plays a crucial causative role in the chemotherapeutic failure of GC. In this paper, we aimed to study the biological function of circNRIP1 and its underlying molecular mechanism in hypoxia-induced 5-FU resistance, and to develop novel therapeutic approaches for combating hypoxia-induced 5-FU resistance in GC.

Accumulating evidence suggests that circRNAs play a crucial role in the occurrence of chemoresistance.[22] One

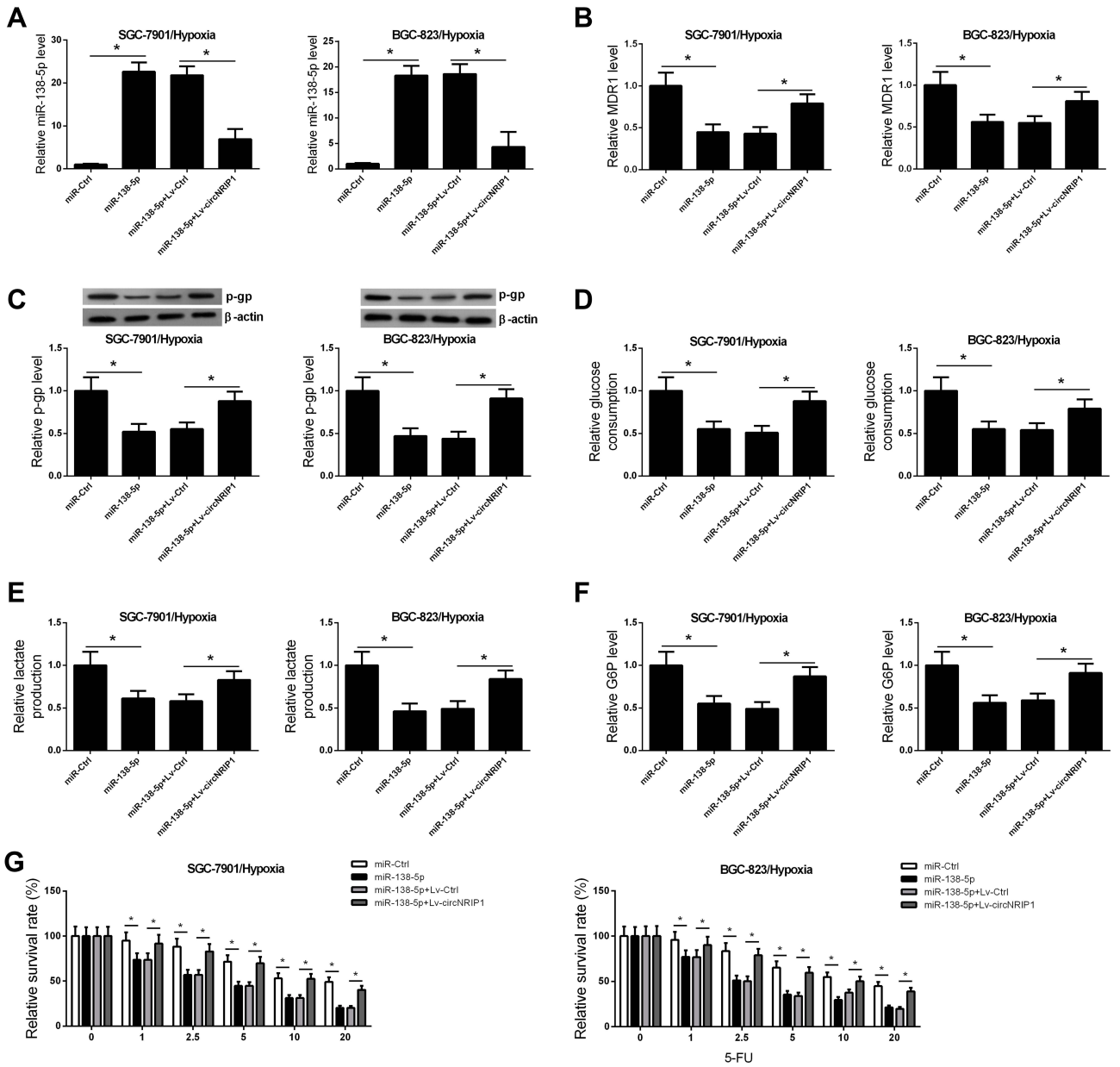

Figure 5 circNRIP1 regulated hypoxia-induced 5-FU resistance in GC cells by sponging miR-138-5p. SGC-7901 and BGC-823 cells were co-transfected with miR-138-5p or miR-ctrl and Lv-NRIP1 or Lv-ctrl, and then incubated under a hypoxic condition. (**A**) qRT-PCR analysis of miR-138-5p expression in SGC-7901 and BGC-823 cells. (**B**) qRT-PCR analysis of MDR1 levels in SGC-7901 and BGC-823 cells. (**C**) Western blot analysis of p-gp levels in SGC-7901 and BGC-823 cells. (**D**) The glucose consumption and (**E**) lactate production were measured in SGC-7901 and BGC-823 cells. (**F**) qRT-PCR analysis of G6P levels in SGC-7901 and BGC-823 cells. (**G**) SGC-7901 and BGC-823 cells were co-transfected with miR-138-5p or miR-ctrl and Lv-NRIP1 or Lv-ctrl, and incubated under a hypoxic condition, followed by incubation with different doses (0, 1, 2.5, 5, 10 and 20 μM) of 5-FU, and cell survival was measured by MTT assay. *$P < 0.05$.

example of this is that circRNA-MTO1 was upregulated in monastrol-resistant breast cancer cells and its upregulation could restrain cell viability and converse the resistance of monastrol-resistant MCF-7 and MDA-MB-231 cells to monastrol by modulating the tumor necrosis factor receptor associated factor 4/Eg5 axis.[23] Similarly, circRNA PVT1 has been stated to maintain the resistance of osteosarcoma cells to doxorubicin and cisplatin through modulating the expression of ATP-binding cassettes B1.[24] However, the participation of circNRIP1 in hypoxia-induced chemoresistance is still obscure. Herein, we found that hypoxia induced the resistance of GC cells to 5-FU. Notably, circNRIP1 was upregulated in GC cells and its downregulation enhanced the sensitivity of GC cells to 5-FU under a hypoxic condition, suggesting a potential role of circNRIP1 in hypoxia-induced 5-FU resistance.

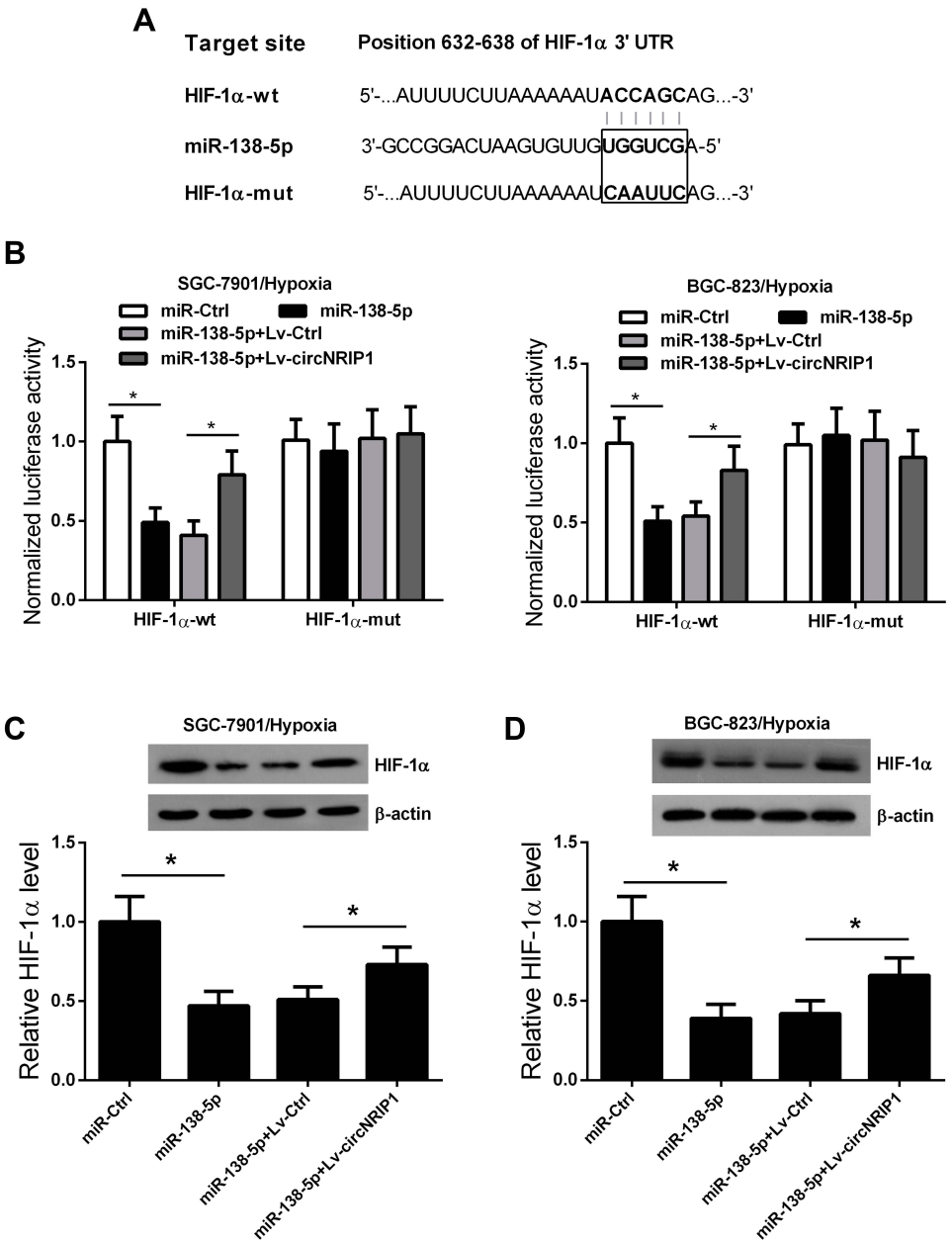

Figure 6 circNRIP1 regulated HIF-1α expression by targeting miR-138-5p. (**A**) Sequence complementarities of miR-138-5p and its target HIF-1α. (**B**) Luciferase reporter assay showing the interaction between miR-138-5p and HIF-1α in SGC-7901 and BGC-823 cells exposed to hypoxia. (**C** and **D**) SGC-7901 and BGC-823 cells were cotransfected with miR-138-5p or miR-ctrl together with Lv-NRIP1 or Lv-ctrl, and then incubated under a hypoxic condition. The expression of HIF-1α was measured using Western blot. *$P < 0.05$.

The participation of circNRIP1 in hypoxia-induced chemoresistance has been elucidated in this research, but the downstream regulatory signaling of circNRIP1 in hypoxia-induced chemoresistance is still poorly defined. It was predicted that the circNRIP1 sequence contains a putative miR-138-5p-binding site. Interestingly, miR-138-5p was reportedly implicated in the development of chemoresistance. As an example, miR-138-5p was down-regulated in cisplatin-resistant non-small-cell lung cancer tissues and its inhibitor could abrogate the inhibitory effect of tripartite motif-containing 65 silencing on cell autophagy and the resistance of cisplatin-resistant A549 cells to cisplatin.[25] In addition, miR-138-5p induced the downregulation of enhancer of zeste homolog 2, which could enhance the sensitivity of docetaxel-resistant prostate cancer cells to docetaxel by inhibiting docetaxel-triggered cancer stem cells populations.[26] Nevertheless, uncertainty still remains as to the mechanism by which circNRIP1

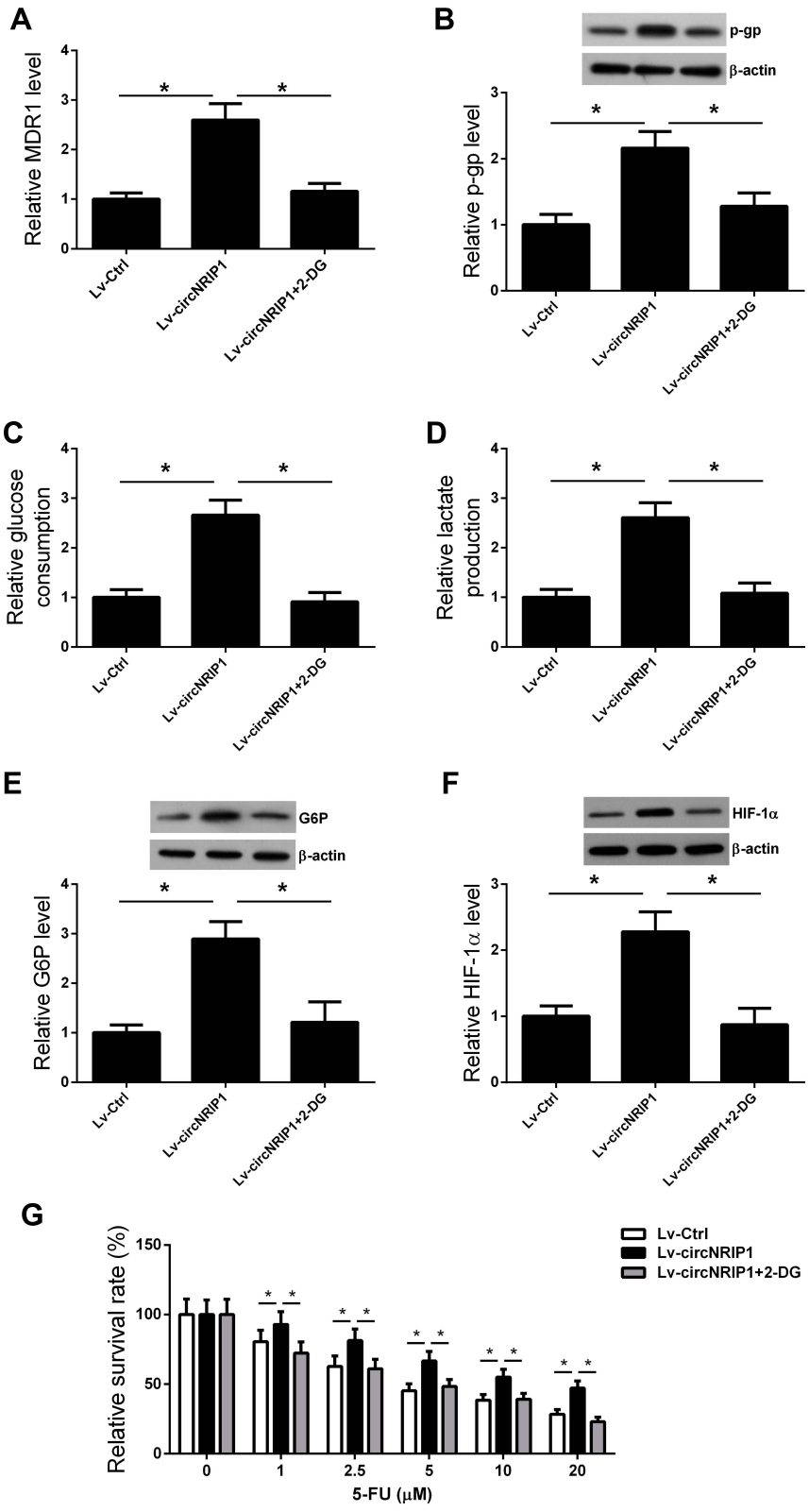

Figure 7 2-DG reversed the effect of circNRIP1 on hypoxia-induced 5-FU resistance in GC cells. BGC-823 cells were transfected with Lv-NRIP1 or Lv-ctrl, followed by treatment with 2-DG under a hypoxic condition. (**A**) qRT-PCR analysis showing 2-DG treatment reversed the circNRIP1-mediated elevation in MDR1 level in BGC-823 cells under a hypoxic condition. (**B**) Western blot analysis showing 2-DG treatment conversed the circNRIP1-mediated elevation in p-gp level in BGC-823 cells under a hypoxic condition. (**C** and **D**) Administration of 2-DG abolished the circNRIP1-mediated increases in glucose consumption and lactate production in BGC-823 cells exposed to hypoxia. (**E**) qRT-PCR analysis showing the circNRIP1-mediated increase in G6P level was blocked by 2-DG treatment in BGC-823 cells exposed to hypoxia. (**F**) qRT-PCR analysis showing the circNRIP1-mediated increase in HIF-1α level was reversed by 2-DG treatment in BGC-823 cells exposed to hypoxia. (**G**) MTT assay showing 2-DG treatment blocked circNRIP1-mediated increase of cell survival in BGC-823 cells stimulated with 5-FU and hypoxia. *$P < 0.05$.

regulates hypoxia-induced 5-FU resistance. In our work, miR-138-5p was found to be a target gene of circNRIP1 and its upregulation could reverse the effect of circNRIP1 on hypoxia-induced 5-FU resistance, inferring that circNRIP1 regulates hypoxia-induced 5-FU resistance by sponging miR-138-5p.

Hypoxia influences the sensitivity of cancer cells to therapeutic agents by multiple mechanisms, such as inhibition of therapeutic agent delivery, induction of EMT, and regulation of the oncogenic signaling pathways.[27–29] Remarkably, the main mechanism by which hypoxia induces chemoresistance is through HIF-1α, which was predicted to be the direct target of miR-138-5p.[30] The contribution of HIF-1α to hypoxia-induced chemoresistance has been demonstrated in numerous human cancers.[31] As an example, HIF-1α has been demonstrated to be a mediator of hypoxia-induced cisplatin resistance in non-small cell lung cancer cells.[32] HIF-1α inhibitor PX-478 could attenuate hypoxia-induced oxaliplatin resistance in colorectal cancer cells by regulating the HIF-1α/miR-338-5p/interleukin-6 feedback loop.[33] Additionally, it is suggested that hypoxic colorectal tumor microenvironment induced resistance to chemoresistance by activating the HIF-1α/transforming growth factor β2-mediated glioma-associated oncogene protein-2 signaling.[34] In this research, we identified that HIF-1α was a downstream target gene of miR-138-5p. More significantly, circNRIP1 was shown to regulate the expression of HIF-1α by sponging miR-138-5p, implying that circNRIP1 may regulate hypoxia-induced 5-FU resistance via the miR-138-5p/HIF-1α axis.

Glycolysis is the main energy producing pathway in hypoxic cancer cells, leading to lactate production from glucose and thereby resulting in the survival of cancer cells under a hypoxic condition.[35] Emerging evidence showed that glycolysis plays a crucial role in the development of hypoxia-induced chemoresistance.[36] Inhibition of the glycolysis pathway by dichloroacetate reportedly mitigated hypoxia-induced 5-FU resistance in GC.[37] HIF-1α is the metabolic major regulator of glycolysis, and pharmacological inhibition of HIF-1α by digoxin could reverse the resistance of gemcitabine-resistant pancreatic cancer cells to gemcitabine.[38] In GC, baicalein has been reported to converse hypoxia-induced 5-FU resistance in AGS cells through inhibition of glycolysis via regulating the phosphatase and tensin homolog/Akt/HIF-1α signaling.[39] In addition, silencing of krüppel-like factor 5 could reverse hypoxia-induced cisplatin resistance in non-small cell lung cancer through modulation of HIF-1α-dependent glycolysis via inhibiting the activation of the phosphoinositol 3-kinase/Akt/mammalian target of rapamycin pathway.[40] Considering all of the above evidence, it seems likely that HIF-1α-mediated glycolysis is emerging as a key driver of hypoxia-induced chemoresistance. However, whether circNRIP1/miR-138-5p axis regulates hypoxia-induced 5-FU resistance through HIF-1α-dependent glucose metabolism is still unclear. In the present work, we found that inhibition of glycolysis by 2-DG could converse the impact of circNRIP1 on hypoxia-induced 5-FU resistance, indicating that circNRIP1 maintained hypoxia-induced resistance to 5-FU by sponging miR-138-5p through HIF-1α-dependent glucose metabolism.

Conclusion

In summary, our findings demonstrated that circNRIP1 maintained hypoxia-induced resistance to 5-FU in GC cells by sponging miR-138-5p through HIF-1α-dependent glucose metabolism. Thus, targeting circNRIP1, in combination with 5-FU, may strongly improve the prognosis of GC patients.

Abbreviations

GC, gastric cancer; circRNAs, circular RNAs; 5-FU, 5-fluorouracil; G6P, glucose-6-phosphate; HIF-1α, hypoxia-inducible factor-1α; miR–138-5p, microRNA-138-5p; ncRNAs, noncoding RNAs; ceRNA, competitive endogenous RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide.

Disclosure

The authors declare that they have no competing interests.

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