

# Impact of RNAi-Targeted Silencing of Survivin and Hypoxia-Inducible Factor-1 $\alpha$ on the Biological Behaviors of Gastric Cancer SGC-7901 Cells *In Vitro*

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## Research Article

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## Abstract

Hypoxia-inducible factor-1 (HIF-1) plays crucial roles in the primary transcriptional responses to hypoxia stress by modulating hypoxia gene expression. Survivin is regarded as an effector downstream of HIF-1 that promotes cell survival under hypoxia conditions. HIF-1 $\alpha$  and survivin are proposed as potential targets in the treatment of gastric cancer. This study was designed to investigate the anticancer effects of RNA interference targeting survivin and HIF-1 $\alpha$  on the gastric cancer cell line, SGC-7901 under hypoxia. In this study, cultured cells were divided randomly into four groups: a blank control group, a scrambled siRNA (SCR) control group, a siRNA-Survivin group (ss group) and a siRNA-Survivin+siRNA-HIF-1 $\alpha$  group (sis+siH group). The siRNA targeting survivin and/or HIF-1 $\alpha$  genes were transfected into SGC-7901 cells. The expression of survivin and HIF-1 $\alpha$  was identified by RT-PCR and Western blotting assay. Apoptosis was determined by flow cytometry. The invasion and migration abilities of the cells were evaluated by transwell and wound healing assays, respectively. Cell growth curves were determined by MTT assay at 48 h indicated that the viability of the SGC-7901 cells was inhibited in a time-dependent manner with a highest inhibitory rate of  $82.1 \pm 0.85\%$  at 48 h. The siRNA targeting the survivin gene decreased the expression of survivin and the combined transfection of siRNA targeting survivin and HIF-1 $\alpha$  down-regulated the expression of both survivin and HIF-1 $\alpha$  ( $P < 0.05$ ). Compared with the blank control group, the combined siRNA groups exhibited obvious features with decreased invasion and migration abilities ( $P < 0.05$ ). The apoptosis rate increased and the cell proliferation decreased following gene silencing ( $P < 0.05$ ). The down-regulation of survivin and HIF-1 $\alpha$  may induce an anticancer effect in SGC-7901 cell lines by enhancing cell apoptosis and decreasing proliferation, migration and invasion ability.

## Introduction

Gastric cancer is the second most common cancer worldwide[1]. Patients with gastric cancer at even early stage usually have distant metastasis and poor prognosis despite the combined therapy with surgery, chemotherapy and radiotherapy [2]. Hypoxia is a shared feature and form of physiological stress on various cancers. Hypoxia stress can facilitate adaptive changes in tumor cells, which eventually leads to increasing tumor growth, invasion and metastasis [3, 4]. Accumulating evidence suggests that hypoxia, resulting from highly active tumor cell proliferation, is related to poor prognosis and development of resistance to chemotherapy and radiotherapy[5, 6]. Hypoxia-inducible factor-1 (HIF-1) is an essential transcriptional regulator, also known as hydrocarbon receptor nuclear translocator, which plays crucial roles in the primary transcriptional responses to hypoxia stress by modulating hypoxia gene expression [6]. Structurally, HIF1 consists of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits [7]. These heterodimeric transcription molecules activate a series of phosphoglycerate kinase and vascular endothelial growth factor (VEGF) genes by response to hypoxia[8]. Survivin, a member of the inhibitor of apoptosis (IAP) family, acts as a key regulator of angiogenesis in addition to tumor progression[9].

RNA interference (RNAi) is a mechanism of transcriptional regulation for specific gene expression silencing in the majority of eukaryotic cells [10, 11]. The process is mediated by small RNA (siRNA) molecules 21–23 nucleotides in length [12, 13]. There is emerging evidence that RNAi plays an important

regulatory role in the articulated molecular mechanism triggered by hypoxia stress. Our previous studies demonstrated that RNAi targeting survivin in BGC-823 cell lines may attenuate the anti-proliferative and apoptosis-inducing effects under hypoxia [14]. However, overexpression of HIF-1 $\alpha$  was associated with the poor prognosis in gastric cancer [15]. Currently, it is well known that HIF-1 transactivates the survivin gene by directly targeting the survivin promoter. Both HIF-1 $\alpha$  and survivin are activated, along with high over expression of VEGF in tumor cells under hypoxia, which results in endothelial cell proliferation, migration and neovascularization. HIF-1 $\alpha$  and survivin are proposed as potential targets in the treatment of gastric cancer. Accordingly, we sought to investigate how RNAi targeting HIF-1 $\alpha$  mediates the growth of gastric cancer cell line SGC-7901 combined with survivin.

## Materials And Methods

### Cell culture and hypoxia treatment

The human gastric cancer cell line SGC-7901 was obtained from the China Center for Type Culture and was maintained in RPMI-1640 medium (Hyclone, Beijing, China) containing 10% fetal bovine serum (Sijiqing Bio. Co., Ltd., Hangzhou, China) and 1% penicillin/streptomycin, in a 5% CO<sub>2</sub> humidified atmosphere at 37 °C incubation. The medium was changed at alternate days and cells were harvested at 70–80% of confluence for experimental purposes. After seeding for 24 h, cells were incubated in normal or hypoxic conditions for a further 48 h (for hypoxic treatment, cells were maintained in a hypoxia incubator of 1% oxygen concentration infused with 5% CO<sub>2</sub> and nitrogen gas mixture).

### RNA interference and groups

Two pairs of siRNAs were designed against human HIF-1 $\alpha$  and survivin, as follows: siRNA-HIF-1 $\alpha$ , 5'-CCAUAUAGAGAUACUCAAATT-3' (sense) and 5'-UUUGAGUAUC UCUAUAU GGTT-3' (antisense); siRNA-survivin, 5'-GGCUGGCUUCAUCCACUGCTT-3' (sense) and 5'- GCAGUGGAUGAAGGCCAGCCTT-3' (antisense).

The sequences 5'-UUGAUGUGUUUAGUCGUATT-3' (sense) and 5'-UAGCGACUAAACACAUCATT-3' (antisense), named scrambled siRNA (SCR) were used as a negative control. 3'-Fluorescein amidite (FAM) fluorescence-labeled SCR was utilized to detect the transfection efficiency. All siRNAs were chemically synthesized by Gen. Co., Ltd. (Shanghai, China).

The cultured cells were randomly divided into four groups: a blank control group, a siRNA-Survivin group (ss group), a siRNA-Survivin+siRNA-HIF-1 $\alpha$  group (sis+siH group) and a SCR control group. For siRNA transfection, SGC-7901 cells were prepared on 6-well plates and incubated overnight to achieve 80-90% confluence. The cells were transfected with 100 nmol/L siRNAs using Hifectin® (Applied Gen Co., Ltd., Beijing, China) according to the protocol provided by the manufacturer. Transfection efficiency rates=cells displaying green fluorescence/total count cells×100%. Subsequently, the exponentially growing SGC-7901 cells were maintained under hypoxic conditions for further experiments. Three independent repeats were conducted for all experiments.

## **Cell viability assay**

The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Funakoshi Co., Tokyo, Japan) assay was performed to evaluate cell proliferation. SGC-7901 cells were seeded on 96-well plates at an optimal density ( $3 \times 10^3$  cells per well) and incubated overnight. After another 48-h incubation in normal or hypoxic conditions, cells were treated with 20  $\mu$ L MTT (5 mg/mL) and 150  $\mu$ L dimethyl sulfoxide (DMSO). The optical density (OD) value of each well was measured at 490 nm using a 2100C ELISA Reader (Rayto Sciences Co., Ltd. Shanghai, China). The cell viability curve of the four groups was drawn. The cell proliferation inhibition rate =  $(1 - \text{average OD value experimental group} / \text{average OD value control group}) \times 100\%$ .

## **Reverse transcription polymerase chain reaction (RT-PCR)**

The primers were as follows: sense, 5'-GCAAGCCCTGAAAGCG-3' and antisense, 5'-GGCTGTCCGACTTG-3' for HIF-1 $\alpha$  (240 bp); Sense, 5'-AACAGCCGAG ATGACCTCC-3' and antisense, 5'-AACTTCAGGTGGATGAGGAGAC-3' for survivin (421 bp); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) sense, 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and antisense, 5'-CATGTGGG - CCATGAGGTCCACCAC-3' for the internal reference (983 bp). Total mRNA was isolated with TRIzol® reagent (Invitrogen Life Technology, USA), and cDNA was prepared with the GoScript™ Reverse Transcription System kit (Promega Biotech Co., Ltd., USA) according to the manufacturer's instructions. The PCR program was as follows: denaturation at 95°C for 5 min; 30 cycles of 94°C for 30 s, annealing at 50°C for 30 s and 72°C for 1 min, with a final step at 72°C for 5 min. PCR products were separated using 2% agarose gel, and the bands were scanned and the relative mRNA expression levels were determined by comparing with the expression of GAPDH.

## **Western blotting analysis**

After treatments, total protein was extracted from cells using radio-immunoprecipitation assay (RIPA) buffer (Beyotime Biotech Co., Ltd., Wuhan, China) and the level of protein was determined using the bicinchoninic acid assay method. Equal amounts of protein lysates (50  $\mu$ g) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (EMD Millipore Co., Hayward, CA, USA). The membranes were blocked with 5% skimmed milk for 1 h and then incubated at 4°C overnight with monoclonal rabbit anti-mouse antibodies against HIF-1 $\alpha$ , GAPDH (Boster Co., Ltd., Wuhan, China) and survivin (Biosynthesis Co., Ltd., Beijing, China). On the following day, the membranes were washed with TBST and incubated for 1 h at room temperature with the secondary peroxidase-labeled goat anti-rabbit antibody (Boster Co., Ltd, Wuhan, China) diluted to 1:2000 in skimmed milk/TBST. The protein bands were visualized by enhanced chemi-luminescence and the band intensity was measured using Quantity One v4.6.2 software (Bio-Rad, California, USA). Quantitative analysis of the relative levels of target proteins was determined using the NIH ImageJ software.

## **Flow cytometry**

For analysis of apoptosis, an annexin V-FITC apoptosis detection kit was used (Invitrogen, USA). After treatment for 48 h, SGC-7901 cells were harvested, washed twice with PBS and resuspended in 500  $\mu$ L of binding buffer. Cell suspensions were then incubated with 5  $\mu$ L of annexin V-FITC and 5  $\mu$ L of propidium iodide (PI) for 10 min at room temperature away from light. The apoptotic rates were calculated based on the number of these transfected gastric cancer cells in the apoptotic state and evaluated immediately by flow cytometry (BD Biosciences, Franklin Lakes, NJ) . The mean fluorescence intensity of annexin-V-FITC/PI was determined by flow cytometry. Then the apoptotic rates were calculated at the mean fluorescence intensity. At last, the results were quantified using WinMDI 2.9 analysis software.

### **Wound healing assay**

Scratch (wound-healing) assays were performed to determine cell migration ability. SGC-7901 cells were seeded in plates at a density of  $3 \times 10^5$  cells/well and incubated overnight before being treated as described above. After 24 hours, cells were maintained in normoxic or hypoxic conditions and scratched using a sterile 200- $\mu$ L tip to create a wound. Cells were then washed three times with PBS to remove debris, and were cultured in a serum-free medium for further 24 hours. The width of the wound area was monitored and measured at more than three positions per scratch by using microscopy to compare the migration ratios among the groups.

### **Invasion assay**

Matrigel invasion assays were employed to assess the invasion of SGC-7901 as previously described. Treated cells were incubated overnight in the serum-free medium. Then, 50  $\mu$ L Matrigel (BD Biosciences, San Jose, CA) was overlaid and maintained at 37 °C for 1 hour inside transwell filters with a membrane pore size of 8.0  $\mu$ m. Next,  $5 \times 10^4$  of treated cells suspended in the serum-free RPMI-1640 medium were added in the upper chambers and the bottom chamber of each well contained only RPMI-1640 medium with 10 % FBS. After incubation in normoxic or hypoxic conditions for 12 h, cells on the upper surface were removed using a cotton swab. Cells on the lower surface of the membrane were fixed (4 % paraformaldehyde) and stained with 0.1 % crystal violet. Cells on the lower surface of the filter were visualized and photographed under the microscope, and the relative numbers were counted (five distinct fields per insert).

### **Statistical analysis**

Data were expressed as mean values with standard error of the mean ( $\pm$  SEM). Statistical analysis was performed using Student's *t* test with SPSS11.0 for Windows. All experiments were performed in triplicate. Differences were considered statistically significant at  $P < 0.05$ .

## **Results**

### **Rates of siRNA transfection**

We observed the expression of transfection efficiency of siRNA under fluorescence microscopy for 6 h in SGC-7901 cells. The results showed a higher transfection efficiency with more than 80% of cells displaying green fluorescence due to the fluorescent-labeled siRNA in both ss group and sis + siH group compared with a SCR control group and blank control group (Fig. 1).

### **Effect of HIF-1 $\alpha$ or/and survivin RNAi on SGC-7901 cell viability under hypoxia**

Cell growth curves determined by MTT assay at 48 h showed that cell viability was inhibited in a time-dependent manner with the highest inhibitory rate at  $82.1 \pm 0.85\%$  after 48 h in the siH + sis group compared with the control group and the SCR control group ( $P < 0.05$ , Fig. 2).

### **Individual inhibitory effects of siRNAs targeting HIF-1 $\alpha$ and survivin under hypoxia**

We further evaluated the silencing effects of siRNA-HIF-1 $\alpha$  and siRNA-survivin in SGC-7901 cell. After transfection, cells were maintained under hypoxia. After 48 h, the expression levels of HIF-1 $\alpha$  and survivin were determined by RT-PCR and Western blotting analysis. The findings showed that the expressions of HIF-1 $\alpha$  and survivin were inhibited in the siH and sis groups at the mRNA and protein levels. The expression levels of HIF-1 $\alpha$  and survivin mRNA and protein were significantly reduced simultaneously compared to control cells ( $P < 0.05$ , Fig. 3 and Fig. 4).

### **Effect of HIF-1 $\alpha$ or/and survivin RNAi on SGC-7901 cell apoptosis under hypoxia**

Apoptosis was assessed for 48 h after transfection by staining cells with Annexin V/PI and analyzed using an FCM. The strongest apoptotic signals were identified in the siH + sis groups and the percentages of apoptotic cells were  $11.5 \pm 2.5$ . The results indicated that the apoptosis rates of SGC-7901 cells in the siH + sis groups were higher than those in the control group ( $P < 0.05$ , Fig. 5).

### **Effect of HIF-1 $\alpha$ or/and survivin RNAi on migration ability of SGC-7901 cells under hypoxia**

Woundhealing assays were performed and the width of the wounded area measured at more than three positions per scratch by microscopy at 12 h and 24 h. A significant difference was found in the migration ratios amongst the groups. The migration of SGC-7901 cells of the siH + sis group ( $198.6 \pm 14.80$  mm) decreased by 45.48% ( $P < 0.05$ ) compared with control group ( $464.0 \pm 15.04$  mm), whilst the SCR group showed no significant difference ( $497.4 \pm 25.57$  mm,  $P > 0.05$ , Fig. 6).

### **Effect of HIF-1 $\alpha$ or/and survivin RNAi on invasive ability of SGC-7901 cells under hypoxia**

In the meanwhile, the invasion assay revealed that the invasive number of SGC-7901 cells on the control and SCR groups were  $91.33 \pm 4.055$  and  $85.33 \pm 4.256$  respectively, whereas the invasion cells were  $24.41 \pm 2.41$  in the siH + sis groups ( $P < 0.05$ , Fig. 7).

## **Discussion**

RNAi technique has been widely used in the functional analysis of mammalian genes using synthetic 19-23-nucleotides double-stranded RNAs as siRNA [15, 16, 17]. RNAi as a target-specific gene suppression technology has provided a promising way for gene therapy in various diseases, particularly in cancer [18, 19, 20]. The efficiency of RNAi on the same target usually shows great difference. One of the critical preconditions for RNAi therapy is the appropriate siRNA that can efficiently knock down the expression of target genes [21, 22]. While the activated oncogene mRNA was effectively inhibited by RNAi technology, the tumor cell growth, proliferation, invasion, vascularization and extravasation were also inhibited [23, 24, 25]. Tamura et al. reported that intraperitoneal administration of ST6GalNAc I siRNA-liposome significantly inhibited peritoneal dissemination of gastric cancer in vitro [26]. Li et al. reported that the survivin-targeting siRNA (siRNA-survivin) in three cancer cell lines, survivin protein expression markedly decreased whereas apoptotic rates noticeably increased [27].

In this study, we employed two pairs of siRNAs targeting survivin and HIF-1a mRNA separately which were then transfected into SGC-7901 cells. On the basis of relative expression of mRNA and protein levels, our findings confirmed that the two siRNAs were highly efficient in suppressing survivin and HIF-1a expression in SGC-7901 cells. The confirmation of efficient siRNA targeting human survivin and HIF-1a in this study warrants further research on survivin and HIF-1a. Our studies have shown that under hypoxic conditions, the expression of HIF-1a was higher in SGC-7901 cells and the results indicated a high-efficiency transfection with more than 80% of cells displaying green fluorescence. HIF-1a, as a major transcriptional factor activated by hypoxia, triggers a series of oncogenes in tumor development [28, 29]. By activation of a large number of downstream target genes, tumor cells adapt to the hypoxia settings and continue to survive, followed by malignant proliferation, metastasis and even resistance to chemotherapy [30, 31]. HIF-1a-survivin pathway provides a means whereby gastric cancer cells can survive and grow under hypoxia conditions, suggesting the pathway as a therapeutic target for the gene treatment of gastric cancer [32, 33]. This finding was in an agreement with the publication of others [34, 35].

We therefore explored the impact of further knockdown on the expression of survivin and HIF-1a in the gastric epithelial cell line SGC-7901. Compared with the SCR and the blank control group, the inhibitory rate of SGC-7901 had significantly lower expression levels of survivin and HIF-1a on the sis + siH. Compared with the blank control group, the expression levels of survivin and HIF-1a mRNA were significantly decreased along with survivin and HIF-1a proteins in the sis + siH group. The flow cytometry results showed that the apoptotic rate of SGC-7901 cells in the sis + siH group was significantly higher than those in the SCR and blank control groups. The results of the transwell migration assay indicated that the numbers of migration cells in the sis + siH group were significantly lower than those in the control group.

There are some limitations to our study. We did not detect the Bcl-2/Bax ratio. More experiments are needed to determine the expression of Bcl-2 and Bax and the exact mechanism underlying this pathway.

## Conclusion

Our findings suggest that RNAi targeting survivin and HIF-1a dramatically reduced cell growth and increased apoptosis in gastric cancer cell *in vitro*. RNAi against survivin and HIF-1a may be a potentially novel approach for the prevention and treatment of gastric cancer. However, the precise underlying mechanisms of survivin and HIF-1a RNAi on proliferation and apoptosis in SGC-7901 cells remain to be fully determined and require further investigation.

## Declarations

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### Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

The authors alone are responsible for the content and writing of the paper.

**Ethical approval** No applicable.

**Informed consent** All authors consent to the submission of this manuscript to the Journal, Molecular Biology Reports.

**Author contributions** Runhong Mu designed the experiments and wrote a draft manuscript. Binxian Li and Mingcheng Li analyzed, interpreted the results of the experiments and revised the manuscript. Yinlin Ge performed the experiments. All authors read and approved the final manuscript.

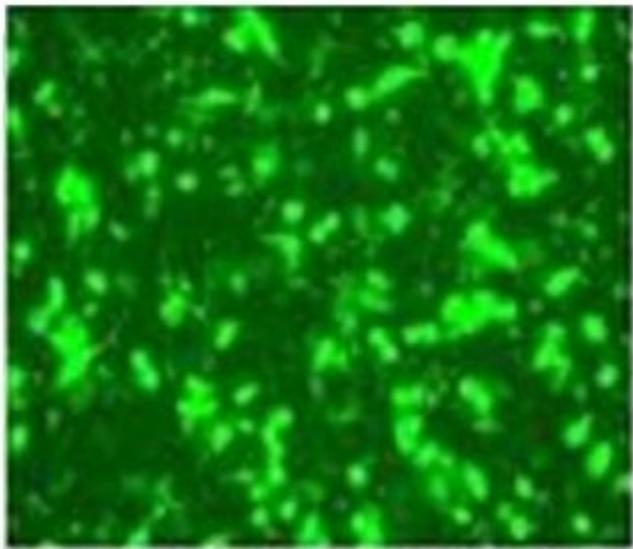
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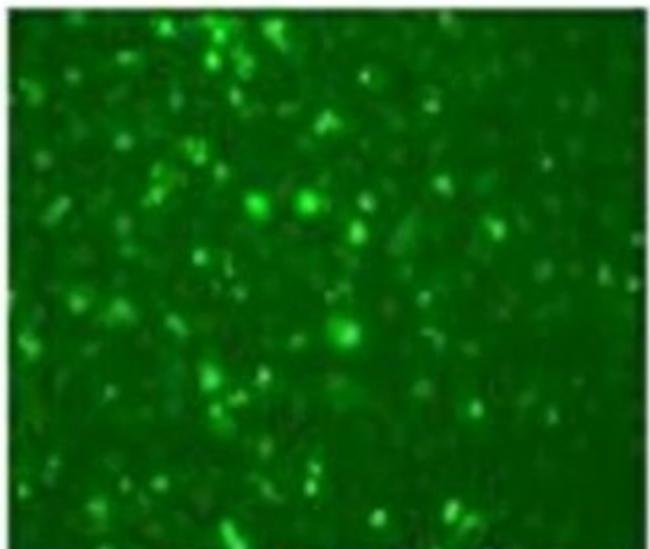
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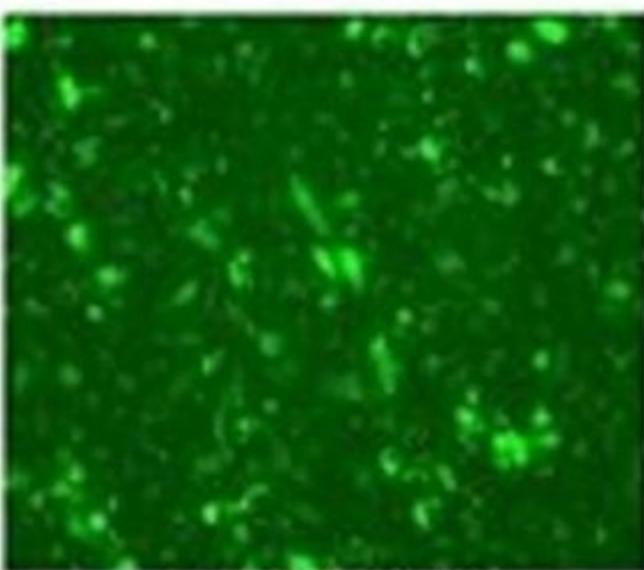
## Figures



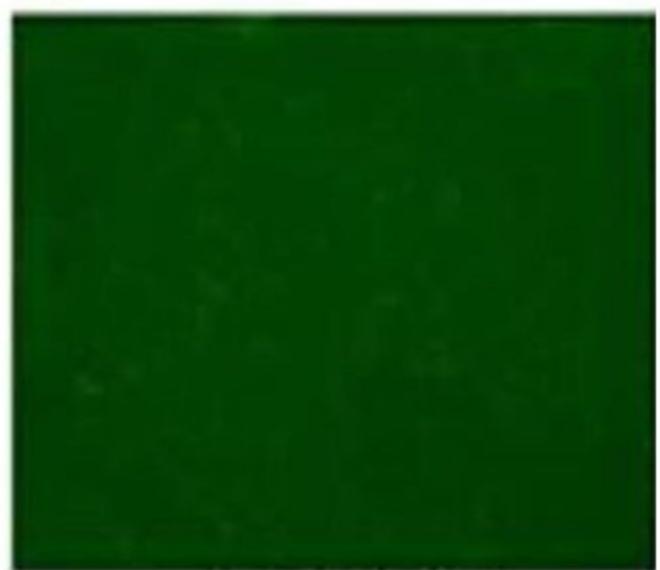
ss group



sis+siH group



SCR control



Blank control

**Figure 1**

Expression of green fluorescence in SGC-7901 cells after transfection for 6 h due to the fluorescent-labeled siRNA ( $\times 200$ ). Note: Data are representative images (magnification  $\times 200$ ) in individual groups of cells from three separate experiments.

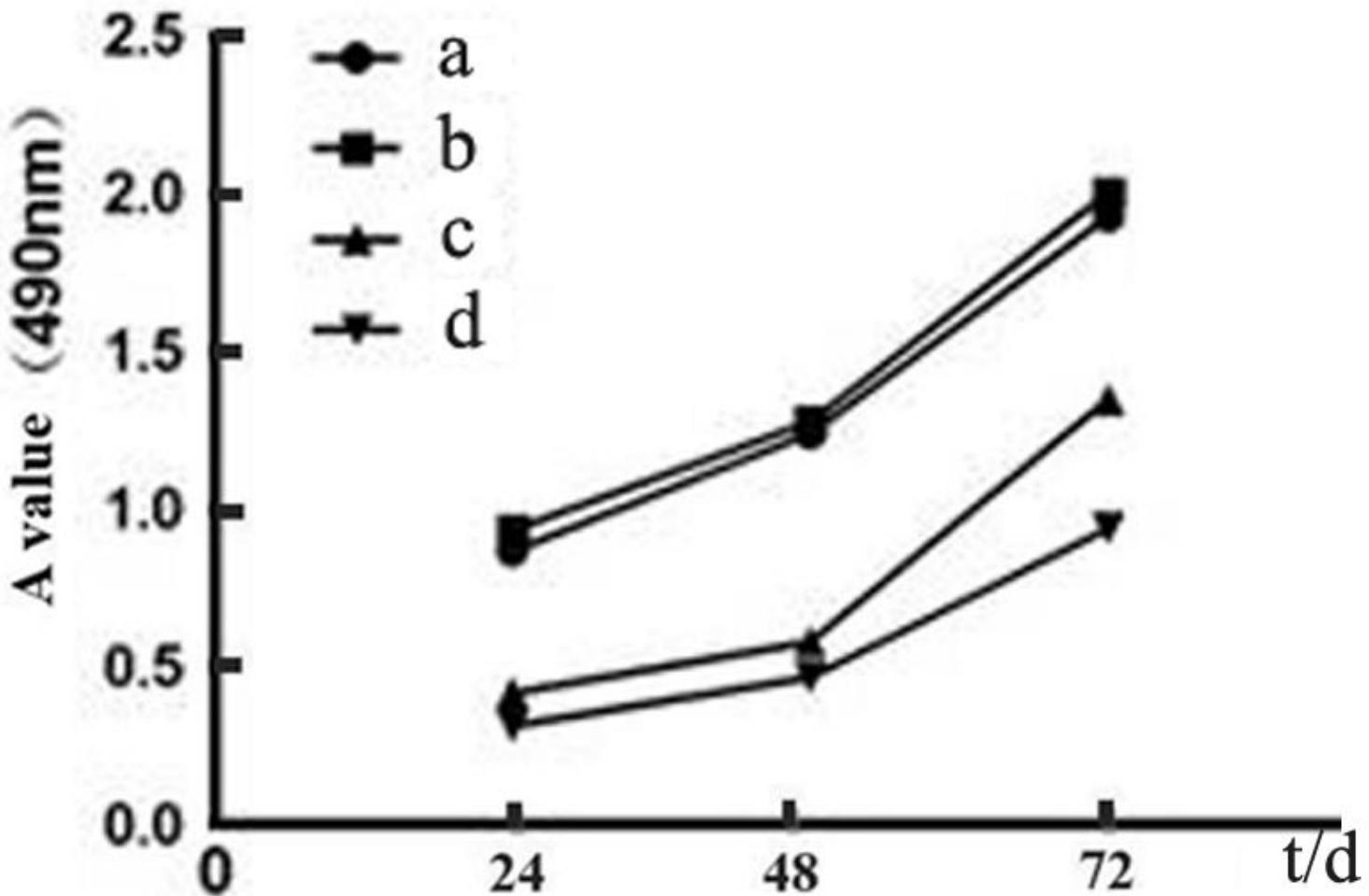
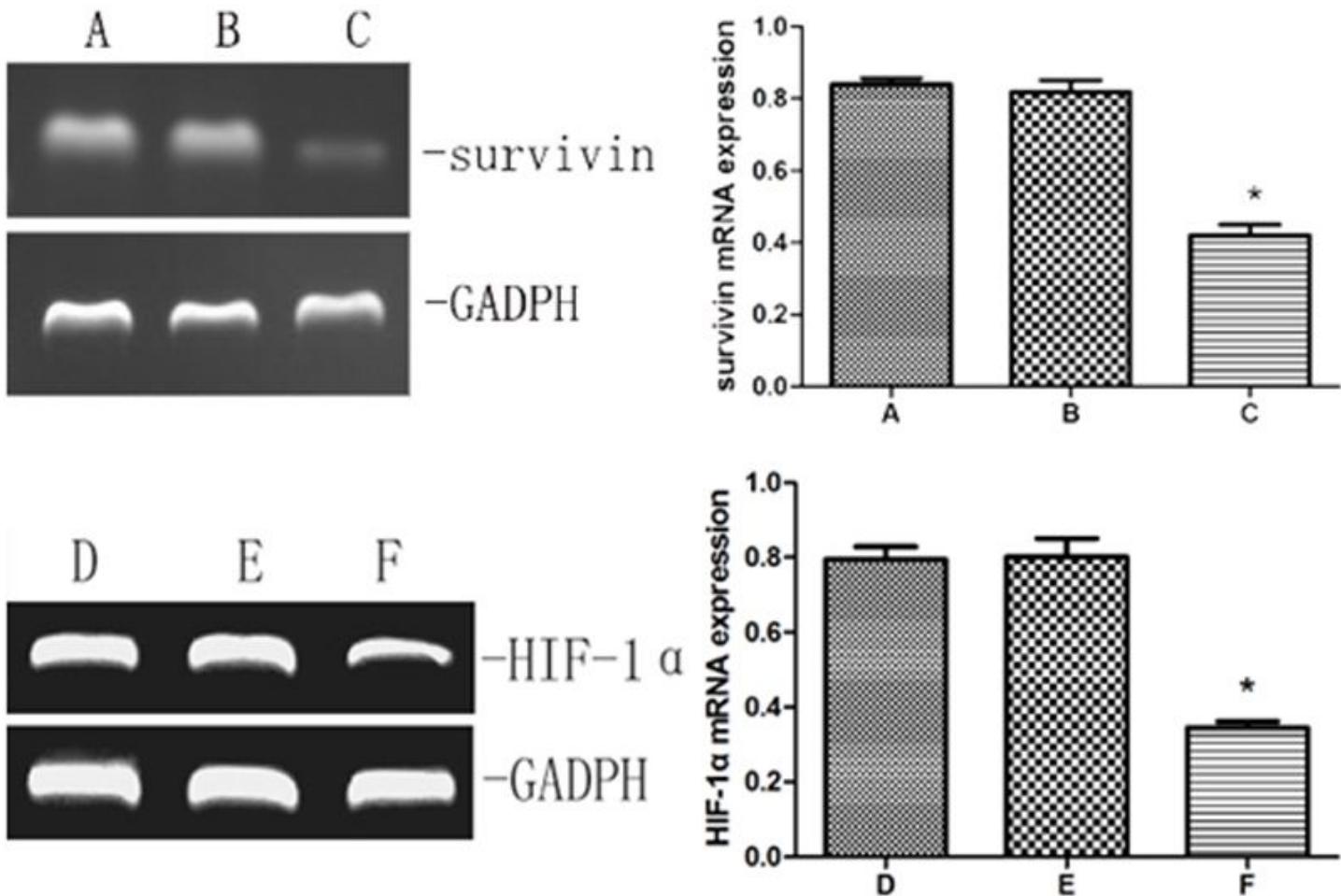


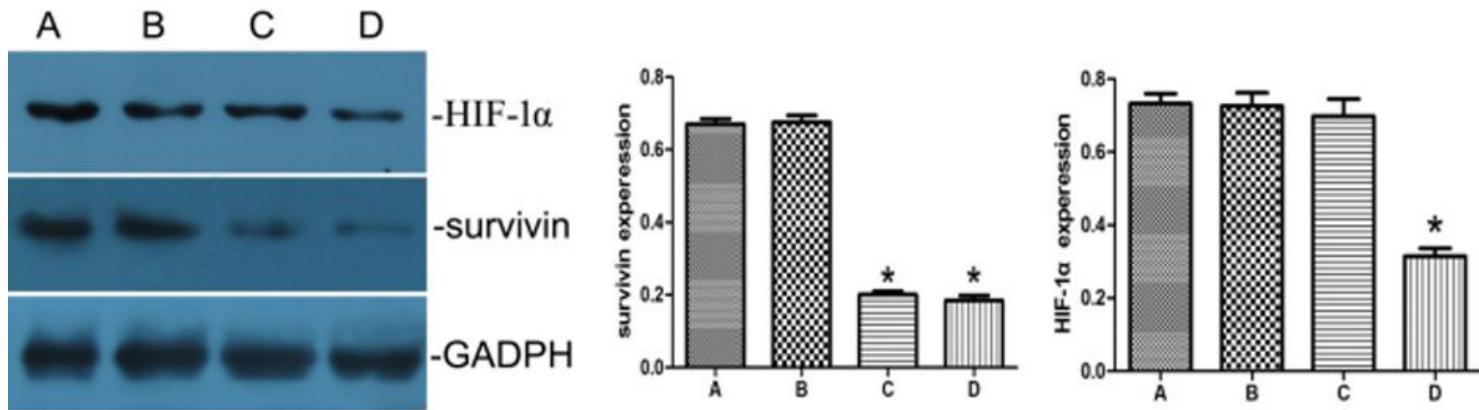
Figure 2

Effect of siRNA on the proliferation of SGC-7901 cells via the MTT assay Note: Line a: Blank control group; Line b: SCR control group; line c: siRNA-Survivin group (ss group); line d: siRNA-Survivin+siRNA-HIF-1 $\alpha$  group (sis+siH group). \*P < 0.05 vs. the control.



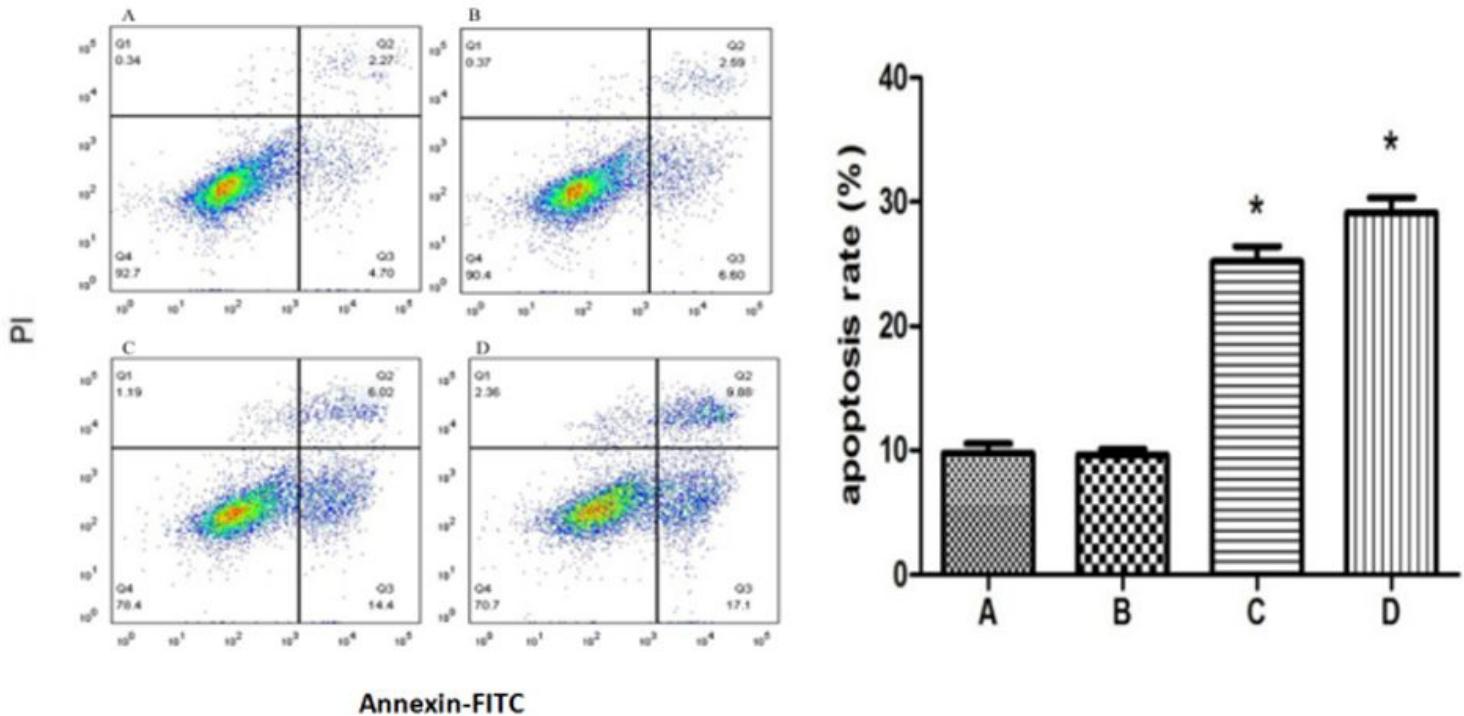
**Figure 3**

Individual inhibitive effects of siRNAs on HIF-1 $\alpha$  and survivin mRNA levels under hypoxia Note: GAPDH was used as internal control and normalized to 100%. The results were similar in three separate experiments. A, D:Blank control group; B, E: SCR control group C, F: siRNA-Survivin+siRNA-HIF-1 $\alpha$  group (sis+siH group). \*P < 0.05 vs. the control.



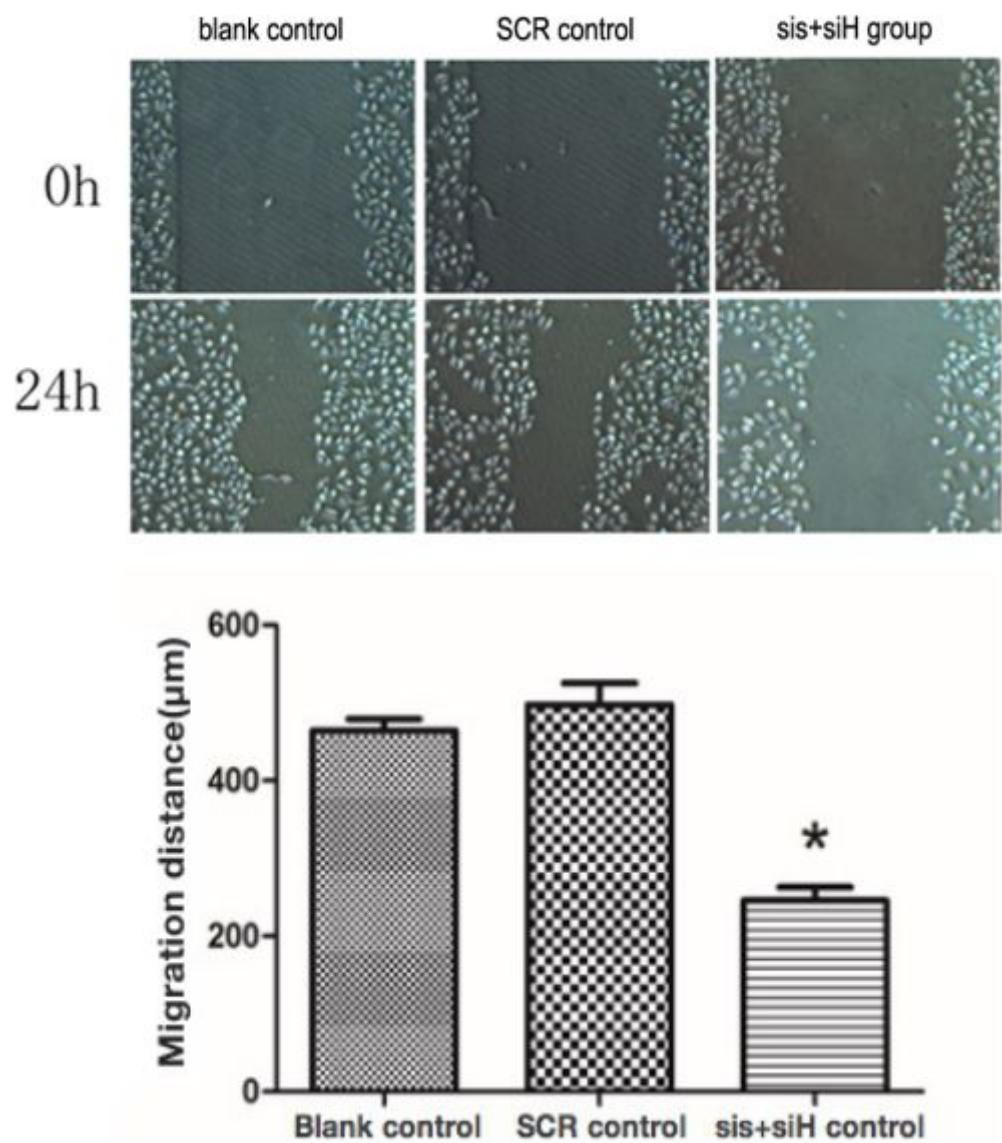
**Figure 4**

Inhibitory effects of siRNAs on HIF-1 $\alpha$  and survivin proteins under hypoxia Note: GAPDH was used as internal control and normalized to 100%. The results were similar in three separate experiments. A:Blank control group; B: SCR control group; C: siRNA-Survivin group (sis group); D: siRNA-Survivin+siRNA-HIF-1 $\alpha$  group (sis+siH group) .\*P < 0.05 vs. the control.



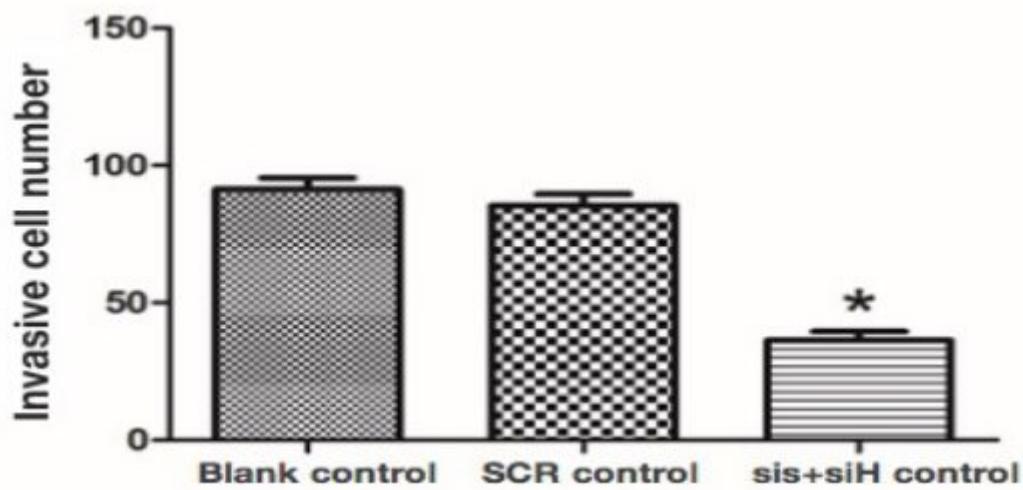
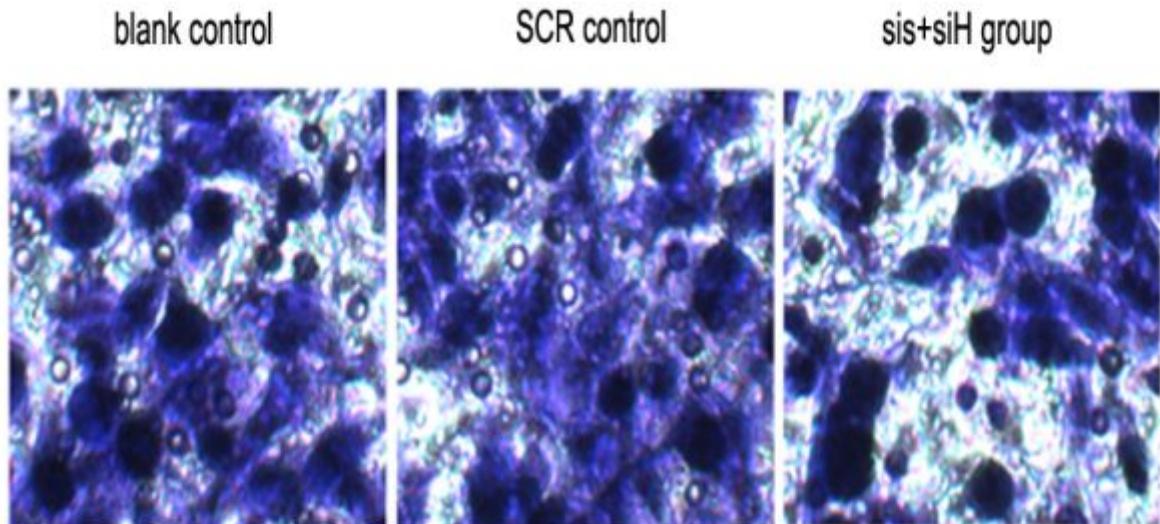
**Figure 5**

Effects of siRNA silencing of HIF-1 $\alpha$  and survivin on apoptosis in SGC-7901 cells Note: Cell apoptosis was assayed by flow cytometry. PI was used for cell nucleus staining and annexin V-FITC for cytomembrane staining. Ratio of apoptosis cells was presented as the standard error of the mean ( $\pm$  SEM). \*P < 0.05 vs. the control.



**Figure 6**

Effect of HIF-1 $\alpha$  or/and survivin RNAi on the migration abilities of SGC-7901 cells under hypoxia Note: \*P < 0.05 vs. the control.



**Figure 7**

Effect of HIF-1 $\alpha$  or/and survivin RNAi on the invasion abilities of SGC-7901 cells under hypoxia Note: \*P < 0.05 vs. the control.