

## Original Article

# RNAi silencing targeting SOX4 inhibits 5-fluorouracil resistance in human colorectal cancer

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**Abstract:** 5-fluorouracil (5-FU) resistance has become a great challenge to successful medical treatment of colorectal cancer (CRC). The purpose of our study is to determine the role of SOX4 in human CRC with 5-FU resistance. RNA interference technology was employed to silence the expression of SOX4 in 5-FU-resistant SW1116 cells. The expression of SOX4 was investigated by qRT-PCR and western blot. Cell viabilities in response to 5-FU treatment were assessed using MTT assay, whereas cell cycle and apoptosis were measured by flow cytometric assay. A mouse xenograft model was used to assess the impact of SOX4 on the efficacy of 5-FU in vivo. We found that SOX4 was highly expressed in 5-FU-resistant SW1116 cells, and silencing SOX4 attenuated 5-FU resistance. These data suggested a crucial role of SOX4 in the progress of 5-FU resistance of CRC cells and silencing SOX4 might be considered as a potential therapeutic strategy for overcoming 5-FU resistance in CRC.

**Keywords:** Colorectal cancer, SOX4, 5-fluorouracil, chemoresistance, viability, cell cycle

## Introduction

Worldwide, colorectal cancer (CRC) is among the most common human gastrointestinal malignancies with increasing incidence and mortality over the past several decades [1]. Despite recent encouraging advances in treatment and understanding the molecular mechanisms of CRC, the 5-year survival rate of CRC patients remains unsatisfactory [2]. Chemotherapy shows high efficacy in decreasing tumor growth and suppressing metastatic progression. However, both intrinsic and acquired drug resistance remains a serious clinical hindrance to successful treatment of CRC and other tumors in the clinic [3].

Up to now, the 5-FU-based chemotherapy regimens are still widely adopted for CRC treatment. 5-fluorouracil (5-FU), a classic anti-metabolite for cancer treatment, was originally introduced in 1957 [4]. As one of the most important pyrimidine antagonists, 5-FU serves its anticancer roles by blocking the activity of thymidylate synthase (TS) and inhibiting RNA or

DNA synthesis [5]. Thus, 5-FU induces cell cycle arrest and/or apoptosis in tumor cells [6]. It was reported that 5-FU-based chemotherapy was a safe and effective treatment for elderly patients with advanced CRC [7]. However, clinical applications of 5-FU have been limited by the rapid development of chemotherapy resistance, which may often result from deficient drug uptake, activation of DNA repair pathways, resistance to apoptosis, and other serious problems. Accordingly, identifying novel therapeutic approaches is of critical importance to overcome chemoresistance.

Gender determining region Y-box 4 (SOX4), a 47-kDa protein member of SOX family, is expressed in a wide range of tissues and has been shown to play an essential role in the regulation of embryonic development and cell fate specification [8]. SOX4 expression has also been reported to be highly expressed in a number of human tumors, including hepatocellular carcinoma [9], oral squamous cell carcinoma [10] and gastric cancer [11]. Ample evidence reveals that the deregulated expression

of SOX4 leads to alterations of oncogenic phenotypes, including inhibition of cell apoptosis, and promotion of cell invasion and metastasis [12]. Recent studies demonstrated that SOX4 in tumor tissues were involved in the regulation of cancer chemoresistance. In the present study, we sought to further verify the speculation that SOX4 serves a crucial role in promoting 5-FU resistance of human CRC cells.

### Materials and methods

#### *Cell lines and culture*

Human CRC cells SW1116, obtained from the American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% penicillin-streptomycin (Gibco, China) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

#### *Generation of the 5-FU-resistant cell line*

5-FU-resistant CRC cell line SW1116/5-FU were generated from parental SW1116 by exposure to gradually increasing doses of 5-FU (Zhenzhou Huawen Chemical Co., Ltd., Zhenzhou, China) in our laboratory. After successive treatments for three months, cells with 5-FU resistance were harvested and used for all subsequent experiments in this study. SW1116/5-FU cells were maintained in medium containing 5-FU to maintain resistance.

#### *Transfection of siRNA*

The siRNAs (small interfering RNAs) against SOX4 (si-SOX4) and negative control (si-NC) were designed and synthesized by Genepharma Company (Shanghai, China). Transfection of cells was performed by using Lipofectamine™ 2000 (Invitrogen, USA) until a final concentration of 50 nM according to the manufacturer's instruction. After transfected and cultured for 48 h, cells were collected for further experiments.

#### *Cell viability assay*

Cells were seeded in 96-well culture plates, and then transfected with si-SOX4 or si-NC for 48 hours. In some experiments, after 48 hours of transfection, cells were treated with indicat-

ed doses of 5-FU for another 48 hours. Cell viability was detected via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT; Sigma-Aldrich) assay on a plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

#### *Flow cytometry analysis of cell cycle and apoptosis*

For cell cycle analysis, the cells were fixed with 75% ethanol, stained with propidium iodide (PI) using the Cycle TEST™ PLUS DNA reagent kit (BD Biosciences), and then subjected to a flow cytometry (FACSCanto II, BD Biosciences) with ModFit 3.0 software (BD Biosciences).

The apoptosis rate of cells was detected using AnnexinV/propidium iodide detection kit (Keygene, Nanjing, China). Following double staining with Annexin V-FITC and PI, cells were analyzed by flow cytometry using CellQuest software (BD Biosciences).

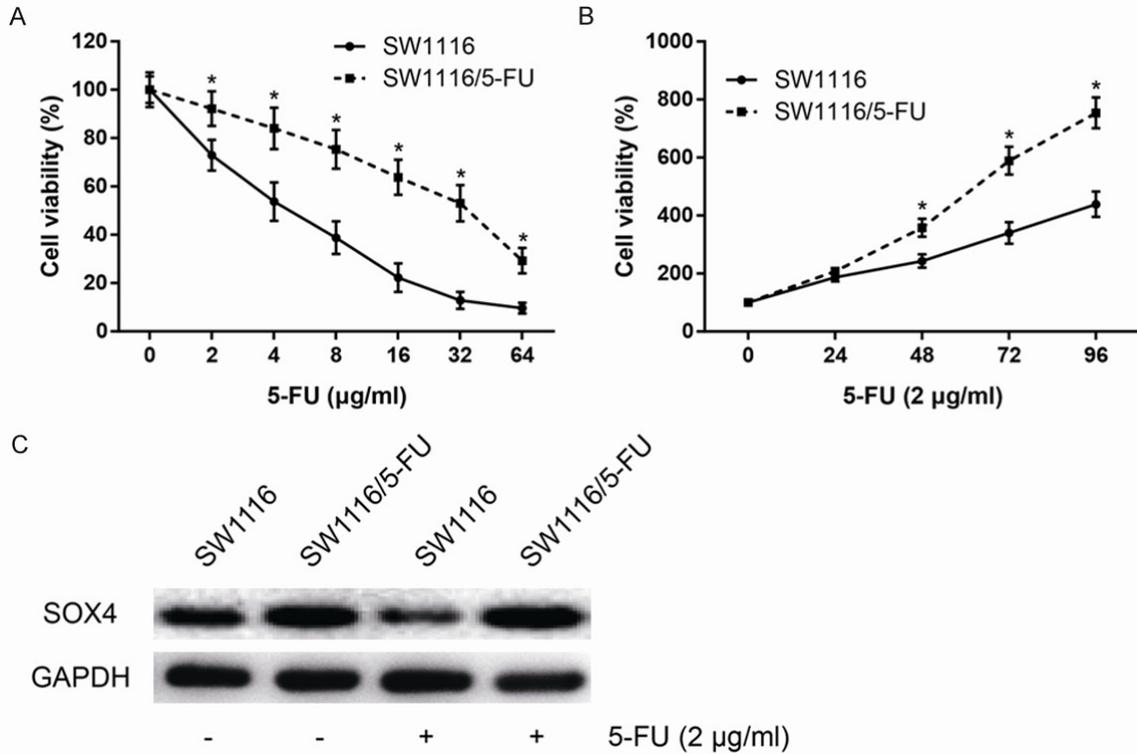
#### *Quantitative real-time PCR*

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA). RNA concentration and purity were determined using NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription of mRNA was performed using the PrimeScript RT Master Mix (Takara Biotechnology, Dalian, China). Quantitative real-time PCR (qRT-PCR) were performed by using SYBR Green PCR Kit (Takara, Japan) on Applied Biosystems 7500 (Life Technologies, California, U.S.A) according to the manufacturer's instructions. The expression of SOX4 mRNA was normalized to GAPDH. The relative expression of genes was calculated using the 2<sup>-ΔΔCt</sup> method [13, 14]. Primer sequences were as follows: SOX4 forward: 5'-GTGAGCGAGATGATCTCGGG-3'; SOX4 reverse: 5'-CAGGTTGGAGATGCTGGACTC-3'; GAPDH forward: 5'-ACAGTCAGCCGCATCTTCTT-3'; GAPDH reverse: 5'-GACAAGCTTCCCGTTCTCAG-3'.

#### *Western blot*

Cells were extracted from cells using radio immunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitor (Beyotime, Shanghai, China). Protein samples were separated by SDS-PAGE and transferred to

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**Figure 1.** SOX4 expression increased in 5-FU-resistant CRC cells. A. MTT assay was performed to analyze the cell viability of SW1116/5-FU cells and parental SW1116 cells in response to 5-FU at various concentrations for 48 h. B. MTT assay was performed to analyze the cell viability of SW1116/5-FU cells and parental SW1116 cells in response to 2 µg/ml 5-FU for 24, 48, 72 and 96 h. C. Western blot analysis was performed to detect SOX4 protein expression in SW1116/5-FU cells and parental SW1116 cells. GAPDH was used as an internal control. The results are expressed as the mean  $\pm$  SD from at least three independent experiments. \* $P < 0.05$  compared with SW1116/5-FU cells.

PVDF membranes (Millipore, Bedford, MA, USA). After being blocked in 5% (w/v) nonfat milk for 2 h at room temperature, the membrane was then probed with the specific primary antibodies (Abcam, Cambridge, UK) overnight at 4°C. The membrane was washed and then incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals were developed using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Buckinghamshire, UK) and analyzed by Quantity One software (Bio-Rad). GAPDH was used as the internal control.

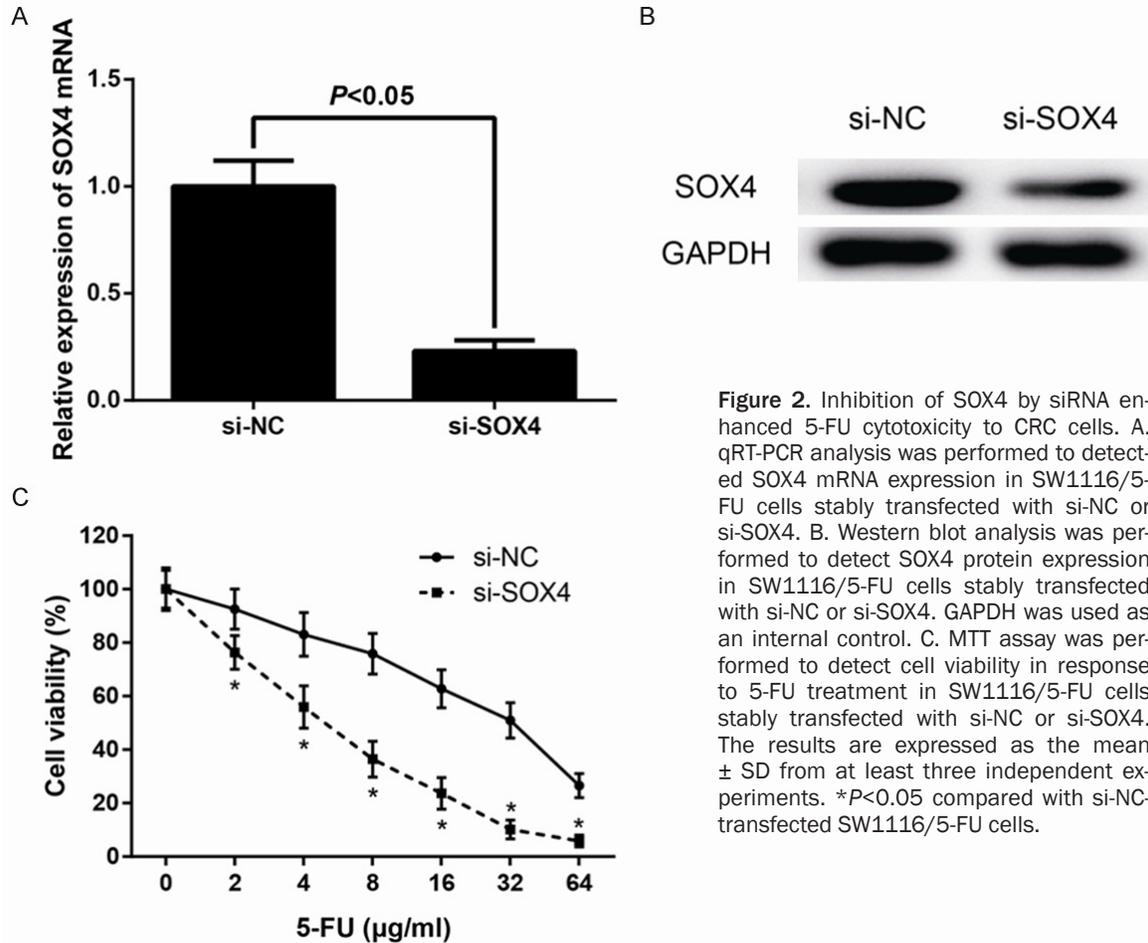
### *In vivo tumorigenesis assay*

A total of ten male nude mice (BALB/c-null, 6-week-old) were purchased from SLAC laboratory animal company (Shanghai, China), and bred in special pathogen-free (SPF) condition. All animal procedures were approved by the

Experimental Animal Ethical Committee of 3201 Hospital.

SW1116/5-FU cells ( $1 \times 10^6$ ) were harvested and re-suspended in 100 µL of serum-free medium, and injected subcutaneously into the right upper back of each nude mouse. When palpable tumors with an average volume of 80-90 mm<sup>3</sup> were formed (at day 14), mice were randomized into two groups with five mice per group such that each group was treated either with si-NC and 5-FU together or si-SOX4 and 5-FU together. 5-FU (50 µg/g) was inoculated through the tail vein, while the siRNAs were injected intratumorally at 3-day intervals for three times before the mice were killed at day 23. Tumor sizes were measured using vernier caliper every three days from the 14th day of injection, and tumor volumes were calculated according to the formula: volume =  $0.5 \times (\text{Length} \times \text{Width}^2)$ . Tumor-bearing mice were sacri-

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**Figure 2.** Inhibition of SOX4 by siRNA enhanced 5-FU cytotoxicity to CRC cells. A. qRT-PCR analysis was performed to detect SOX4 mRNA expression in SW1116/5-FU cells stably transfected with si-NC or si-SOX4. B. Western blot analysis was performed to detect SOX4 protein expression in SW1116/5-FU cells stably transfected with si-NC or si-SOX4. GAPDH was used as an internal control. C. MTT assay was performed to detect cell viability in response to 5-FU treatment in SW1116/5-FU cells stably transfected with si-NC or si-SOX4. The results are expressed as the mean  $\pm$  SD from at least three independent experiments. \* $P < 0.05$  compared with si-NC-transfected SW1116/5-FU cells.

ficed on the 23th day after injection, and tumors were dissected and weighted.

### Statistical analysis

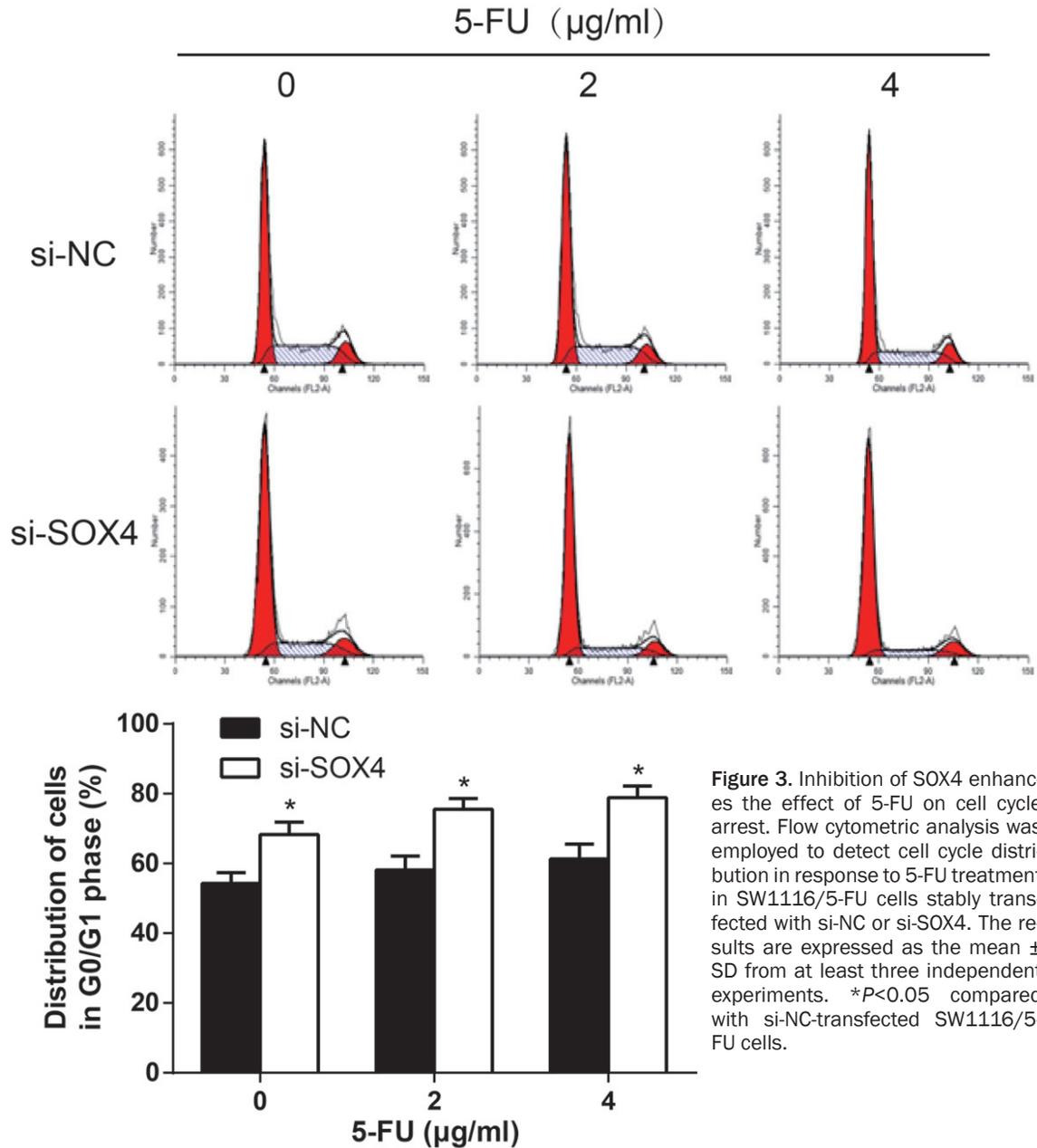
Data were expressed as the mean  $\pm$  standard deviation (SD) according to the results of at least three independently repeated experiments. Statistical significance was analyzed with two-tailed Student's *t*-test. All statistical analyses were calculated using Statistical Package for the SPSS 17.0 (SPSS, Chicago, USA) and Graphpad Prism (version 6.01) software (GraphPad software, La Jolla, CA, USA). A value of  $P < 0.05$  indicated a significant difference.

### Results

#### SOX4 expression increased in 5-FU-resistant CRC cells

To mimic the pathophysiological effect of long-time exposure to 5-FU, which is the firstline chemotherapeutic drug for CRC treatment, we

established SW1116/5-FU cell lines through transforming human CRC SW1116 cells by exposure to elevated dose of 5-FU for three months. To confirm resistance, parental cells and 5-FU-resistant cells were treated with 5-FU at various concentrations for 48 h. Cell viability assays shown that SW1116/5-FU cells tolerated remarkably higher concentrations of 5-FU compared with parental SW1116 cells (**Figure 1A**). The  $IC_{50}$  was 10.6  $\mu$ g/ml for SW1116 cells and 39.1  $\mu$ g/ml for SW1116/5-FU cells. Furthermore, cell proliferation rate in the presence of 2  $\mu$ g/ml 5-FU was investigated at different time point, we observed that SW1116/5-FU showed more resistance to 5-FU treatment compared to parental SW1116 cells (**Figure 1B**). The results of western blot indicated that SOX4 protein expression increased in SW1116/5-FU cells when compared to parental SW1116 cells. After 5-FU treatment, SOX4 protein decreased markedly in parental SW1116 cells, but increased in SW1116/5-FU cells (**Figure 1C**).

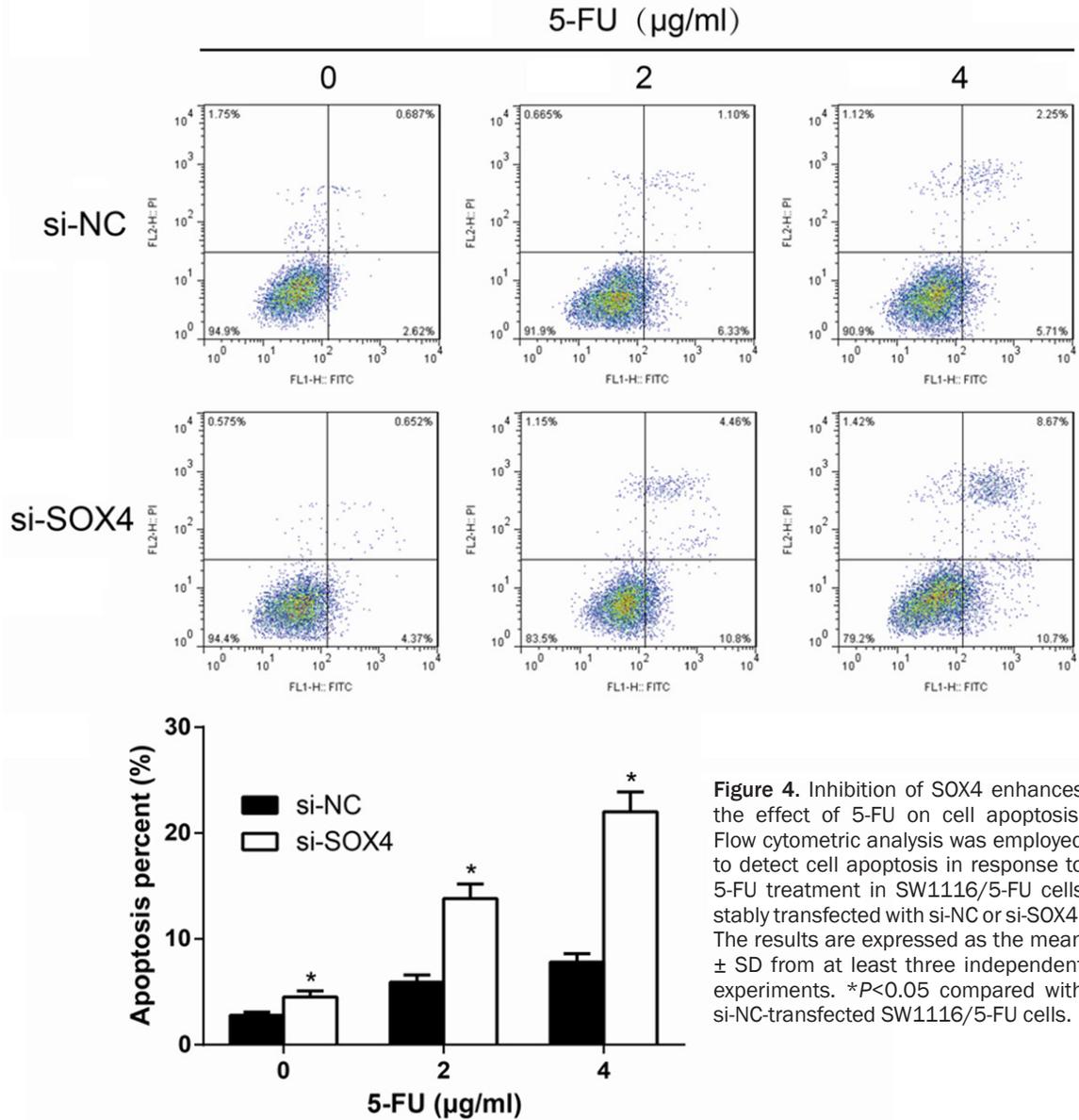


**Figure 3.** Inhibition of SOX4 enhances the effect of 5-FU on cell cycle arrest. Flow cytometric analysis was employed to detect cell cycle distribution in response to 5-FU treatment in SW1116/5-FU cells stably transfected with si-NC or si-SOX4. The results are expressed as the mean  $\pm$  SD from at least three independent experiments. \* $P < 0.05$  compared with si-NC-transfected SW1116/5-FU cells.

*Inhibition of SOX4 by siRNA enhanced 5-FU cytotoxicity to CRC cells*

To determine whether SOX4 inhibition enhances the cytotoxicity of 5-FU in the CRC cells, 5-FU-resistant cells were treated with siRNA to silence SOX4 expression. Analysis of SOX4 mRNA expression with qRT-PCR indicated that in SW1116/5-FU cells with SOX4 siRNA transfection, SOX4 mRNA decreased evidently when compared to SW1116/5-FU cells with si-NC transfection (Figure 2A). Moreover, SOX4 protein expression detected through

western blot showed the SOX4 protein level noticeably reduced after SOX4 knockdown (Figure 2B). After siRNA transfection, SW1116/5-FU cells were treated with 5-FU at various concentrations for cell viability assay. The  $IC_{50}$  of 5-FU was 10.0 μg/ml for si-SOX4-transfected SW1116/5-FU cells, and 37.1 μg/ml for control si-NC-transfected SW1116/5-FU cells (Figure 2C). The cell viability of SW1116/5-FU cells after SOX4 knockdown decreased more rapidly, as compared to SW1116/5-FU cells transfected with si-NC.



**Figure 4.** Inhibition of SOX4 enhances the effect of 5-FU on cell apoptosis. Flow cytometric analysis was employed to detect cell apoptosis in response to 5-FU treatment in SW1116/5-FU cells stably transfected with si-NC or si-SOX4. The results are expressed as the mean ± SD from at least three independent experiments. \*P<0.05 compared with si-NC-transfected SW1116/5-FU cells.

*Inhibition of SOX4 enhances the effect of 5-FU on cell cycle arrest and apoptosis*

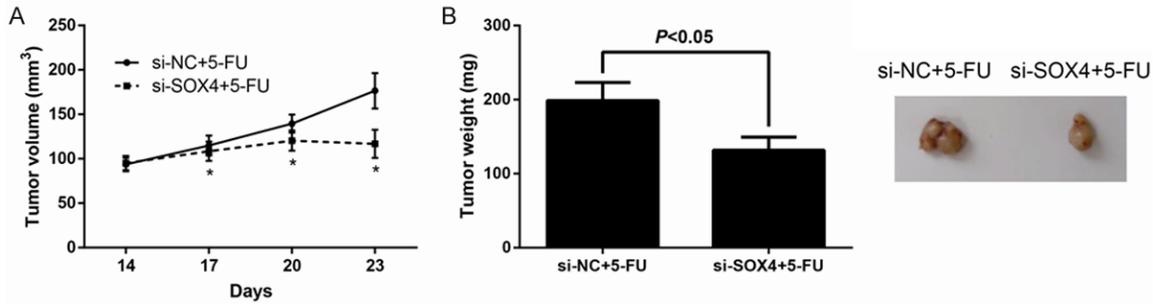
5-FU treatment induces cell cycle arrest and apoptosis [15]. We examined these cellular processes in SW1116/5-FU cells with or without SOX4 silencing to determine their involvement in SOX4-mediated resistance to 5-FU treatment at various concentrations for 48 h. SOX4 knockdown significantly enhanced the induction of cell cycle arrest at the G0/G1 phase by 5-FU treatment (Figure 3). The increase of cell population in G0/G1 phase was accompanied by a concomitant reduction in S phase in si-SOX4-transfected SW1116/5-FU

cells. Moreover, SOX4 knockdown significantly enhanced the induction of apoptosis observed following 5-FU treatment (Figure 4). These findings indicated a role of SOX4 in 5-FU-induced apoptosis and cell cycle arrest.

*Inhibition of SOX4 enhances 5-FU cytotoxicity in vivo*

To confirm the proof-of-principle that SOX4 knockdown could potentially increase the cytotoxic effect of 5-FU *in vivo*, a mouse CRC xenograft model was established by subcutaneously inoculating SW1116/5-FU cells in nude mice. We observed that si-SOX4-injected

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**Figure 5.** Inhibition of SOX4 enhances 5-FU cytotoxicity *in vivo*. A. Tumor volume was calculated every three days following injection of SW1116/5-FU cells stably transfected with si-NC or si-SOX4 after 5-FU treatment. B. After 23 days, Tumor weights are plotted according to treatment groups. Representative photographs of tumors from each group were also shown. The results are expressed as the mean  $\pm$  SD from at least three independent experiments. \* $P < 0.05$  compared with si-NC-transfected SW1116/5-FU cells.

tumors had approximately 4-fold decrease in SOX4 mRNA levels compared with the NC group (Data not shown), indicating that delivery of si-SOX4 into the tumors was successful. As shown in **Figure 5A**, when 5-FU was combined with si-SOX4, it led to greater suppression of tumor growth in comparison to 5-FU treatment with si-NC. The average weight of si-SOX4 and 5-FU treated tumors were decreased to 66.2% of the NC group at day 23 (**Figure 5B**). Representative images of tumors at day 23 were also shown. Therefore, these results provided novel evidence that SOX4 inhibition can enhance the effectiveness of 5-FU on tumor growth *in vivo*.

### Discussion

Up to now, chemotherapy has gradually become one of the most critical therapeutic approaches for CRC patients, particularly for those who experience serious metastases. 5-FU is one of the first-line chemotherapeutic drugs against CRC. However, the overall efficacy of 5-FU treatment is somehow limited in clinic because of the development of acquired drug-resistance [16]. Over the years, as a major clinical problem for chemotherapy, resistance of chemotherapy drugs has been intensively studied and the precise mechanisms for 5-FU resistance in CRC remain elusive. In this study, we have identified that SOX4 regulates the response of CRC cells to 5-FU treatment.

Accumulating evidences demonstrated that, as an oncogene, SOX4 serves a crucial role in improving resistance to chemotherapy in many types of malignancies, such as oral squamous

cell carcinoma [17] and cervical cancer [18]. However, little is known regarding the impact of SOX4 in human CRC and chemotherapy resistance. In the current study, we established 5-FU-resistant cell line from human CRC SW-1116 cells to seek an understanding of the molecular mechanisms of 5-FU resistance. At first, we found that SOX4 was up-regulated in 5-FU-resistant cancer cells and this might be a cellular adaptive response to cytotoxicity. Subsequently we validated the emerging roles of SOX4 by knockdown of SOX4 expression in 5-FU-resistant CRC cells. Downregulation of SOX4 enhanced the cytotoxic effect of 5-FU in 5-FU-resistant CRC cells through affecting cell cycle transition and inducing apoptosis enhancement.

To our best knowledge, this is the first study showing that SOX4 expression was increased upon acquisition of drug-resistance to 5-FU in CRC cell. Previous studies also indicated that SOX4 might exert functions as an oncogene in CRC by modulating cancer-related processes such as cell proliferation, apoptosis, invasion and migration. The SOX4 mRNA and protein expression were markedly higher in CRC tissues compared with adjacent normal mucosa tissues [19], and CRC patients with a higher SOX4 expression level experienced a significantly higher incidence of recurrence and a shorter recurrence-free survival [20], indicating the possible role of SOX4 in CRC progression. Besides, SOX4 expression can be controlled by miRNAs. For example, some miRNAs, such as miR-363-3p [21] and miR-320 [22], exert their anti-oncogenic functions in CRC through targeting SOX4.

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Furthermore, it is also critical to detect both the toxicity and potency of SOX4 silencing in chemotherapy in vivo. Patient-derived xenograft models could serve as a link between clinical research and in vitro studies in cell lines. We found that tumor volume in SOX4 silenced mice treated with 5-FU was markedly decreased, indicating that knockdown of SOX4 enhanced the effect of 5-FU on the suppression of subcutaneous tumor growth.

Chemoresistance remains one of the most significant issues to successful treatment in the majority of human tumors, including CRC [23]. Accordingly, screening for targets to overcome drug resistance is of critical significance for cancer therapy. The present study provided novel indications that SOX4 is implicated in the 5-FU-resistance ability of human CRC cells. Thus SOX4 might be a potential therapeutic target for those 5-FU-resistant CRC patients in the future.

### Disclosure of conflict of interest

None.

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