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Reversal of Multidrug Resistance in Gastric Cancer Cells by E2F-1 Downregulation In Vitro and In Vivo

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ABSTRACT

Transcription Factor E2F-1 plays a critical role in cell cycle regulation and other biological processes in cells. However whether or not it is involved in the multi-drug resistance (MDR) process of gastric cancer has not been fully elucidated yet. To explore the role of E2F-1 in the MDR process of gastric cancer in vitro and in vivo, a cisplatin-resistant gastric cancer cell line with stable downregulation of E2F-1 was established. E2F-1 shRNA led to downregulation of endogenous E2F-1 mRNA and protein. It significantly promoted the sensitivity of SGC7901/DDP cells to cisplatin, doxorubicin, and fluorouracil. Flow cytometry confirmed that the percentage of apoptotic cells increased after E2F-1 downregulation. This notion was further supported by the observation that downregulation of E2F-1 blocked entry into the S-phase of the cell cycle. Furthermore, downregulation of E2F-1 significantly increased intracellular accumulation of doxorubicin. In addition, we determined the in vivo effects of E2F-1 small interfering RNA (shRNA) on tumor size, and apoptotic cells in tumor tissues were detected by deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling and hematoxylin and eosin staining. In molecular studies, semiquantitative RT-PCR and western blotting revealed that E2F-1 downregulation could inhibit expression of MDR1, MRP, Bcl-2/Bax, c-Myc, Skp2, Survivin, and Cyclin D1. In conclusion: E2F-1 may be involved in regulating multiple signaling pathways in reversing MDR, suggesting that E2F-1 may represent a novel target for gastric cancer therapy. J. Cell. Biochem. 115: 34–41, 2014.

KEY WORDS: E2F-1 TRANSCRIPTION FACTOR; RNA INTERFERENCE; GASTRIC CANCER; MULTIDRUG RESISTANCE; MURINE MODEL

Gastric cancer is one of the most frequent cancers, and it ranks the third most common cancer in China [Wu et al., 2002]. Although surgical techniques and chemoradiotherapy have effectively promoted advancements in its treatment, its overall survival rate is still low [Yang et al., 2012]. Due to the importance of chemotherapy in treating gastric cancer, the development of multidrug resistance (MDR) becomes a serious obstacle to effective chemotherapy. Mechanisms involved in MDR include decreased drug accumulation in tumor cells, altered intracellular drug distribution, increased detoxification, and uncoupled pathways linking cellular damage with apoptosis [Shi et al., 2008]. Although the molecular mechanisms of the MDR have not yet been demonstrated in gastric cancer cells, some studies have reported that the mechanisms of MDR were associated with the over-expression of P-glycoprotein (P-gp) encoded by MDR1 gene in tumor cells [Han et al., 2006; Song et al., 2012; Wang et al., 2012], thus, the inhibition of P-gp expression in tumor cells could be one of the most effective ways to reverse MDR and make tumor cells resensitize to chemotherapy [Chen et al., 2012]. In addition, Apoptosis was a common pathway that finally mediated the killing functions of anticancer drugs, which was an important cause of MDR [Hong et al., 2005].

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E2F is a family of transcription factors implicated in cell cycle control. To date, 8 E2F subunits have been identified. Thus far, E2F-1 is the best characterized member. It promotes cell cycle progression by regulating the expression of critical regulator genes involved in DNA replication and G1/S transition [Han et al., 2006]. Most importantly, it plays a most important role in malignant phenotypes of some cancers. Some previous studies reported that E2F-1 could affect the cell proliferation and apoptosis and it may be involved in regulating MDR in some cancers [Han et al., 2003; Lu et al., 2012]. E2F-1 could affect the cell cycle and apoptosis. Furthermore, apoptosis is just one of the most important mechanisms of reversal MDR. Consequently, E2F-1 may play a crucial role in the control of reversal MDR. We assumed that E2F-1 may influence the MDR of gastric cancer cells through regulating the expression of the MDR1 gene and/or with other genes associated with apoptosis. The aim of this study was to explore the relationship between E2F-1 and MDR of gastric cancer cells.

MATERIALS AND METHODS

REAGENTS

Fluorouracil, cisplatin, and doxorubicin were purchased from Sigma–Aldrich (St Louis, MO). Cell culture medium RPMI-1640 was purchased from Invitrogen–Gibco (Carlsbad, CA). Fetal bovine serum (FBS) was from Invitrogen–Gibco. Trypsin, streptomycin, and penicillin were obtained from Sunshine Biotechnology (Nanjing, China). E2F-1, Bax, Bcl-2, c-Myc, Skp2, Survivin, MDR1, MRP, and GAPDH antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest commercial grade available.

CELL CULTURE

The cisplatin-resistant gastric cancer cell line, SGC7901/DDP, was supplied by the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI 1640 supplemented with 10% FBS (Sijiqing Biotec, Co. Ltd., Hangzhou, China), antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in a humidified 5% CO_2 atmosphere at 37.8°C. For SGC7901/DDP cells, 0.6 μg/ml cisplatin was supplemented in the medium to maintain the drug-resistance phenotype.

GENE TRANSFECTION

Recombinant lentiviral vector for E2F-1 gene (E2F-1 shRNA) and null vector (NC shRNA) were stored in our laboratory [Xie et al., 2009b]. SGC7901/DDP cells were seeded in six-well plates with antibiotic-free medium. After 24 h incubation, cells were infected with viral supernatant at a multiplicity of infection of 100 PFU per cell (MOI = 100), and the stable-transfected cell lines were obtained by culturing transfected cells in the presence of 700 mg/ml G418 (Invitrogen, Carlsbad, CA) for 3–4 weeks. The cells were divided into three groups: SGC7901/DDP + LV-E2F-1-RNAi, SGC7901/DDP + LV-NC, and SGC7901/DDP.

MEASUREMENT OF CELL VIABILITY

In 96 well plates, cells were seeded in 100 μl PRMI-1640 medium supplemented with 10% FBS at 5×10^3 cells/well. Then chemotherapeutic agents were added in normal growth medium supplemented with FBS. After 48 h incubation, 10 μl Cell Counting Kit-8 (CCK-8) was added and culture was continued for 1 h in humidified atmosphere containing 5% CO_2. Absorbances at 450 nm were measured by Microplate Reader (Bio-Tech Company). The relative drug resistance folds were analyzed by compared with IC50.

MEASUREMENT OF PUMP RATE OF DOXORUBICIN BY FLOW CYTOMETRY

The cells were inoculated into six-well plates and 4 mg/ml doxorubicin was added, and all wells were placed at 37°C for 30 min. Flow cytometry was used to measure the fluorescent intensity of doxorubicin in cells with an excitation wavelength of 488 nm and emission wavelength of 575 nm. The cells were then washed twice with fresh culture medium and incubated with the new medium at 37°C for 1 h to detect the retained doxorubicin. Subtraction of the fluorescence retained from the total fluorescence was the fluorescent index of doxorubicin. The procedure was repeated three times and an average value was obtained to calculate the pump rate of doxorubicin. The pump rate of the drug from the cells = (accumulated quantity of doxorubicin−retained quantity of doxorubicin)/accumulated quantity of doxorubicin.

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

SGC7901/DDP cells (1×10^6) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4°C for 30 min. The cell pellet was incubated in a solution containing 50 μg/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACScan (Becton–Dickinson, Mountain View, CA). The data was analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA).

SEMIQUANTITATIVE REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from SGC7901/DDP + LV-E2F-1-RNAi cells, SGC7901/DDP + LV-NC cells, and SGC7901/DDP cells using TRIzol Reagent (Invitrogen). All gene segments were amplified and verified by semiquantitative RT-PCR. cDNAs were reverse-transcribed from 2 μg total RNA. The PCR primer sequences were shown in Table I. The products obtained from PCR amplification were separated and photographed in 1% agarose gel. Densitometry was made using the UVP gel image analysis system (BIO-RAD) and the ratio of target to control PCR products was determined by dividing the densitometric volume of the target band by that of the GAPDH band.

WESTERN BLOTTING

Cell lysates were prepared in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris–HCl (pH 7.6), 1 mmol/L EDTA (pH 8.0), 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three-buffer system. The membranes were incubated with a dilution of primary antibody (anti-E2F-1: 1:500, anti-c-myc: 1:1000, anti-survivin: 1:1,500, anti-MDR1: 1:1,000, anti-MRP:

TABLE I. The Primer Sequences for Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

	Primer sequence	Product length (bp)
E2F-1	Upstream: 5′-CCC AAC TCC CTC TAC CCT-3′ Downstream: 5′-CTC CCA TCT CAT ATC CAT CCT G-3′	217
c-Myc	Upstream: 5′-TTC TCT CCG TCC TCG GAT TC-3′ Downstream: 5′-GTA GTT GTG CTG ATG TGT GG-3′	282
Survivin	Upstream: 5′-AAA TGC ACT CCA GCC TCT GT-3′ Downstream: 5′-TGT CGA GGA AGC TTT CAG GT-3′	311
MDR1	Upstream: 5′-ACC AAG CGG CTC CGA TAC A-3′ Downstream: 5′-TCA TTG GCG AGC CTG GTA GTC-3′	110
MRP	Upstream: 5′-GGA CCT GGA CTT CGT TCT CA-3′ Downstream: 5′-CGT CCA GAC TTC TTC ATC CG-3′	292
Skp2	Upstream: 5′-GCT GCT AAA GGT CTC TGG TGT-3′ Downstream: 5′-AGG CTT AGA TTC TGC AAC TTG-3′	291
Bax	Upstream: 5′-CCA AGA AGC TGA GCG AGT GT-3′ Downstream: 5′-CCG GAG GAA GTC CAA TGT C-3′	269
Bcl-2	Upstream 5′-GAC TTC GCC GAG ATG TCC AG-3′ Downstream: 5′-CAT CCC AGC CTC CGT TAT CC-3′	259
CyclinD1	Upstream: 5′-CCC TCG GTG TCC TAC TTC AA-3′ Downstream: 5′-GGG GAT GGT CTC CTT CAT CT-3′	237
GAPDH	Upstream: 5′-ACC ACA GTC CAT GCC ATC AC-3′ Downstream: 5′-TCA CCA CCC TGT TGC TGT A-3′	450

1:1,000, anti-Bax: 1:2,000, anti-Bcl-2: 1:1,500, anti-Skp2: 1:2,000, anti-CyclinD1: 1:200), overnight at 4°C. The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary antibody (1:1,000) (Santa Cruz Biotechnology) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL), according to the manufacturer's recommendations. Western blot film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA). After development, the membrane was stripped and reprobed with antibody against GAPDH (1:1,000) or β-actin (1:1,500) to confirm equal sample loading.

EFFECT OF E2F-1 shRNA on Reversing MDR of Human Gastric Cancer In Vivo

BALB/c 3-week-old male nude mice (Guangxi Animal Center, Nanning, China) were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines. All experimental studies were approved by the Guangxi Medical University Animal Care and Use Committee. SGC7901/DDP cells were used for tumor implantation. Approximately 2×10^6 tumor cells were harvested, resuspended in 100 μl PBS, implanted subcutaneously into the flanks of the BALB/c nude mice, and resulting tumor was named as SGC7901/DDP tumor. After 7 days, when the SGC7901/DDP tumor measured 3–5 mm in diameter, these nude mice were randomly divided into the following three groups (10 mice/per group): SGC7901/DDP + LV-E2F-1-RNAi, SGC7901/DDP + LV-NC, and SGC7901/DDP. The animals were administered an intratumoral injection of LV-siRNA-E2F-1 or LV-siRNA-NC at a titer of 5×10^6 TU in 100 μl PBS, and injection of an equal volume of PBS was used as a blank control. After the first injection, the animals were administered a similar injection every 2 days. DDP was administered by intraperitoneal injection at a dose of 25 mg/kg. After the first injection, the animals were administered a similar injection every 2 days. The tumors were monitored every day and measured every 2 days with a caliper, and the diameters were recorded. The tumor volume (TV) was calculated by the formula: $TV = W^2 \times L/2$, where L is the length and W is the width of the tumor. The relative tumor volume (RTV) was calculated by the formula: $RTV = V_t/V_0$ (V_0 is the TV at the day when the chemicals were given, and V_t is the TV of subsequent measurement). The animals were sacrificed at 21 days after tumor injection and the tumors were analyzed.

HEMATOXYLIN AND EOSIN (HE) STAINING AND DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED DUTP-BIOTIN NICK END LABELING (TUNEL) ASSAY

For HE staining tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin wax. Tissue sections were dewaxed and rehydrated according to a standard protocol. Sections were stained with HE. For the TUNEL assay, apoptotic cells in sections of mouse tumor tissue were detected using an in situ apoptosis detection kit (KEYGEN, Nanjing, China) as instructed by the manufacturer. Cells were visualized with a light microscope (Olympus IX70, Tokyo, Japan). The apoptotic index was calculated as follows: the apoptotic index = number of apoptotic cells/total number of cells. The in vivo experiments strictly obeyed the ethical principles and guidelines for scientific experiments on animals.

STATISTICAL ANALYSIS

Data are expressed as mean ± SE. Statistical significance was determined using χ^2 test, Student's *t*-test, or one-way analysis of variance (ANOVA). Statistical analysis were carried out using SPSS version 13.0 (Chicago, IL) or Origin 7.5 software programs (OriginLab, Northampton, MA). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

E2F-1 shRNA Inhibits E2F-1 mRNA and Protein Expression

To test the hypothesis that E2F-1 shRNA could down-regulate E2F-1 mRNA and protein expression in SGC7901/DDP, we treated SGC7901/DDP cells with Lv-shRNA-E2F-1 and E2F-1 negative control, we found that the transfection of Lv-shRNA-E2F-1 into SGC7901/DDP cells led to remarkable inhibition of E2F-1 mRNA (Fig. 1A) and protein expression (Fig. 1C). Densitometry analysis showed that E2F-1 mRNA (Fig. 1B) and protein (Fig. 1D) in SGC7901/DDP + LV-E2F-1-RNAi cells were about three and fourfold lower, respectively, than those in SGC7901/DDP + LV-NC cells and SGC7901/DDP cells ($P < 0.05$), while no differences were found between SGC7901/DDP + LV-NC cells and SGC7901/DDP cells. These results suggested that E2F-1 shRNA could down-regulate E2F-1 mRNA and protein expression in SGC7901/DDP.

E2F-1 shRNA Reversing the Multidrug Resistance

To rule out the critical roles of down-regulate E2F-1 on chemotherapeutic sensitivity, we measured the IC50 values of SGC7901/DDP cells exposed to some clinical chemotherapeutic drugs (cisplatin, doxorubicin, and fluorouracil). SGC7901/DDP + LV-E2F-1-RNAi cells exhibited significantly decreased IC50 values for cisplatin,

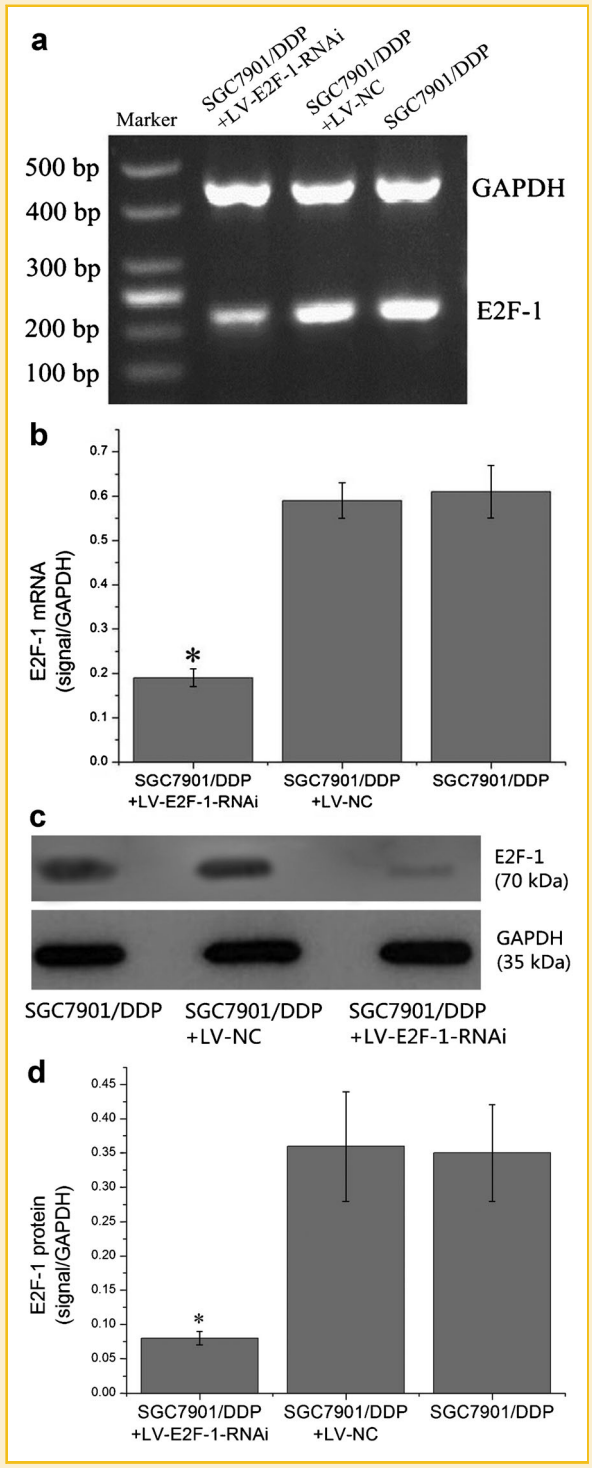

Fig. 1. mRNA and protein expressions of E2F-1 after RNA interference. A,B: Expression level of E2F-1 mRNA was determined by semiquantitative reverse-transcriptase polymerase chain reaction; (C,D) Expression level of E2F-1 protein was determined by Western blotting. mRNA results were expressed as the ratio of E2F-1 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting results were expressed as the ratio of optical density of E2F-1 bands to GAPDH bands. All values are mean ± SE. $^*P < 0.05$, SGC7901/DDP + LV-E2F-1-RNAi cells versus SGC7901/DDP + LV-NC cells and SGC7901/DDP cells.

doxorubicin, and fluorouracil (Fig. 2A). We concluded that lower level of E2F-1 expression was correlated with decreased capacity of cells to resist the clinical chemotherapeutic drugs.

EFFECTS OF E2F-1 shRNA on Pump Rates of Doxorubicin

To dissect the possible way leading to the effect of E2F-1 shRNA reversing the MDR in SGC7901/DDP cells, we examined the potential changes of pump rate of doxorubicin. Pump rates of doxorubicin in the three groups is shown in Figure 2B. SGC7901/DDP + LV-NC and SGC7901/DDP group showed no significant difference in the pump rate of doxorubicin, but were all higher than that of SGC7901/DDP + LV-E2F-1-RNAi group ($P < 0.05$). This suggests that SGC7901/DDP + LV-NC and SGC7901/DDP group had a stronger ability to transport doxorubicin than the SGC7901/DDP + LV-E2F-1-RNAi group, doxorubicin can be moved out quickly, causing less injury to cells, thus having a higher drug tolerance.

EFFECT OF E2F-1 shRNA on Cell Cycle Control

We used flow cytometry to determine whether the effect of E2F-1 shRNA on SGC7901/DDP cell reversing the MDR was mediated, at least in part, through affecting cell cycle progression. We found that SGC7901/DDP + LV-E2F-1-RNAi cells were 39.61% were in G0/G1phase and 47.90% in S phase, with a 33.62% and 33.57% increase in the G0/G1 phase cell population, and 38.78% and 34.73% decrease in the S phase cell population, compared to SGC7901/DDP + LV-NC cells and SGC7901/DDP cells ($P < 0.05$) (Fig. 2C). These data indicate that the resistance to anticancer drugs was reversed is associated with cell cycle arrest at the G0/G1 phase and suggest that restoring sensitivities of anticancer drugs by controlling the G0/G1 and S checkpoints and inducing a specific block in cell cycle progression (Fig. 2D).

E2F-1 shRNA Induces Cellular Apoptosis

We postulated that the effect of E2F-1 shRNA on SGC7901/DDP cells was related to its impact on apoptosis, to test this assumption, cells were stained with Annexin V-FITC and 7-AAD, and then subsequently analyzed by flow cytometry (Fig. 2E), the apoptotic percentage of SGC7901/DDP + LV-E2F-1-RNAi cells was 33.82 ± 1.26%, which was significantly higher than that of SGC7901/DDP + LV-NC cells (17.34 ± 0.81%) and SGC7901/DDP cells (13.16 ± 1.06%) (Fig. 2F). This result demonstrates that down-regulation E2F-1 is able to induce apoptosis in gastric cancer SGC7901/DDP cells.

E2F-1shRNA Influenced the Expression of MDR1, MRP, Bcl-2/Bax, c-Myc, Skp2, Survivin, and Cyclin D1

To investigate the mechanism by which E2F-1 shRNA induces reversing the MDR in SGC7901/DDP cells, we detected expression levels of MDR genes and several apoptotic family members including MDR1, MRP, Bcl-2/Bax, c-Myc, Skp2, Survivin, and Cyclin D1 by semi-quantitative RT-PCR and Western blotting analysis. Densitometry analysis (Fig. 3) indicated that MDR1, MRP, Bcl-2/Bax, c-Myc, Skp2, Survivin, and Cyclin D1 mRNA and protein expression of SGC7901/DDP + LV-E2F-1-RNAi cells were lower than that in SGC7901/DDP + LV-NC cells and SGC7901/DDP cells ($P < 0.05$), while no differences were found between SGC7901/DDP + LV-NC cells and SGC7901/DDP cells.

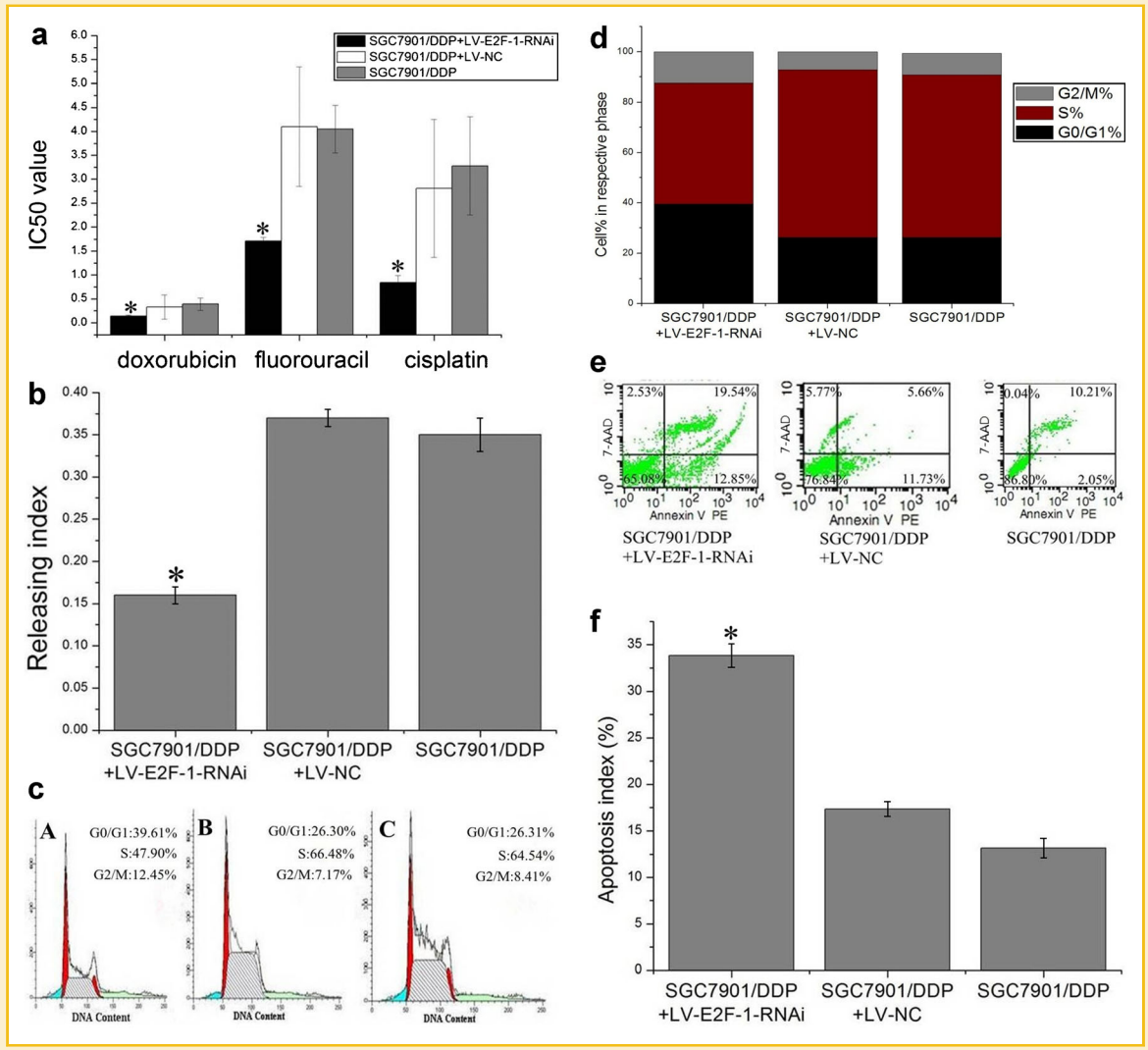

Fig. 2. Effect of downregulation of E2F-1 on IC50, cell pump rate of doxorubicin, cell cycle, and apoptotic rate in SGC7901/DDP cells after RNA interference. A: The IC50 of three cells in cisplatin, doxorubicin, and fluorouracil were assessed by CCK-8 assay. B: Pump rate of doxorubicin in SGC7901/DDP cells after RNAi was analyzed by flow cytometry. C: Cell cycle in SGC7901/DDP cells after RNAi was analyzed by flow cytometry. D: Proportion of cell cycle of three cells. E: E2F-1 shRNA induced apoptosis in SGC7901/DDP cells. F: Apoptotic rate in SGC7901/DDP cells after RNA interference. $^{*}P < 0.05$, SGC7901/DDP + LV-E2F-1-RNAi cells versus SGC7901/DDP + LV-NC cells and SGC7901/DDP cells.

EFFECT OF E2F-1 SHRNA ON REVERSING MULTIDRUG RESISTANCE OF HUMAN GASTRIC CANCER IN VIVO

We also utilized murine model to support our observation in cell lines. We examined the effect of E2F-1 shRNA on growth of SGC7901/DDP cells in vivo by an intratumoral injection of LV-E2F-1-RNAi and LV-NC into SGC7901/DDP tumor. We detected expression levels of E2F-1 in vivo by semi-quantitative RT-PCR and Western blotting. The mRNA (Fig. 4A) and protein (Fig. 4B) expression level of E2F-1 in SGC7901/DDP + LV-E2F-1-RNAi group was lower than that in SGC7901/DDP + LV-NC group and SGC7901/DDP group. Three weeks after intratumoral injection, TV from SGC7901/DDP + LV-E2F-1-RNAi group was $0.74 \pm 0.17\ cm^3$, which was significantly less than $1.63 \pm 0.59\ cm^3$ from SGC7901/DDP group, and $1.98 \pm 0.542\ cm^3$ from SGC7901/DDP + LV-NC group ($P < 0.05$) (Fig. 4C), the tumor growth curves indicate the significant growth inhibition in SGC7901/DDP + LV-E2F-1-RNAi group ($P < 0.05$). The percent of apoptotic tumor cells in SGC7901/DDP + LV-E2F-1-RNAi cells was $7.5 \pm 1.7\%$, which was more than $3.1 \pm 1.1\%$ in SGC7901/DDP + LV-NC cells and $3.0 \pm 1.2\%$ in SGC7901/DDP cells, as determined by the hematoxylin and eosin (HE) staining analysis (Fig. 4D) and TUNEL method (Fig. 4E).

DISCUSSION

The development of MDR to cancer chemotherapy is a major obstacle to the effective treatment of gastric cancer [Fan et al., 2000]. However, the mechanism of MDR remains obscure. P-gp was the first molecule identified as a modulator of MDR. After that, various other molecules were shown to be involved, including transporters that

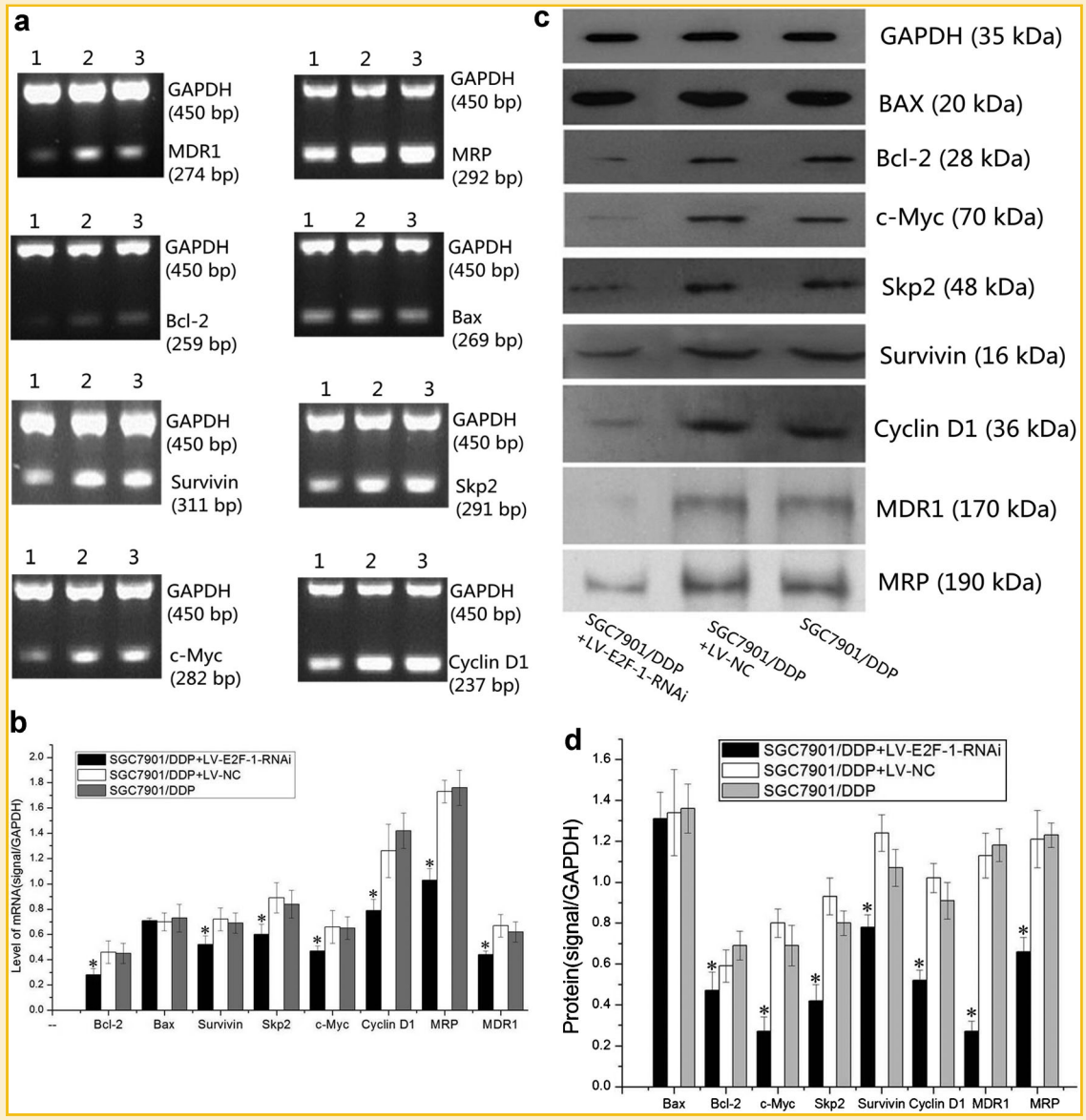

Fig. 3. E2F-1 shRNA regulates reversal of multidrug resistance through some pathways. A: Semi-quantitative RT-PCR analysis of MDR1, MRP, Bcl-2, Bax, c-Myc, Skp2, Survivin, and Cyclin D1 mRNA expression in SGC7901/DDP cells, GAPDH was used as the internal control. Lane 1: SGC7901/DDP + LV-E2F-1-RNAi cells; Lane 2: SGC7901/DDP + LV-NC cells; Lane 3: SGC7901/DDP cells. B: MDR1, MRP, Bcl-2, Bax, c-Myc, Skp2, Survivin, Cyclin D1, and GAPDH mRNA levels were measured at three groups, normalized to those of GAPDH. C: Western blot analysis of MDR1, MRP, Bcl-2, Bax, c-Myc, Skp2, Survivin, Cyclin D1, and GAPDH in SGC7901/DDP cells from three groups, respectively, GAPDH: internal control protein. D: MDR1, MRP, Bcl-2, Bax, c-Myc, Skp2, Survivin, Cyclin D1, and GAPDH protein levels were measured at three groups, normalized to those of GAPDH. $^{*}P < 0.05$, SGC7901/DDP + LV-E2F-1-RNAi cells versus SGC7901/DDP + LV-NC cells and SGC7901/DDP cells.

eject anticancer drugs from cells, such as MDR-associated protein (MRP) [Chuman et al., 1996], genes regulating apoptosis, such as p53 [Matsuhashi et al., 2005], PKC [Han et al., 2002], and Bcl-2 family [Xiao et al., 1998]. Recently, the distribution of drugs in cancer cells was also considered to play a part in MDR [Minchinton and Tannock, 2006], but there may be other mechanisms that control MDR of gastric cancer cells [Fan et al., 2000].

Transcription factor E2F-1 has been largely studied as a promoter of S phase transition in the cell cycle and as a regulator of apoptosis, E2F-1 has been shown to eliminate latent neoplastic cells through apoptosis in vitro [Li et al., 2009]. Downregulation of E2F-1 significantly inhibits the growth of prostate cancer cell lines and leiomyosarcoma [Nguyen et al., 2005], and it is linked to low proliferation and longer survival of different types of carcinoma cells in vivo and in vitro [Evangelou et al., 2007]. Our previous study reported that E2F-1 plays diverse roles in cells ranging from regulating cell cycle progression to promoting cell death by apoptosis in gastric cancer cells in vitro and in vivo [Xiao et al., 2007; Xie et al., 2009a,b; Wang et al., 2011]. The present study is believed to be the first to correlate E2F-1 with MDR of gastric cancer cells, and we

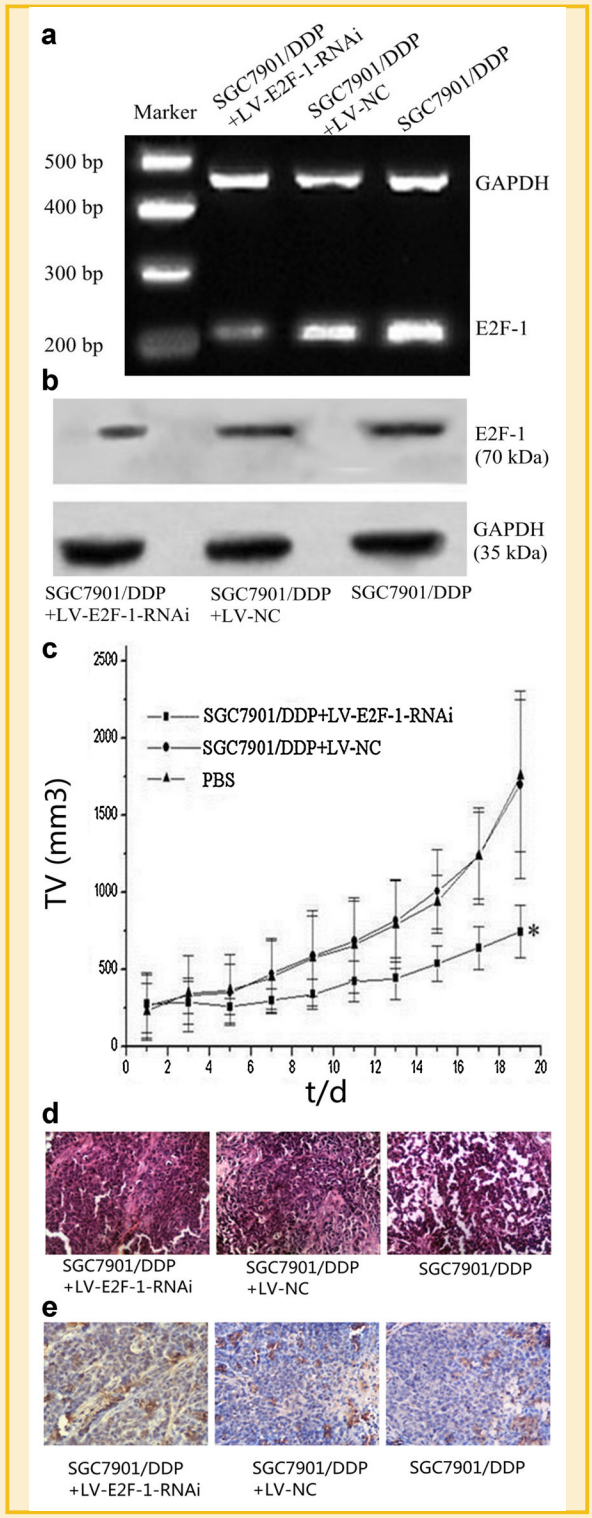

Fig. 4. Effect of E2F-1 shRNA-mediated inhibition of E2F-1 mRNA and protein expression and downregulation of E2F-1 on apoptosis in vivo. A: mRNA expression level of E2F-1 was determined by semiquantitative RT-PCR. B: Protein expression level of E2F-1 was determined by western blotting. C: Tumor volume of nude mice in each group is presented. D,E: Tumor cell apoptosis was assessed by HE (D) staining and TUNEL assay (E). $^{*}P < 0.05$, SGC7901/DDP + LV-E2F-1-RNAi cells versus SGC7901/DDP + LV-NC cells and SGC7901/DDP cells.

found that expression of E2F-1 regulated drug efflux pumping, the cell cycle, and apoptosis. The multiple changes conferred by E2F-1 on gastric cancer cells are not surprising, given the involvement of E2F-1 in a wide range of biochemical reactions, and E2F-1 is a transcription factor that contributes to reversing MDR.

Our study indicated that E2F-1 shRNA led to downregulation of E2F-1 mRNA and protein expression in SGC7901/DDP cells, caused cell cycle arrest in the G0/G1 phase, and induced cell apoptosis. Furthermore, downregulation E2F-1 in SGC7901/DDP cells enhanced the sensitivity of SGC7901/DDP cells to cisplatin, doxorubicin, and fluorouracil. The ability to pump doxorubicin was reduced significantly, moreover, a strong anti-tumor effect of E2F-1 shRNA in vivo was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when E2F-1 mRNA and protein was downregulation by E2F-1 shRNA. These findings suggest that E2F-1 shRNA reversed MDR of human gastric cancer cells.

MDR is mainly due to the overexpression of P-gp (which is encoded by MDR1) and MRP, they function as a drug efflux pump which actively transports drugs from the inside to the outside of cancer cells and prevents the intracellular accumulation of anticancer drugs inside cancer cells necessary for cytotoxic activity, it should be noted that P-gp-mediated drug efflux was not the only mechanism involved in drug resistance. Previous studies have shown that the effect of P-gp on drug resistance is closely related to cell cycle distribution and apoptosis [Kornmann et al., 1999; Sicari et al., 2012; Tsubaki et al., 2012]. S phase kinase-associated protein 2 (skp2) is a key regulator of cell cycle control, c-Myc is required for Skp2 to trigger S phase entry, Skp2-Myc connection is important for S phase progression, skp2 recently has been shown to be a direct downstream target gene of c-myc [Kornmann et al., 1999], in addition, suppression of cyclin D1 (a regulatory kinase of cell cycle distribution) levels has been shown to potentiate the response of human pancreatic cancer cells to cisplatinum [Kornmann et al., 1998]. Besides regulation of cell cycle distribution, apoptosis is a common pathway that finally mediates the killing effects of anticancer drugs, which is an important cause of MDR. Bcl-2 family, which includes anti- and pro-apoptotic members such as Bcl-2 and Bax [Igney and Krammer, 2002; Tait and Green, 2010; Sicari et al., 2012], Bcl-2 binds to the mitochondrial membrane, competitive binding with Bax and forming Bcl-2/Bax heterodimer, which leads to closing mitochondrial permeability transition pore and preventing the release of cytochrome C, thereby inhibiting apoptosis via the Bcl-2/Bax signal pathway, moreover, Survivin induces mitochondrial fragmentation, and reduces mitochondrial respiration [Hagenbuchner et al., 2012], it is closely related to apoptosis. Therefore, in the present study, inhibition of E2F-1 expression may have decreased MDR1, MRP, Bcl-2/Bax, c-Myc, Skp2, Survivin, and Cyclin D1 expression directly or indirectly, which was responsible for reversal of MDR in human gastric cancer cells in vitro and in vivo. Further studies are needed to confirm our results.

The term MDR was originally coined to define a condition enabling a disease-causing organism or cancer cells to resist distinct drugs or chemicals with a wide variety of structure and function, targeted at eradicating the organism/cancer cell. Much routine chemotherapy cannot achieve good therapeutic effects because of MDR [Lu et al., 2012]. In this study, we showed that E2F-1 plays a critical role in reversing MDR. Downregulation of E2F-1 using RNAi reversed the

progression of MDR in gastric cancer SGC7901/DDP cells in vitro and in vivo. In conclusion, this study lays the foundation for treatment of MDR in gastric cancer through manipulation of E2F-1 expression.

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