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Forkhead Box R2 Knockdown Decreases Chemoresistance to Cisplatin via MYC Pathway in Bladder Cancer

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Background: Bladder cancer is a very common urological cancer globally, and cisplatin- or gemcitabine-based chemotherapy is essential for advanced bladder cancer patients. Many patients with bladder cancer have a relatively poor response to chemotherapy, leading to failure of clinical treatment. We mined the GSE77883 GEO dataset, identifying FoxR2 as being a significantly upregulated gene in T24 chemoresistant cells. Herein, we assessed how FoxR2 functions in bladder cancer cell chemoresistance.

Material/Methods: Cisplatin-resistant T24 (T24/DDP) cells were constructed by administering increasing concentrations of cisplatin, and differences in expression of FoxR2 were examined in T24/DDP and T24 cells. FoxR2 loss- and gain-of-function cells models were established in T24/DDP and T24 cells, respectively. Cell survival, clone formation, cell cycle, and cell apoptosis were assessed, and the MYC pathway was verified.

Results: FoxR2 was significantly upregulated in T24/DDP cells compared to T24 cells. Knockdown of FoxR2 in T24/DDP cells, survival rate, and clone formation were decreased, G1/S phase transition was suppressed, and cell apoptosis was promoted. These results were reversed by restoration of FoxR2 levels in T24 cells. We found that FoxR2 knockdown enhanced sensitivity to cisplatin, whereas MYC overexpression antagonized chemosensitivity in T24/DDP cells.

Conclusions: FoxR2 knockdown decreases chemoresistance to cisplatin via the MYC pathway in bladder cancer cells, and this may be a target for overcoming chemoresistance in bladder cancer.

MeSH Keywords: Cisplatin • Drug Resistance • Forkhead Transcription Factors • Urinary Bladder Neoplasm

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Background

Bladder cancer is very common, with an incidence of around 8 people per in 100 000 population, and this rate is rising steeply in China [1]. More than 7 in 10 bladder cancers are non-muscle-invasive bladder cancer (NMIBC), which can be treated via immunotherapy and chemotherapy or by surgical resection. However, up to 80% of NMIBC patients have recurrence within 2 years, and around 30% of these patients progress to advanced bladder cancer, with high aggressiveness and mortality [2]. At present, cisplatin- or gemcitabine-based neoadjuvant chemotherapy before radical cystectomy is used for treating advanced bladder cancer [3]. However, drug resistance inevitably occurs in most patients due to chemoresistance, leading to unsatisfactory therapeutic effects. Thus, it is crucial to explore other effective targets in order to overcome chemoresistance.

Forkhead box R2 (FoxR2) is a novel Fox transcription factor discovered in 2004 [4]. FoxR2 has a highly-conserved C-terminal forkhead domain [4]. FoxR2 has been shown to be a pro-oncogene in several cancers and is closely linked to tumorigenesis in lung cancer [5], hepatocellular carcinoma [6], colorectal cancer [7], prostate cancer [8], medulloblastoma, and glioma [9,10]. Furthermore, several studies have shown many Fox family members to be involved in cancer chemoresistance [11–13]. However, it remains unclear whether FoxR2 mediates cancer chemoresistance. Mining of Gene Expression Omnibus (GEO) data for GSE77883 revealed increased expression of FoxR2 in T24-chemoresistant cells compared with parent cells, suggesting that FoxR2 is involved in bladder cancer chemoresistance. Multidrug resistance in bladder cancer is attributed to resistance to cisplatin (DDP) and gemcitabine [14–16]; therefore, we hypothesized that FoxR2 is also involved in cisplatin resistance.

We established cisplatin-resistant bladder cancer cells (T24/DDP) using a concentration gradient increment method to explore how FoxR2 affects bladder cancer chemoresistance. We found that FoxR2 enhanced chemoresistance via activation of MYC transcription. This shows that FoxR2 may be a viable target for therapy of chemoresistance in bladder cancer.

Material and Methods

Cell culture and cisplatin resistance

T24 cells came from the Chinese Academy of Sciences Culture Collection (Shanghai, China). RPMI-1640 containing 10% FBS (Gibco, USA) at 37°C was used for cell growth in a 5% CO_2 incubator. Wang et al. [17] reported that cisplatin-resistant T24/DDP was produced via stepwise exposure to escalating concentrations (0.2 μg/ml to 2.0 μg/ml) of DDP (Selleck, USA) for >6 months.

qRT-PCR

Total RNA was extracted via Trizol (Life Technologies, USA) and DNase I was used to eliminate DNA contamination. The purified RNA was reversely transcribed using a First Strand cDNA Synthesis Kit (TaKaRa Bio, Japan) following the provided directions. The LightCycler480 system (Roche, Germany) was used for reactions with SYBR Green Premix (Toyobo Co., Japan). β-actin was used as the housekeeping gene for normalizing. Primers used for FoxR2 and β-actin were:
FoxR2, 5'-GTGCATGAGTCAGCCAGAGTT-3' (forward),
5'-AAGTAGGCACAGTAAGGAAGG-3' (reverse);
β-actin, 5'-TTCCTTCCTGGGCATGGAGTC-3' (forward),
5'-TCTTCATTGTGCTGGGTGCC-3' (reverse).
Triplicate reactions were conducted. Amplification productions were calculated via the $2^{-\Delta\Delta Ct}$ method.

Cell transfection

The siRNAs and scramble control were from GenePharma (Shanghai, China). FoxR2 cDNA and c-MYC cDNA ORF expressional plasmids were purchased from Sino Biological (Beijing, China). Lipofectamine 2000 (Life Technologies) was used for transfection.

Cell viability assay

T24 and T24/DDP cells were plated for 24 h in 96-well plates in 100 μl media. A range of DDP was used to treat cells, and MTT assay (Sigma, USA) was used to assess viability. We added 100 μl MTT (5 mg/ml) to each well for 4 h at 37°C. Supernatants were then replaced using 150 μl DMSO for 15 min. Finally, the OD of each well was determined at 570 nm.

Colony-formation assay

We placed 1000 cells into 35-mm dish (in triplicate) and maintained them in RPMI-1640 with or without DDP. After 2 weeks, 4% paraformaldehyde was used for cell fixation prior to crystal violet staining. Colony formation was calculated based on: (number of colonies/number of seeded cells)×100%.

Western blot analysis

RIPA was used to lyse cells, and a BCA Protein Assay Kit (ThermoFisher, USA) was used for quantification. Equal amounts of proteins were separated on 10% SDS-PAGE gels prior to transfer to membranes (Millipore, USA). Blots were blocked for 3 h in 5% non-fat (Mengniu, Hohhot, China), primary antibodies against FoxR2 (1: 500, Proteintech), MDR1 (1: 500, Proteintech), MRP (1: 1000, CST), MYC (1: 1000, CST), CDK4 (1: 500, Proteintech), CCND1 (1: 1000, Proteintech), and P21 (1: 500, Proteintech) overnight at 4°C. Then, an HRP-conjugated

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secondary antibody was used to probe blots for 2 h. Signals were then detected using enhanced chemiluminescence reagent (ThermoFisher, USA). Image-Pro plus 6.0 was used for densitometric analyses, with GAPDH used for normalization.

Cell apoptosis assay

After treatment, cells were collected and spun at 2000 rpm for 5 min. The Annexin V-FITC/PI Apoptosis Detection Kit (Bioleng, USA) was then used according to the provided directions, followed by suspending cells in 500 µl binding buffer, then adding 5 µl Annexin V-FITC. Next, 5 µl PI was added for 10 min in the dark, and cells were assessed via flow cytometry (CytoFlex, Beckman, USA) within 1 h.

Cell cycle analysis

After treatment, 70% ethanol was used to fix cells overnight at 4°C. PBS was then used for washing, followed by 30-min staining with PI in a buffer containing RNase A and Triton X-100 at 4°C and flow cytometric analysis (CytoFlex, Beckman, USA).

Statistical analysis

SPSS 18.0 was used for analyses. All the described experiments were performed at least 3 times, the data are means±SD. Data were compared via *t* tests and one-way ANOVA with Tukey's multiple comparison test for 2 and >2 groups, respectively. $p<0.05$ was significant.

Results

FoxR2 is significantly upregulated in cisplatin-resistant T24 cells

Cisplatin-resistant T24 cells (T24/DDP) generated using increasing concentrations of DDP were used to investigate the mechanisms involved in chemoresistance in bladder cancer. The cell survival rate of T24/DDP cells and parental T24 cells decreased dose-dependently in response to DDP (0–15 µg/mL), and T24/DDP cells showed significantly greater resistance to cisplatin compared with T24 cells (Figure 1A). Half maximal inhibitory concentration (IC_{50}) values were higher in T24/DDP cells (6.18±0.76 µg/mL) compared with T24 cells (1.63±0.13 µg/mL) (Figure 1B). Furthermore, expression levels of the drug-resistant markers MDR1 and MRP were significantly elevated in T24/DDP cells compared with that of T24 cells (Figure 1C). Both at protein and mRNA levels, FoxR2 expression was significantly upregulated in T24/DDP cells compared with the parental T24 cells (Figure 1D, 1E), which was consistent with the GEO data (GSE77883) from microarray analysis of T24 chemoresistant cells (Figure 1F). These results showed that FoxR2 may be linked with bladder cancer chemoresistance.

Knockdown of FoxR2 sensitises T24/DDP cells to cisplatin

As FoxR2 was upregulated in resistant T24/DDP cells, we knocked down FoxR2 using siRNA to evaluate the effect of FoxR2 on bladder cancer cisplatin resistance. Figure 2A shows the interference efficiency of FoxR2. Expression of FoxR2 was significantly suppressed in the FoxR2 siRNA groups relative to the scrambled siRNA control group. As siRNA-2 showed the greatest knockdown effect, it was selected for use in subsequent assays. MTT assays revealed that FoxR2 knockdown alleviated resistance to DDP compared with the scrambled siRNA control group (Figure 2B), and the IC_{50} value was slightly decreased in si-FOXR2 cells (5.08±0.14 µg/mL) compared with the scrambled group (6.86±0.17 µg/mL) (Figure 2C). Colony-formation assays demonstrated that FoxR2 knockdown decreased the rate of colony formation in the absence of DDP. Knockdown of FoxR2 sensitised T24/DDP cells to treatment with 6 µg/mL DDP, as demonstrated by a significant decrease in colony formation (Figure 2D). We then investigated whether FoxR2 was linked to progression through the cell cycle in bladder cancer cells. Downregulation of FoxR2 led to more cells in the G_0/G_1 phase relative to the scrambled siRNA group (Figure 2E, Supplementary Figure 1A), suggesting that knockdown of FoxR2 inhibited G_1/S phase transition in response to DDP. Finally, we investigated whether FoxR2 affected DDP sensitivity by regulating cisplatin-induced cell apoptosis. Flow cytometry results revealed an increased rate of apoptosis in FoxR2 knockdown cells after DDP treatment (Figure 2F, Supplementary Figure 1B).

Restoration of FoxR2 enhances cisplatin resistance in T24 cells

As FoxR2 was downregulated in T24 cells, we overexpressed FoxR2 in this cell line (Figure 3A). MTT assays revealed that overexpression of FoxR2 led to mildly increased resistance to cisplatin relative to the control group (Figure 3B), and the IC_{50} value was slightly increased in the FOXR2-overexpressed group (2.72±0.15 µg/mL) compared with the negative control group (NC, cells transfected with empty vectors) (1.95±0.08 µg/mL) (Figure 3C). Furthermore, upregulation of FoxR2 promoted colony formation in the absence of DDP. FoxR2 overexpression led to resistance of T24/DDP cells to treatment with 1.5 µg/mL DDP, as shown by a significant increase in colony formation (Figure 3D). Cell cycle analysis revealed that upregulation of FoxR2 resulted in higher numbers of cells in S phase versus the control group (Figure 3E, Supplementary Figure 2A), suggesting that enhancement of FoxR2 promotes G_1/S phase transition. Flow cytometry results showed a reduced cell apoptosis rate in FoxR2 restoration cells after DDP treatment (Figure 3F, Supplementary Figure 2B).

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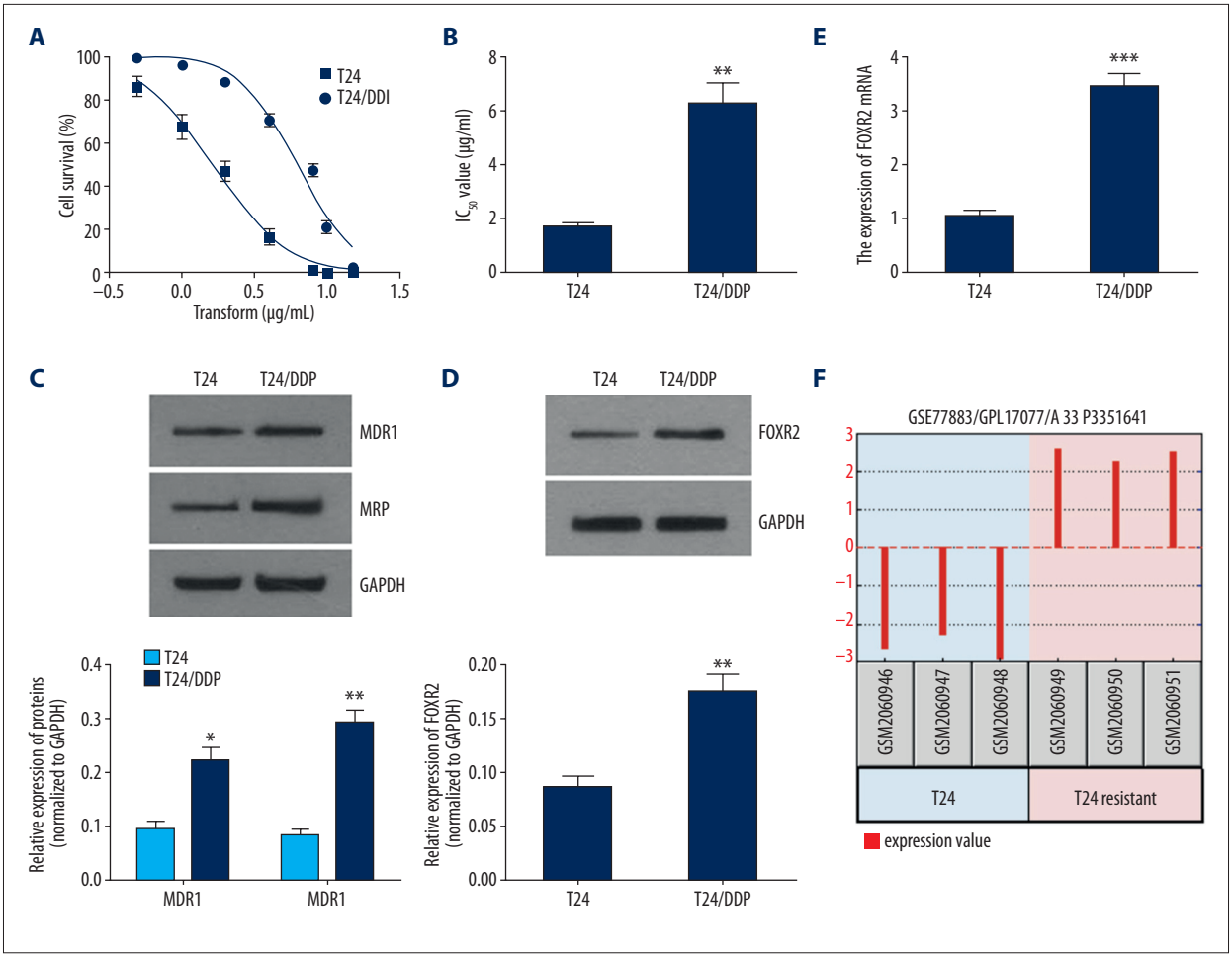

Figure 1. FoxR2 is significantly upregulated in DDP-resistant T24 cells. (**A**) Cell survival was assessed in T24 and T24/DDP cells by MTT assay. (**B**) IC_{50} values of cisplatin in T24 and T24/DDP cells. (**C**) Drug resistance-related genes – MDR1 and MRP – were analysed by Western blotting. FoxR2 expression in T24 and T24/DDP cells was analysed by Western blotting (**D**) and qRT-PCR methods (**E**). (**F**) FoxR2 mRNA expression in GEO data (GSE77883). All data represent the mean±SD, and all experiments were conducted in triplicate. * $P<0.05$, ** $P<0.01$.

FoxR2 mediates cisplatin resistance via the MYC pathway

To identify how FoxR2 mediates chemoresistance in bladder cancer, STRING software (*https://string-db.org/*) was used to construct integrated gene network maps. The results showed that the most common oncogene was MYC (Figure 4A). Previous studies have shown that MYC is involved cancer chemoresistance; therefore, we investigated whether FoxR2 mediated cisplatin resistance via the MYC pathway. In T24/DDP cells, FoxR2 knockdown suppressed expression of MYC and its target genes CDK4 and CCND1, but upregulated another target gene, p21. Overexpression of FoxR2 in T24 cells showed the opposite results (Figure 4B). FoxR2 knockdown enhanced sensitivity to DDP, whereas MYC overexpression antagonized chemosensitivity in T24/DDP cells (Figure 4C). Meanwhile, enhancement of MYC abolished the effects of FoxR2 knockdown on cell colony formation (Figure 4D), cell cycle arrest (Figure 4E, Supplementary Figure 3A), and cell apoptosis (Figure 4F, Supplementary Figure 3B) in T24/DDP cells. These results suggest that FoxR2 mediates cisplatin resistance via the MYC pathway.

Discussion

FoxR2 is a Fox family member involved in dysregulation in multiple cancers. Xu et al. reported overexpression of FoxR2 in prostate cancer, and FoxR2 knockdown decreased growth and metastasis of tumors [8]. In ovarian cancer, upregulation of FoxR2 has been frequently reported and is associated with poor survival as well as promotion of metastasis and growth [18]. Wang et al. confirmed that FoxR2 contributes to NSLC proliferation and invasion [5]. These findings show that FoxR2 is an oncogene that contributes to tumorigenesis and cancer progression. However, the role of FoxR2 has not been previously reported in bladder cancer.

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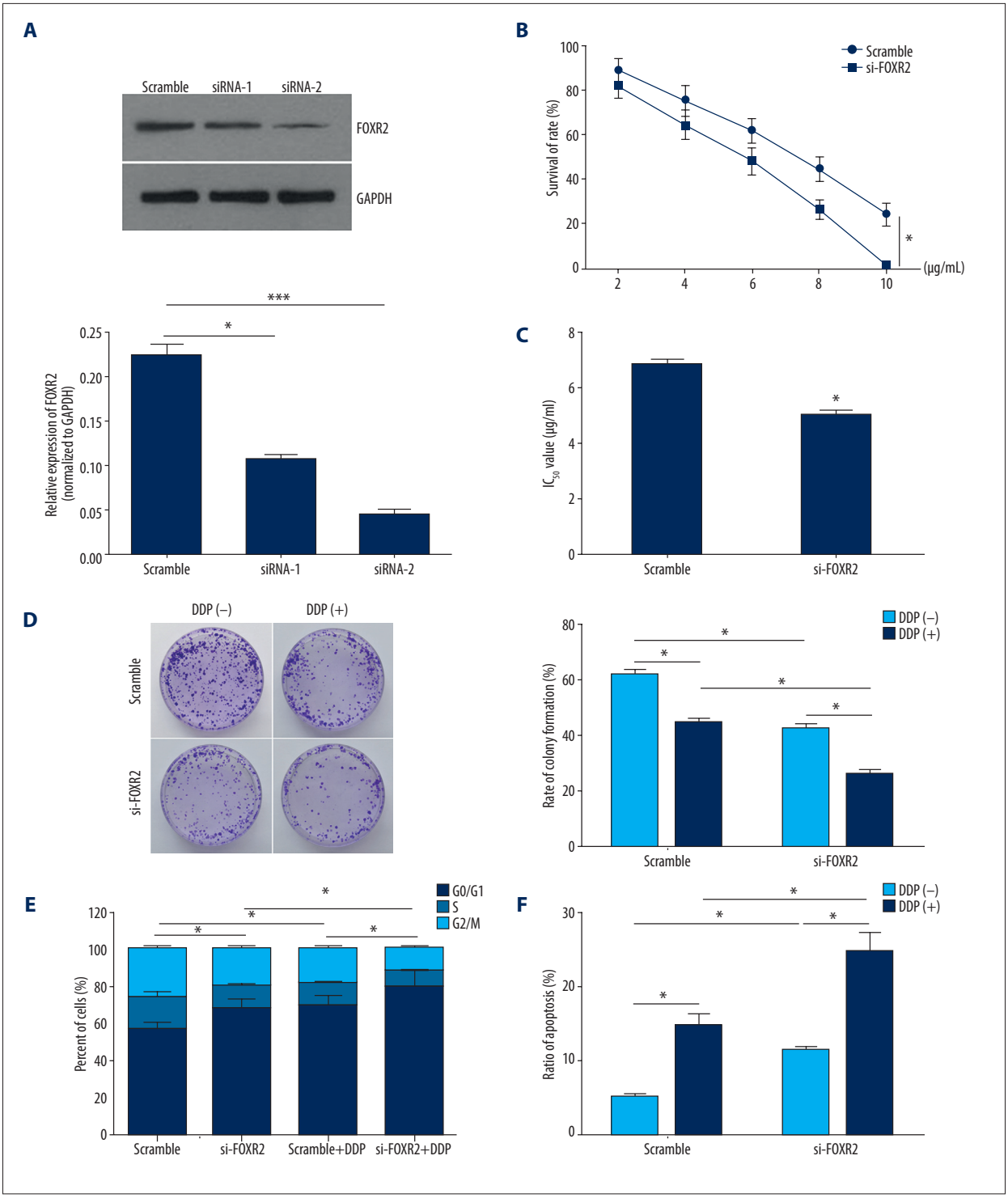

Figure 2. FoxR2 knockdown sensitises T24/DDP cells to cisplatin. (**A**) FoxR2 expression was analysed in T24/DDP cells transfected with FoxR2 siRNA. (**B**) Cell survival rates of FoxR2 knockdown cells treated with DDP (0–10 μg/mL) were determined by MTT assay. (**C**) The IC_{50} values of cisplatin in si-FOXR2 cells and scrambled group. (**D**) Colony formation results show the number of colonies of T24/DDP cells transfected with FoxR2 siRNA in the presence or absence of 6 μg/mL DDP. (**E**) Knockdown of FoxR2 inhibited G_1/S phase transition in response to treatment with 6 μg/mL DDP. (**F**) Flow cytometry analysis was used to assess apoptosis in T24/DDP cells transfected with FoxR2 siRNA in the presence or absence of 6 μg/mL DDP. All data represent the mean±SD of 3 replications. * $P<0.05$, *** $P<0.001$.

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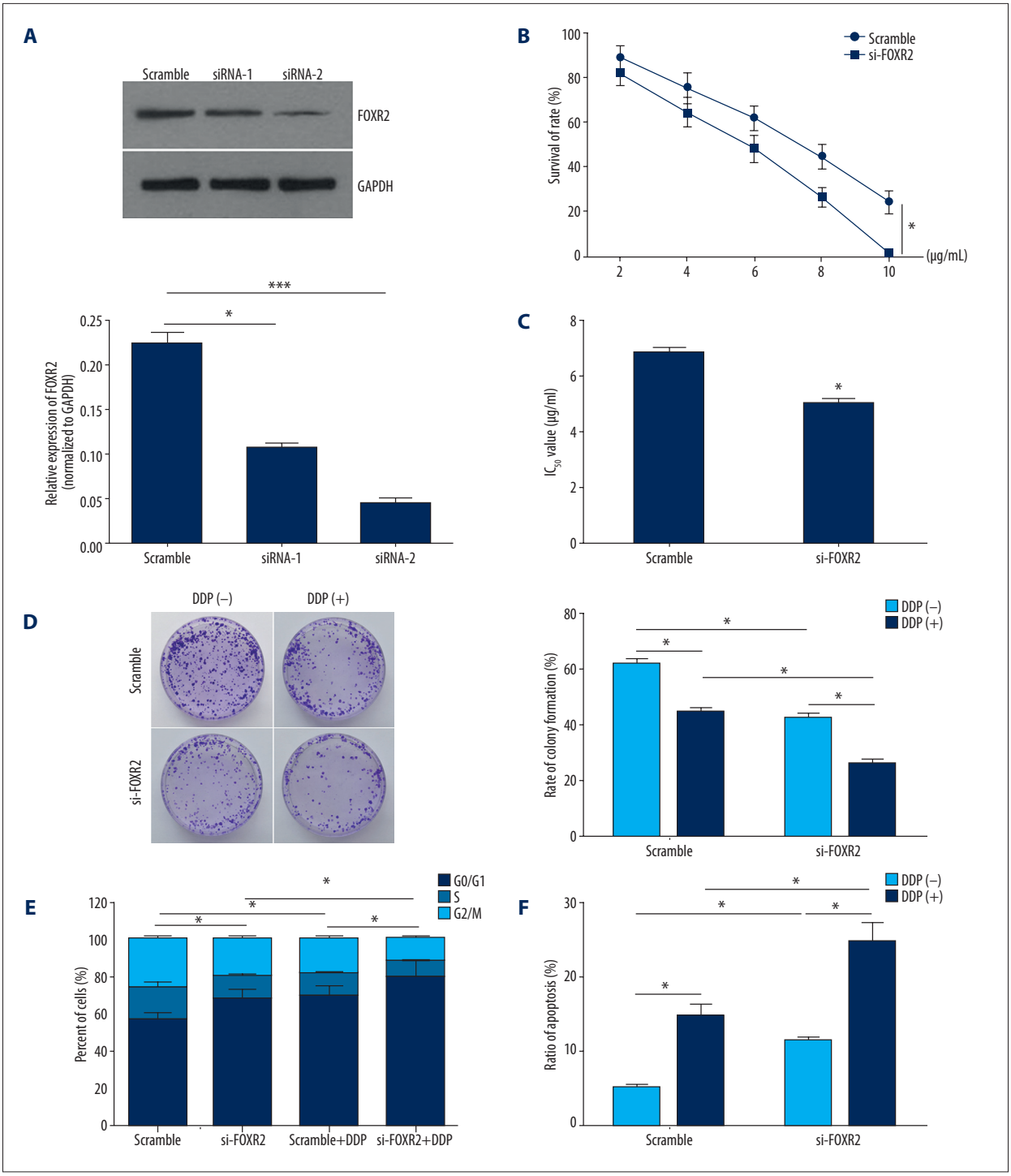

Figure 3. Overexpression of FoxR2 enhances cisplatin resistance in T24 cells. (**A**) FoxR2 expression was determined in T24 cells transfected with FoxR2 cDNA expression plasmid. (**B**) Cell survival rates of cells overexpressing FoxR2 treated with DDP (0–5 μg/mL) were determined by MTT assay. (**C**) The IC_{50} values of cisplatin in FOXR2-overexpressing cells and NC group. (**D**) Colony formation results show the number of colonies of T24 cells transfected with FoxR2 cDNA expression plasmid in the presence or absence of 1.5 μg/mL DDP. (**E**) Overexpression of FoxR2 promoted G_1/S phase transition in T24 cells. (**F**) Flow cytometry analysis was used to assess cell apoptosis in T24 cells transfected with cDNA expression plasmid in the presence or absence of 1.5 μg/mL DDP. NC – negative control, cells transfected with empty vectors. All data represent the mean±SD of 3 replications. * $P<0.05$, *** $P<0.001$.

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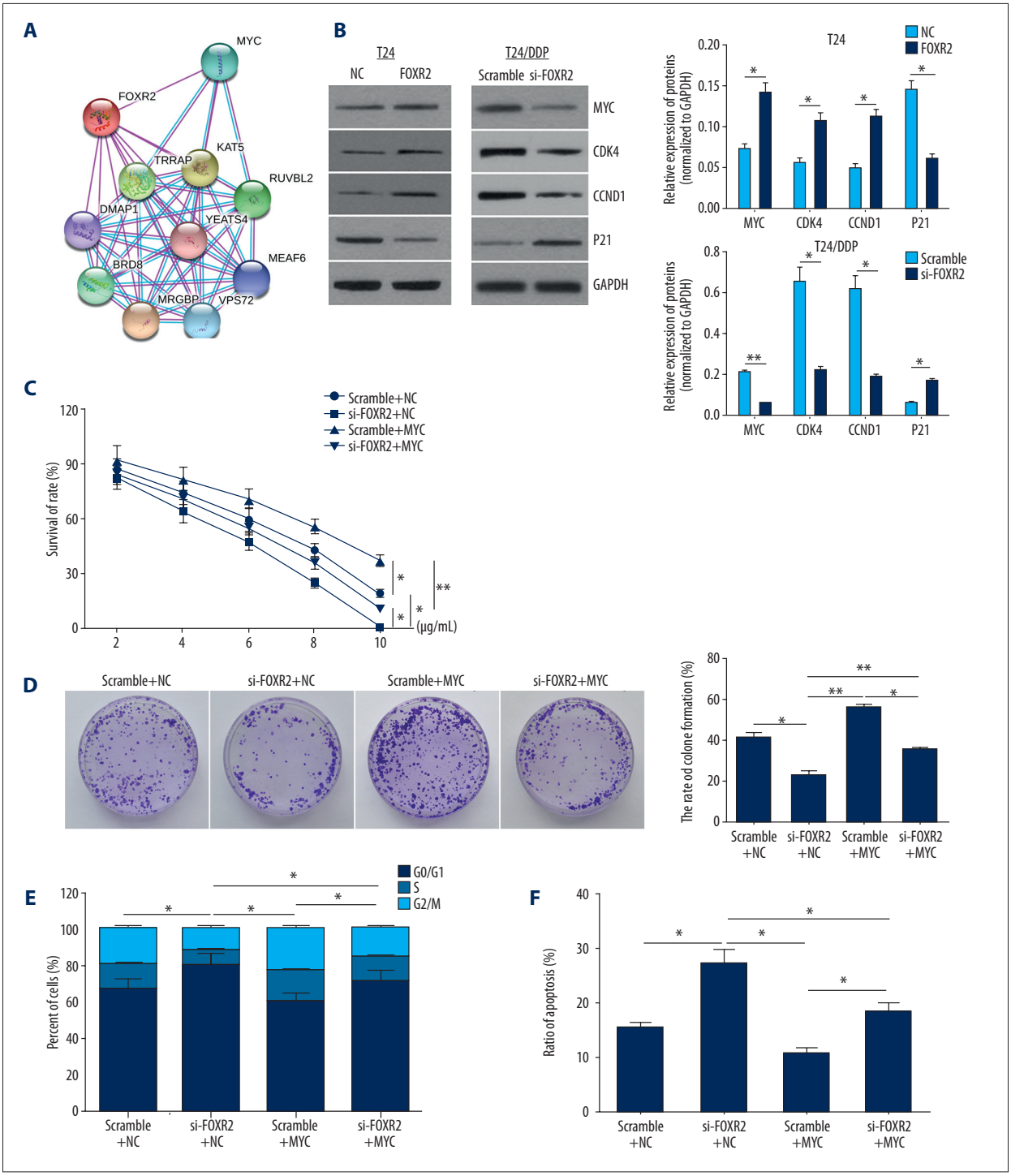

Figure 4. FoxR2 mediates cisplatin resistance via the MYC pathway. (**A**) STRING software was used to construct integrated gene network maps for FoxR2. (**B**) MYC and its target genes were detected in FoxR2 loss- and gain-of-function cell models. (**C**) Cell survival rates of T24/DDP cells cotransfected with FoxR2-overexpressing plasmids and MYC-overexpressing plasmids or empty vectors (NC) were measured by MTT after treatment with DDP (0–10 μg/mL). (**D**) Colony formation was assessed in T24/DDP cells cotransfected with FoxR2-overexpressing plasmids and MYC-overexpressing plasmids or empty vectors (NC) in the presence DDP (6 μg/mL). Cell cycle (**E**) and apoptosis (**F**) were analysed in T24/DDP cells cotransfected with FoxR2-overexpressing plasmids and MYC-overexpressing plasmids or empty vectors (NC) using flow cytometry in the presence of 6 μg/mL DDP. NC – negative control, cells transfected with empty vectors. All data represent the mean±SD of 3 replications. * $P<0.05$, ** $P<0.01$.

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Cisplatin is a first-line chemotherapy drug used to treat advanced bladder cancer, and has favorable effects on preventing bladder cancer recurrence and metastasis [19]. Clinical studies have demonstrated that DDP provides better 5-year cancer-specific survival relative to only surgery in those with bladder cancer [20,21]. However, bladder cancer typically has a relatively poor response to chemotherapy, resulting in failure of clinical treatment. Improvement of therapeutic effects requires a better understanding of the underlying mechanisms of chemoresistance. The involvement of several molecules has been identified in bladder cancer chemosensitivity, such as miR-101, miR-150, maspin, and vitamin D [22–25]. The role played by FoxR2 in cisplatin-based chemosensitivity remains unclear. In the present study, mining of microarray data from bladder cancer chemoresistant cells revealed that FoxR2 expression was higher in resistant cells that in the parent cells. FoxR2 expression in our constructed resistant cell line, T24/DDP, also confirmed these results. Moreover, knockdown of FoxR2 enhanced sensitivity to DDP in T24/DDP cells. Conversely, enhanced FoxR2 expression promoted DDP chemoresistance in T24 cells. Our findings are the first to report on FoxR2-mediated bladder cancer chemoresistance.

The transcription factors of the MYC family are well-known oncogenes, including c-MYC (MYC), N-MYC (MYCN), and L-MYC (MYCL), which have been confirmed to play important roles in tumorigenesis in many malignances [26]. MYC is a common proto-oncogene, and its deregulated expression is associated with oncogenesis [27]. Previous studies have demonstrated that MYC mediates chemoresistance in tongue, lung, colorectal, and bladder cancers [28–30]; therefore, MYC is an alternative chemotherapeutic target. Analysis of functional protein association networks highlighted an interaction between FoxR2 and MYC. Li et al. reported that FoxR2 interacts with MYC to drive proliferation in cancer [31]. Therefore, we hypothesized that FoxR2 mediates bladder cancer cisplatin resistance via MYC. In our rescue experiments, enhanced cisplatin sensitivity induced by knockdown of FoxR2 was attenuated via upregulation of MYC. CDK2, CDK4, and CCND1 are target genes of MYC that control G1/S phase transition [32]. Activation of another target gene, p21, suppressed proliferation and drove apoptosis and cell cycle arrest [26]. The role of MYC and its associated target gene expression in the present study was consistent with previous reports. Thus, FoxR2-mediated chemoresistance in bladder cancer may act via regulation of the MYC pathway.

Conclusions

The present study showed that FoxR2 knockdown decreases chemoresistance to cisplatin via the MYC pathway in bladder cancer. Our findings highlight FoxR2 as a potential target for DDP-based chemotherapy in bladder cancer.

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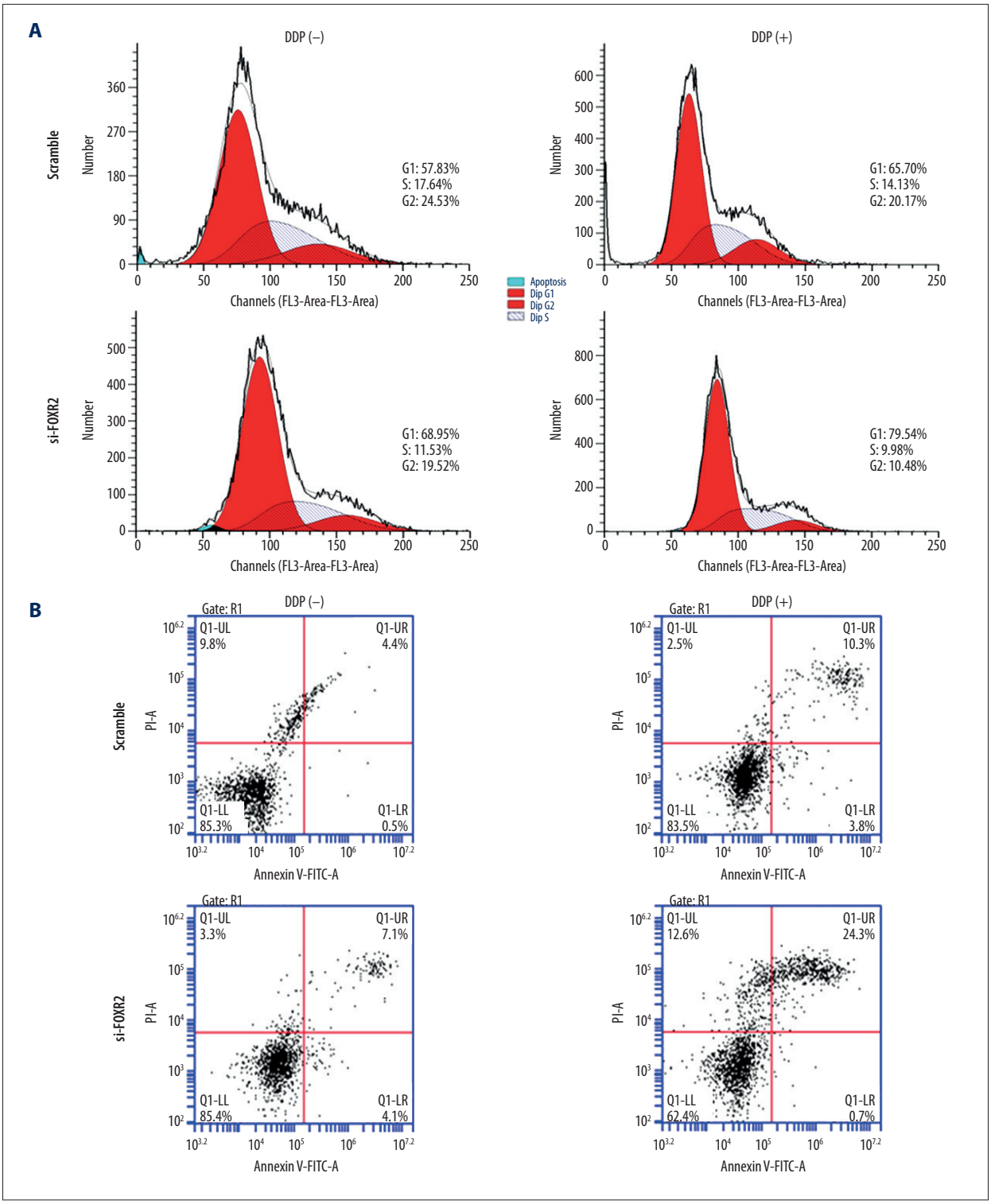
Supplementary Data



Supplementary Figure 1. The effect of FoxR2 knockdown on cell cycle and apoptosis in T24/DDP cells. (**A**) Knockdown of FoxR2 inhibited G_1/S phase transition in the presence or absence of 6 μg/mL DDP. (**B**) Flow cytometry analysis was used to assess apoptosis in T24/DDP cells transfected with FoxR2 siRNA in the presence or absence of 6 μg/mL DDP.

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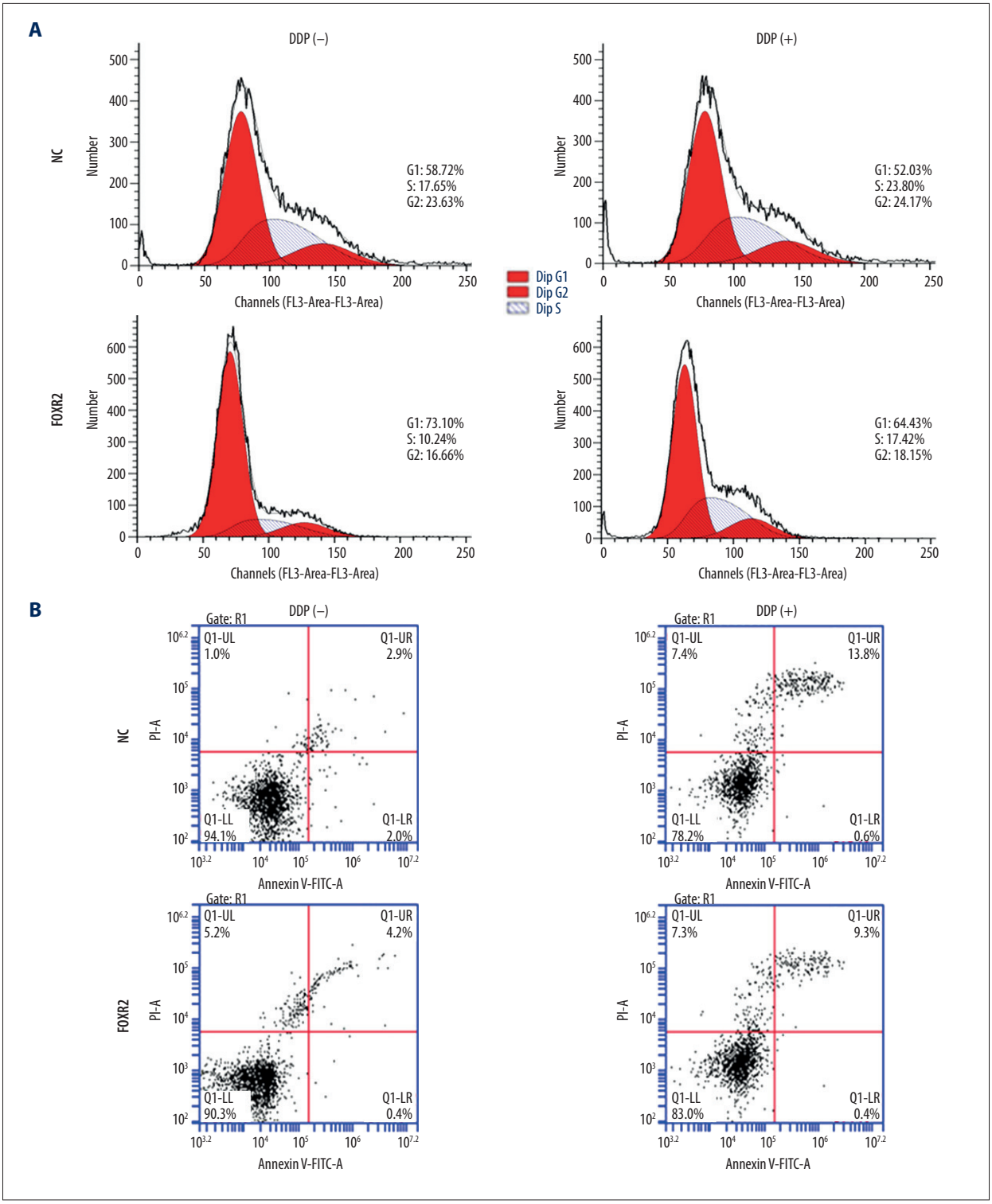

Supplementary Figure 2. The effect of FoxR2 overexpression on cell cycle and apoptosis in T24 cells. (**A**) Upregulation of FoxR2 promoted G_1/S phase transition in the presence or absence of 1.5 μg/mL DDP. (**B**) Cell apoptosis was detected in T24 cells transfected with FoxR2-overexpressing plasmids in the presence or absence of 1.5 μg/mL DDP.

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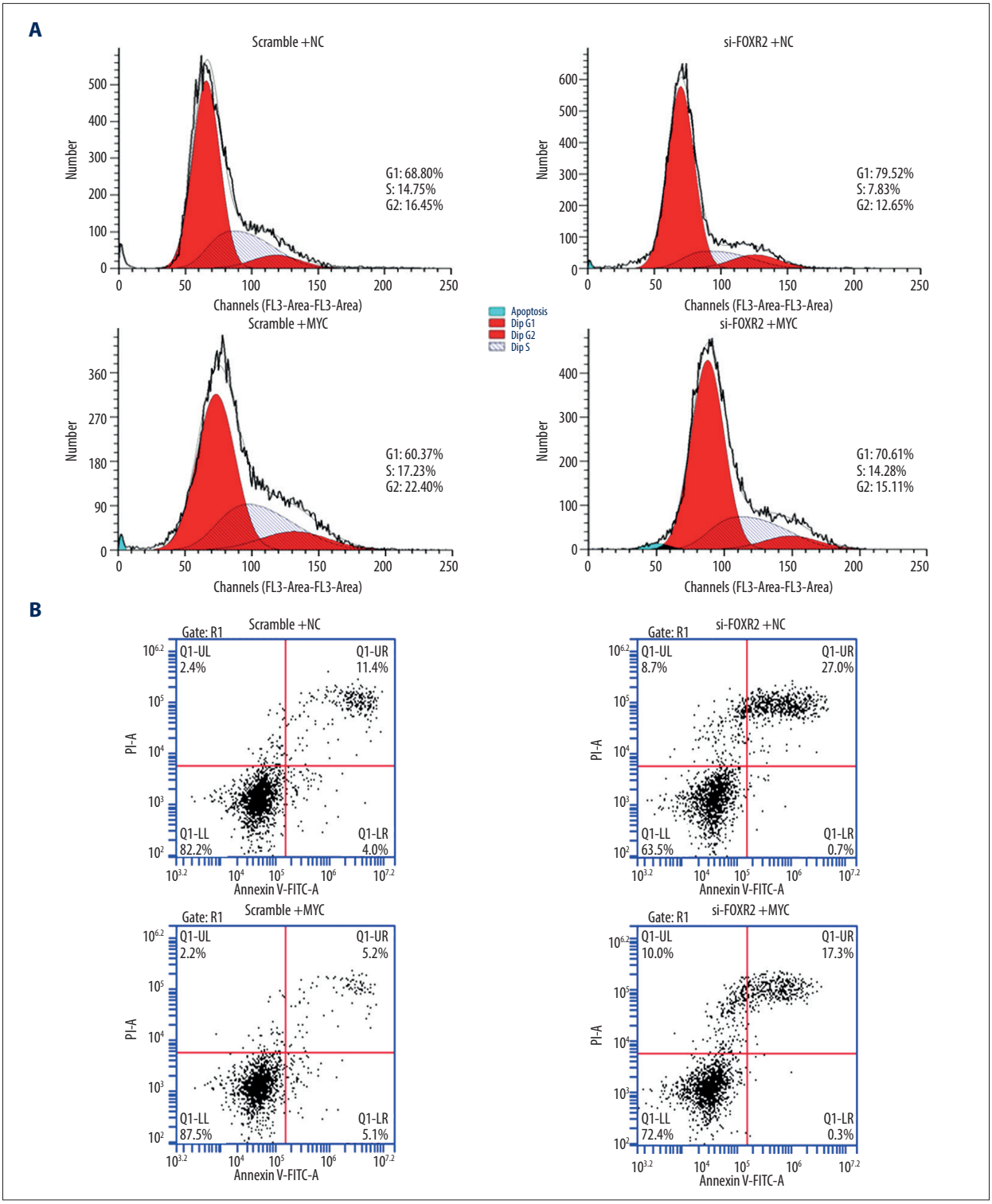

Supplementary Figure 3. FoxR2 regulates cell cycle and apoptosis through MYC. Cell cycle (**A**) and apoptosis (**B**) were analysed in T24/DDP cells cotransfected with FoxR2 overexpressed plasmids and MYC overexpressed plasmids or empty vectors (NC) using flow cytometry in presence of 6 μg/mL DDP.

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