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Original Article

Bladder cancer cells prevent cisplatin-induced oxidative stress by upregulating Nestin1 expression

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Abstract: Objective: Redox adaptation plays a critical role in cancer cells' drug tolerance and sensitivity. The antioxidative response is induced by nuclear factor erythroid 2-related factor 2 (Nrf2), which triggers the transcriptional activation of genes related to chemosensitivity, glutathione synthesis, and cell protection. Although Nestin1 is known to regulate cellular redox homeostasis by regulating Nrf2 in lung cancer cells, its regulatory effect on the antioxidative state of bladder cancer (BC) cells remains unclear. Methods: The oxidative stress levels in two cisplatin-treated BC cell lines (T24 and J82) were examined using 2',7'-dichlorofluorescin diacetate staining and real-time quantitative reverse transcription-PCR (RT-qPCR) assays. The cell viability, growth, and apoptosis were determined using CCK-8, colony formation, and flow cytometric assays, respectively. The mRNA and protein levels of Nestin1, Nrf2, and several antioxidant enzymes were quantified using RT-qPCR and western blot assays. A mouse xenograft model was used to determine the effect of Nestin1 on the T24 tumor growth *in vivo*. Results: Cisplatin treatment induced reactive oxygen species (ROS) generation and antioxidative damage in the T24 and J82 cells, reducing their viability and growth and triggering their apoptosis. Moreover, the Nestin1 and Nrf2 protein levels were enhanced in both treated cell lines. Loss- and gain-of-function assays indicated that Nestin1 expression was positively correlated with the Nrf2 protein expression in the BC cells. Nestin1 overexpression reduced the ROS generation, alleviated the redox disorder, promoted cell viability, and reduced apoptosis, but its silencing had the opposite effects. Importantly, Nestin1 overexpression enhanced the chemoresistance of BC cells to cisplatin *in vivo*, but its knockdown improved the chemosensitivity of the cells and increased their apoptosis. Conclusion: These results provide a theoretical basis for further targeting the transcription factors, including Nestin1 and Nrf2, in the treatment of BC with cisplatin.

Keywords: Bladder cancer, cisplatin, Nestin1, Nrf2, antioxidative response

Introduction

Bladder cancer (BC), the most common malignancy of the urinary system, has a high relapse rate, with over 70% of afflicted patients carrying high-risk, non-muscular invasive tumors [1, 2]. Although radiotherapy and chemotherapy can be useful in some patients with BC, recurrence occurs frequently because of the eventual resistance of the tumor cells to the various treatments. To date, there has been no technological progress or development of innovative strategies for the treatment of this disease due to the lack of knowledge of its pathogenesis. Thus, because BC can easily relapse and has high invasive and metastatic capabilities, there is an urgent need to elucidate the molecular mechanisms underlying its chemoresistance in order to identify new targets for drug therapy.

Reactive oxygen species (ROS) are continuously produced in living cells as a result of oxidative reactions that occur during various physiological processes and can trigger the activation of certain biological pathways. Generally, ROS levels are balanced by a scavenging system that comprises various antioxidant enzymes and molecules. An imbalance between the generation and elimination of ROS can lead to higher ROS levels, which may cause damage to the cellular proteins, DNA, and lipids and ultimately initiate and facilitate carcinogenesis [3-5]. Moreover, excessive ROS production can also induce cell aging and mediate cell death by triggering oxidative stress and disorder (intracellular ROS increase) [6, 7].

Nestin1, an intermediate filament protein, has recently been shown to be a specific neovascu-

larization marker and has been suggested as a therapeutic target for inhibiting angiogenesis [8]. Nestin1 has been used as a specific marker of angiogenesis during tumor development, especially for colorectal carcinomas [9] and prostate cancer [10]. In gastric cancers, no correlation has been found between the density of Nestin1-positive microvessels and patients' clinical prognoses [11]. However, in patients with larger cancers, Nestin1-based microvessel density has a stronger correlation with survival time than CD34-based microvessel density does [11]. Wang et al. demonstrated that Nestin1 binds competitively to the Kelch-like ECH-associated protein 1 (Keap1) Kelch domain to protect nuclear factor erythroid 2-related factor 2 (Nrf2) from Keap1-mediated degradation, which subsequently leads to the upregulation of antioxidant enzyme expression. Nrf2 is one of several 66 kDa transcription factors that can coordinate the cellular stress response to combat oxidative stress [12]. Several lines of evidence have shown that Nrf2 activators prevent not only the development of malignant tumors but also the malignant tumors of a variety of chronic diseases for which both oxidative stress and immunological stress are the key mechanisms of pathogenesis [13, 14]. The activation of the Keap1-Nrf2 system is the main pathway of the oxidative stress response [15]. Nestin1 has been shown to bind directly to both Keap1 and Nrf2 and increases the expression of the latter to modulate oxidative equilibrium in lung cancer [16]. However, the roles of Nestin1 and Nrf2 in BC tumorigenesis remain unknown.

Although the importance of Nestin1 and Nrf2 in maintaining antioxidant homeostasis and chemosensitivity in cancer cells has been confirmed [16], there are no data on the potential cross-talk between these two pathways in chemoresistant carcinoma cell lines that show high antioxidant levels. Therefore, in this study, we examined the expression levels and functions of Nestin1 and Nrf2 in two BC cell lines (T24 and J82) following the treatment of the cells with cisplatin. Furthermore, loss- and gain-of-function experiments were performed to determine the effect of Nestin1 on the chemosensitivity of the BC cells to this cancer drug.

Materials and methods

Cell culture and transfection

The T24 and J82 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Both strains were incubated in Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute-1640 culture medium (Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL. Co. Ltd.). The negative control small interfering (si) RNA (siNC; 60 nM; 5'-AGC CUG AUC GAC GCA CAG-3') and Nestin1 siRNA (siNestin1; 60 nM; 5'-UGG UCC UCU UCU GGA G-3') were purchased from RiboBio (Guangzhou, China). The Nestin1 complementary DNA (cDNA; NCBI reference sequence: NM_006617.2) was cloned into the pcDNA3.1 vector, and the resultant plasmid was named pcDNA3.1-Nestin1. For the transfection of the BC cells, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was mixed with the siRNAs or the plasmids and FBS-free culture medium according to the manufacturer's instructions. The untreated cells were used as controls. The transfection efficiency was monitored using western blot assays.

Measurement of the intracellular ROS levels

The intracellular ROS levels were measured using the 2',7'-dichlorofluorescin diacetate (DCFDA; Applygen, Beijing, China) staining method. In brief, BC cells were stained with 5 μg/L DCFDA in a 37°C incubator for 30 min in the dark and then washed twice with phosphate-buffered saline before an analysis with an automatic microplate reader.

Cell viability assay

To assess the viability of the treated cells, Cell Counting Kit-8 (CCK-8) (96992; Sigma-Aldrich, St. Louis, MO, USA) was used according to the instructions provided by the manufacturer. In brief, after seeding the cells in 96-well plates, 10 μL of CCK-8 reagent was added to each well, and the plates were incubated at 37°C for 2 h. Finally, the optical density (OD) of the cells in each well was recorded with an automatic microplate reader (Infinite M200; Tecan, Mannedorf, Switzerland).

Colony formation assay

The cells were inoculated into 6-well plates (1000 cells/well) and left overnight to adhere to the well surface. At 24 h post-treatment or transfection, the cells were incubated for a further 9-11 days and then fixed and stained with a mixture containing a crystal violet-like solution (Sigma-Aldrich) and methyl alcohol (ratio: 9:1). Images of the colonies were taken, and the number of colonies was counted using the colony counter tool in ImageJ software (National Institutes of Health, Bethesda, MD, USA). Colonies consisting of 50 cells or more were counted. Cells that had not been treated or transfected were used as controls.

RNA extraction and real-time qRT-PCR

The total RNA extraction from the BC cell lines was carried out using the TRIzol reagent (Invitrogen), and the concentration was assessed using a NanoDrop 2000 spectrophotometer (OD_{260}) (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was then reverse transcribed to cDNA using a MMLV First-Strand Kit (Invitrogen) and an oligo(dT)20 primer. PCR amplification of the target genes was then carried out in an ABI 7300 thermocycler (Applied Biosystems, Carlsbad, CA, USA) using the SYBR Premix Ex Taq Kit (Takara, Beijing, China) according to the instructions of the manufacturers. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 40 s. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal reference for the target mRNA expression levels, which were determined using the $2^{-\Delta\Delta CT}$ method. All the tests were conducted in triplicate.

Western blot analysis

The total protein extraction from the BC cells was carried out using standard protocols. The cell lysates were then separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the protein bands were transferred onto polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). The membranes were then incubated with specific primary antibodies and secondary antibodies at 4°C overnight. The primary antibodies used were anti-Nestin1 (1:500 dilution; ab22035; Abcam, Cambridge, UK), anti-Nrf2 (1:1000; ab31163; Abcam), anti-glutamate-cysteine ligase modifier subunit (GCLM) (1:500; ab153967; Abcam), anti-heme oxygenase 1 (HMOX1) (1:500; ab13248; Abcam), anti-NAD(P)H quinone dehydrogenase 1 (NQO1) (1:500; ab2346; Abcam), and anti-GAPDH (1:5000; ab8245; Abcam), and the secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:5000; ab205719; Abcam) and HRP-conjugated goat anti-rabbit antibody (1:5000; ab6721; Abcam). The protein immune response signals were measured and analyzed using an electrochemiluminescence image analysis system (Thermo Fisher Scientific).

Apoptosis detection

Flow cytometry combined with an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Pharmingen, Franklin Lakes, NJ, USA) was used to evaluate the cell death. In brief, the cells were suspended in 20 μL of binding buffer and then treated with 10 μL of annexin V-FITC and 5 μL of PI. The percentage of dead cells was measured using a flow cytometer (FC500; Beckman Coulter, Krefeld, Germany).

In vivo assay of tumor growth using a mouse xenograft model of bladder cancer

Male BALB/c nude mice (age: 5-6 weeks) were provided by the Laboratory Animal Center of the First Affiliated Hospital of Zhengzhou University. To establish the tumor xenografts, T24 cells (1×10^6) that were stably transfected with pcDNA3.1-Nestin1 or siNestin1 were injected subcutaneously into the right flank of each mouse in the respective experimental groups [17]. After 1 week, the mice were administered 5 mg/L (30 mg/kg body weight) cisplatin once a day for 10 days. The tumor sizes were measured once every 3 days following anesthetization of the animals with sodium pentobarbital (50 mg/kg body weight, together with 33 IU heparin, intraperitoneally). The tumor sizes were calculated using the following equation: $0.5 \times ab^2$, where a is the tumor length and b is the tumor width. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was then carried out on 5 μm tissue sections cut from the paraffin-embedded excised tumors, and the signal

detection was achieved using an Alexa Fluor 488-conjugated secondary antibody (Invitrogen). All the animal tests were conducted in accordance with the guidelines approved by the Animal Care and Use Committee of the Laboratory Animal Center of the First Affiliated Hospital of Zhengzhou University.

Statistical analysis

All the values are presented as the means ± standard deviations. One-way analyses of variance followed by Tukey *post hoc* tests were used for the comparisons among multiple groups, but Student *t*-tests were used for the comparisons between two groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

The Nestin1 and Nrf2 levels were upregulated in the cisplatin-treated bladder cancer cells

To determine whether the cisplatin treatment causes an antioxidative response in the BC cells, T24 and J82 cells were treated with 20 mg/L of the cancer drug for 60 h. Subsequent DCFDA staining showed that ROS generation was significantly higher in the cisplatin-treated groups than it was in the control groups (**Figure 1A** and **1B**). RT-qPCR assays were then used to quantify the gene expressions of the antioxidant enzymes GCLM, HMOX1, and NQO1. The results showed that the mRNA and protein expression levels of all three genes in the T24 and J82 cells were clearly increased by the cisplatin treatment (**Figure 1C-F**). Additionally, the results of the CCK-8 and colony formation assays indicated that the viability and growth of the T24 and J82 cells were significantly reduced in response to the cisplatin treatment (**Figure 1G-J**). To assess whether the decrease in cell viability was associated with apoptotic death, a flow cytometry analysis was carried out, which revealed that the cisplatin administration had resulted in a clear elevation in the proportion of annexin V-positive cells (**Figure 1K** and **1L**).

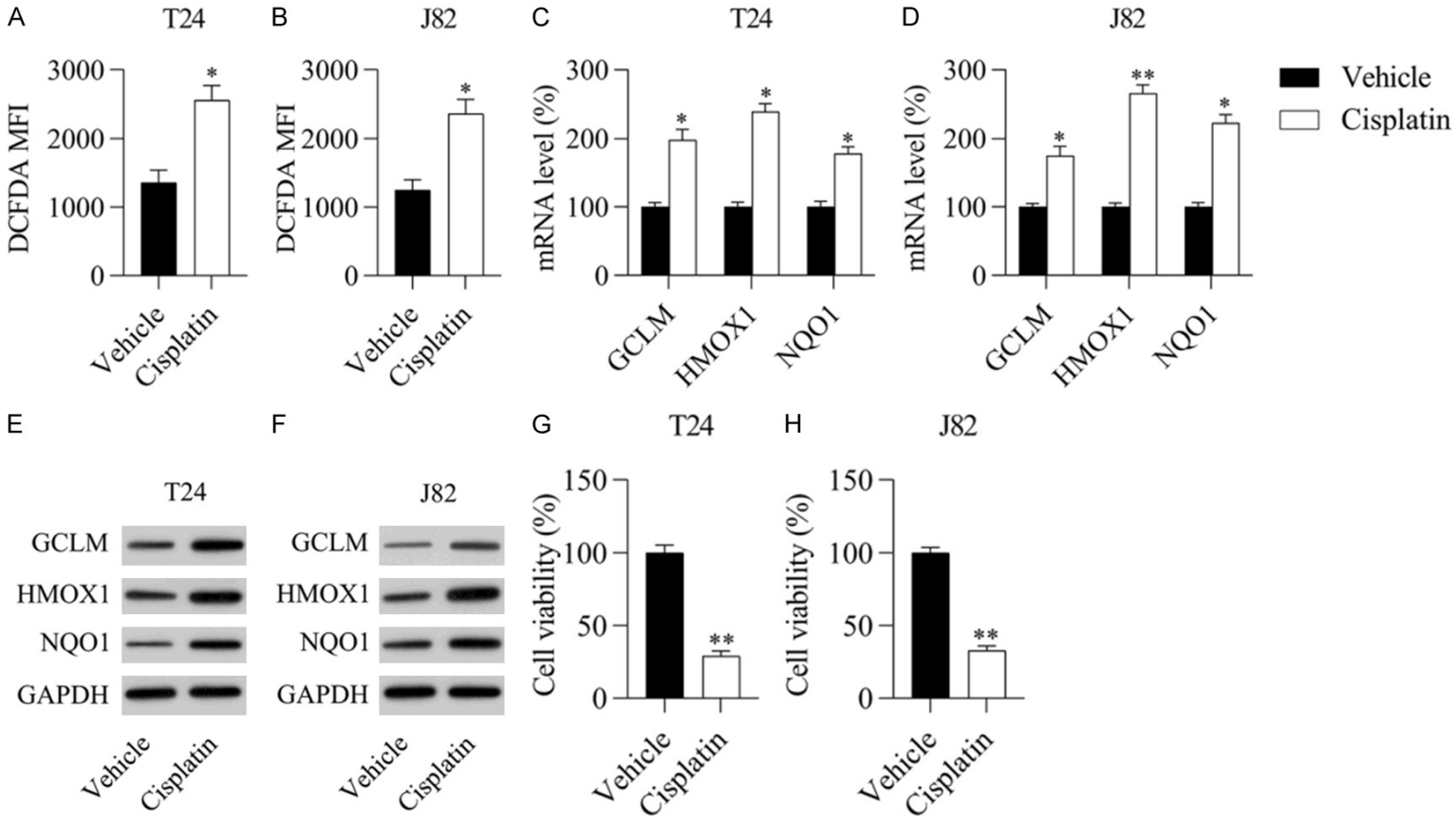
To characterize the involvement of Nestin1 and Nrf2 in the cisplatin-induced ROS production and cell death, we first quantified the levels of these two proteins in the drug-treated T24 and J82 cells. As shown in **Figure 2A** and **2B**, the protein levels of Nestin1 and Nrf2 were significantly increased in both the cisplatin-treated cell lines, suggesting that the Nestin1/Nrf2 axis may be involved in the cisplatin-mediated attenuation of the BC cells.

Nestin1 affected the antioxidative response and cell death of the cisplatin-treated bladder cancer cells

To examine the effects of Nestin1 on the antioxidative response and the deaths of the cisplatin-treated T24 and J82 cells, the cells were transfected with pcDNA3.1-Nestin1 or siNestin1 to upregulate and downregulate the Nestin1 expressions, respectively. The Western blot data verified that the transfection of pcDNA3.1-Nestin1 resulted in the upregulation of the Nestin1 expression in the cells, and the Nrf2 level was also elevated as a result of the increased Nestin1 level (**Figure 3A** and **3B**). By contrast, the transfection with siNestin1 successfully knocked down the Nestin1 expressions in the T24 and J82 cells, and the Nrf2 levels were subsequently reduced as well (**Figure 3A** and **3B**). Our data suggest that the Nrf2 protein level correlates positively with the Nestin1 protein level, which is consistent with the findings of a previous study [16].

To test the effect of Nestin1 upregulation on ROS production and cell injury in BC cells in response to cisplatin treatment, Nestin1-overexpressing cells and control cells were treated with 20 mg/L cisplatin for 60 h. Our quantification of the ROS levels revealed that they were lower in the Nestin1-overexpressing cells than in the empty vector-carrying control cells (**Figure 4A** and **4B**). The mRNA and protein expression levels of the antioxidant enzymes GCLM, HMOX1, and NQO1 were significantly upregulated by the Nestin1 overexpression (**Figure 4C-F**), suggesting that Nestin1 increased the antioxidative response in the cisplatin-treated BC cells. Using the CCK-8 and colony formation assays, we also found that the high Nestin1 overexpression level had partially restored the viability and growth of the cisplatin-treated T24 and J82 cells (**Figure 4G-J**). The flow cytometric results indicated that the number of annexin V-positive BC cells was significantly lower in the Nestin1-overexpressing group than in the empty vector-carrying group (**Figure 4K** and **4L**).

To determine the effect of Nestin1 depletion on the antioxidative response and cell damage in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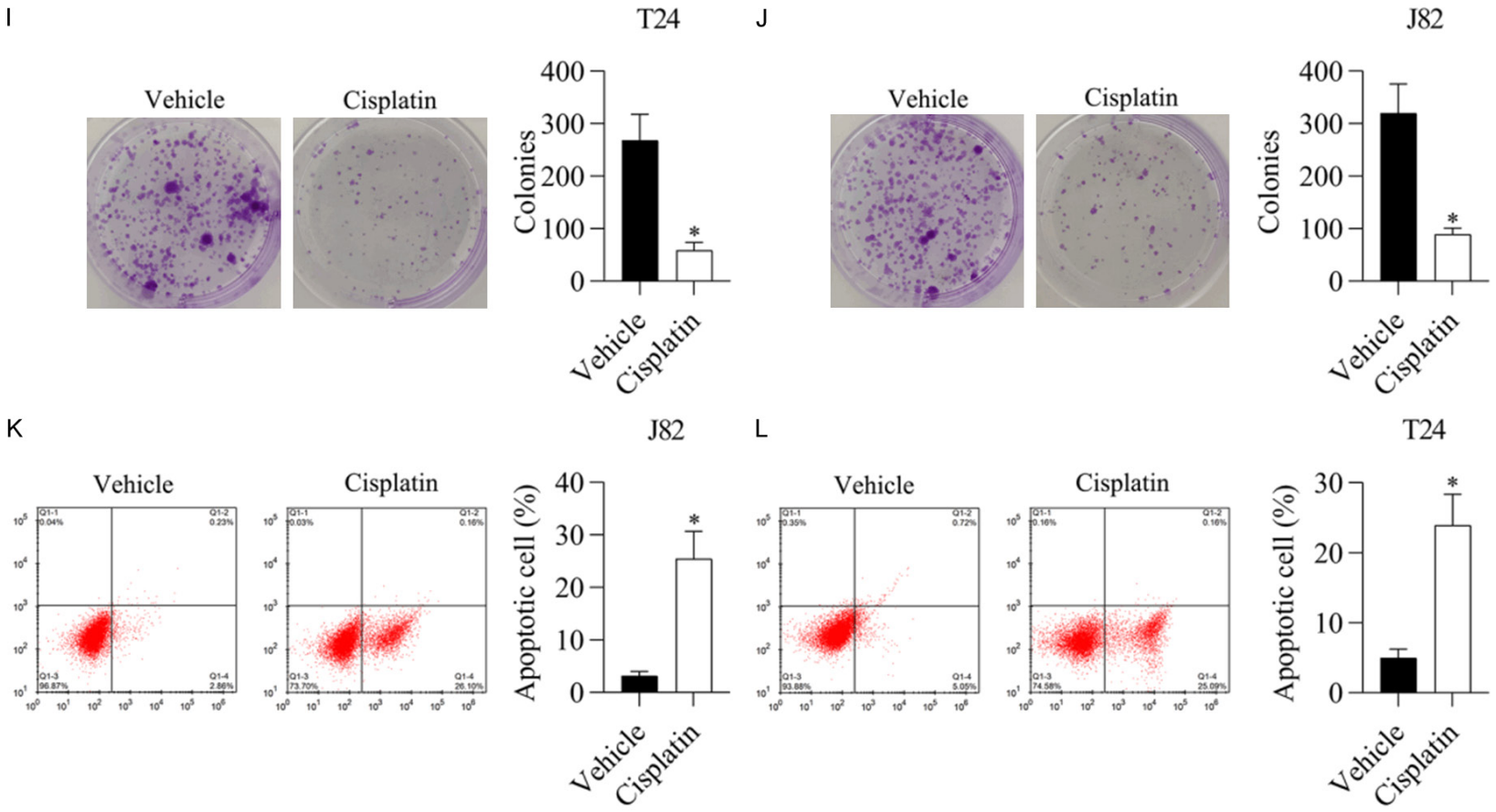


Figure 1. The Cisplatin-induced antioxidative response and cytotoxicity in bladder cancer (BC) cells. The T24 and J82 cell lines were treated with 20 mg/L cisplatin for 60 h (A, B). The cells were stained using an ROS detection probe, and the production of ROS was analyzed using flow cytometry (C-F). The RT-qPCR and WB analyses revealed the effect of cisplatin on the expression levels of the antioxidant-related genes *GCLM*, *HMOX1*, and *NQO1* in the BC cells (G, H). CCK-8 assays were used to determine the cell viability (I, J). The colony formation of the cell lines treated with 20 mg/L cisplatin for 96 h (K, L). Cisplatin (20 mg/L) was added to the cell strains for 24 h, following which the cell apoptosis was detected by annexin V/PI staining and analyzed using flow cytometry. The results are expressed as a percentage of the control values and are presented as the mean ± SD of 3 separate experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. vehicle.

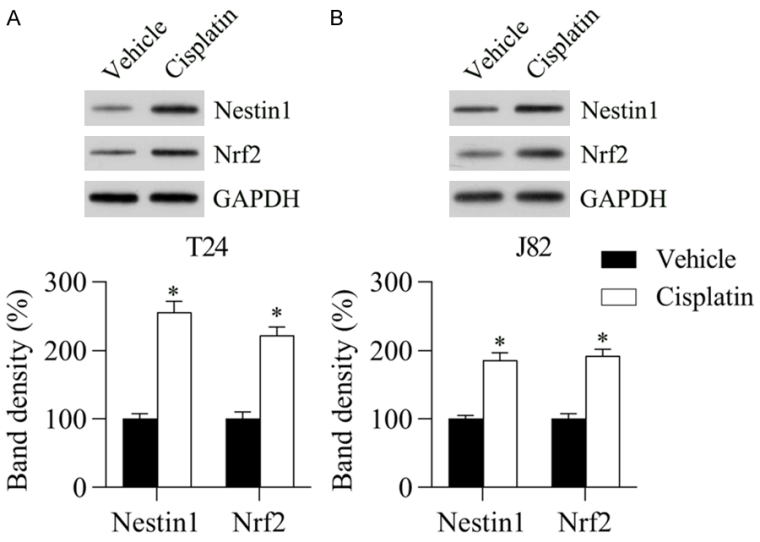


Figure 2. The cisplatin-induced upregulation of the Nestin1 and Nrf2 expression in the bladder cancer cells. T24 and J82 cells were treated with 20 mg/L cisplatin for 60 h (A, B). Western blot analyses was carried out to examine the Nestin1 and Nrf2 protein levels in the T24 and J82 cells. The quantification of the relative band intensities is shown. The results are expressed as a percentage of the control values and are presented as the mean ± SD of 3 separate experiments. $^{*}P \leq 0.05$ vs. the vehicle.

the cisplatin-treated BC cells, T24 and J82 cells with or without Nestin1 silencing were treated with 20 mg/L cisplatin for 60 h. The quantification of the ROS levels revealed that the Nestin1 knockdown had facilitated the ROS generation considerably in the treated cells (**Figure 5A** and **5B**). Moreover, the mRNA and protein expression levels of the three tested antioxidant genes were significantly lower in the Nestin1-knockdown cells than they were in the control cells (**Figure 5C-F**), suggesting that the Nestin1 silencing decreased the antioxidative response in the cisplatin-treated BC cells. Moreover, the Nestin1 knockdown further attenuated the viability of the T24 and J82 cells, as assessed by the CCK-8 and colony formation assays (**Figure 5G-J**). Additionally, Nestin1 depletion resulted in a higher proportion of apoptotic cells, as determined using flow cytometry (**Figure 5K** and **5L**). Collectively, our data suggest that the Nestin1 expression is essential for maintaining the antioxidative response and preventing apoptotic cell death by mediating the Nrf2 expression.

Nestin1 inhibited the chemosensitivity of the xenograft bladder cancer tumors to cisplatin

We applied a mouse xenograft model to further characterize the effect of Nestin1 on the chemosensitization *in vivo*, using cisplatin-treated nude mice bearing xenotransplanted tumors of Nestin1-overexpressing or -silenced T24 cells. We found that the cisplatin treatment significantly repressed the tumor growth in the mice relative to the growth in the untreated control mice. However, the Nestin1 overexpression significantly and partially counteracted the inhibitory effect of the cisplatin on tumor growth, but its depletion further augmented the growth suppressive activity of the drug (**Figure 6A-C**). These results were verified using Western blot, where the Nestin1 and Nrf2 protein levels were significantly higher in the cisplatin-treated control and pcDNA3.1-Nestin1-carrying groups than in the untreated control group but lower in the cisplatin-treated siNestin1-carrying cells (**Figure 6D**). According to the DCFDA staining results, the ROS generation in the tumor samples was upregulated after the cisplatin treatment, but this effect was counteracted in the Nestin1-overexpressing group. By contrast, the ROS generation was significantly increased in the Nestin1-silenced BC tumors (**Figure 6E**). These results were further verified by the TUNEL staining, which indicated a higher number of TUNEL-positive cells in the cisplatin groups than that in the untreated control group. Moreover, the cisplatin-treated Nestin1-silenced group had a significantly higher number of TUNEL-positive cells than the cisplatin-treated Nestin1-overexpressing and cisplatin control groups (**Figure 6F**). These findings suggest that Nestin1 exerts a chemoresistance effect against cisplatin in BC tumors *in vivo*.

Discussion

The ability of tumor cells to develop resistance to various chemotherapeutic drugs remains a significant challenge in the management of patients with BC. Both redox adaptation and an increase in detoxifying molecules are factors that play a significant role in the cell death escape of chemoresistant cancers [18]. ROS

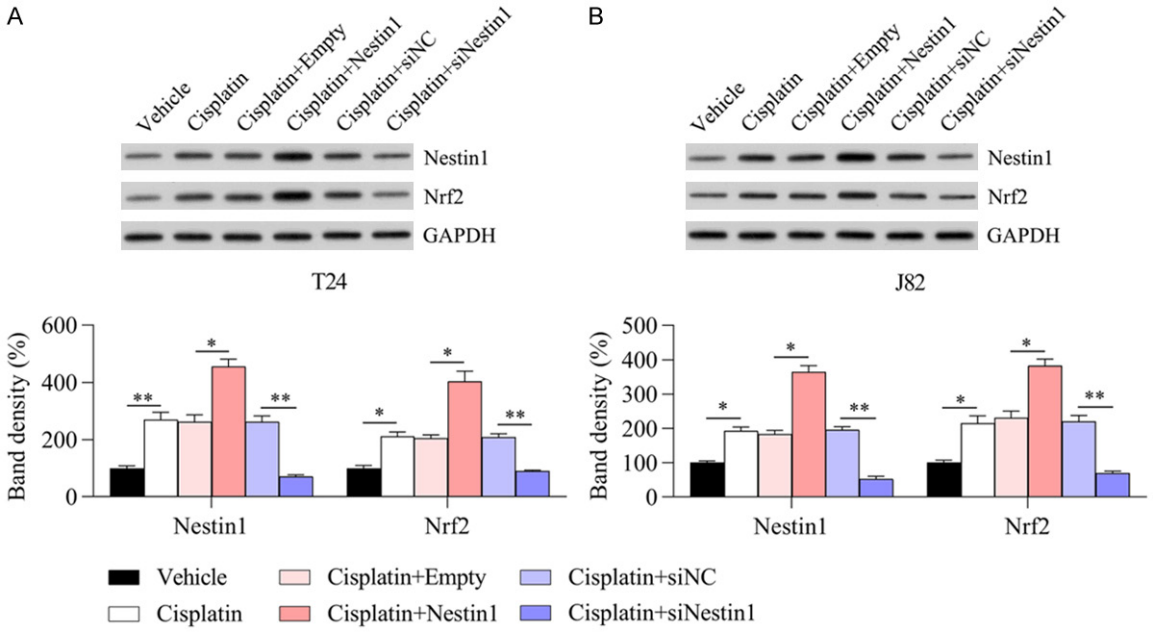
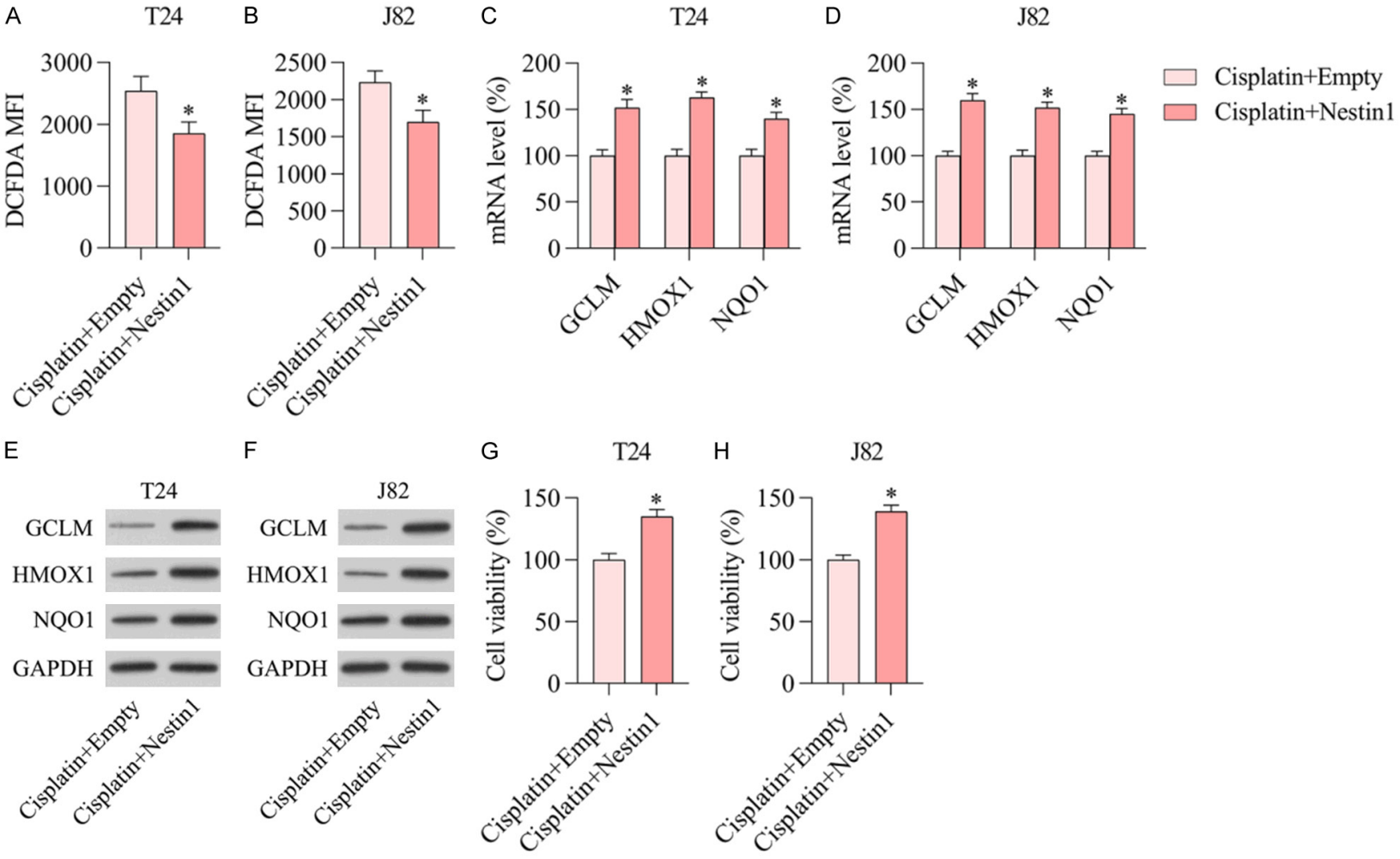


Figure 3. The overexpression and silencing of Nestin1 in the cisplatin-treated bladder cancer cells. The T24 and J82 cells were transfected with pcDNA3.1, pcDNA3.1-Nestin1, normal control siRNA (siNC), or Nestin1 siRNA (siNestin1) and then treated with 20 mg/L cisplatin for 60 h (A, B). Western blot analyses were carried out to examine the Nestin1 and Nrf2 protein levels in the cells. The quantifications of the relative band intensities are shown. The results are expressed as a percentage of the control values and are presented as the mean ± SD of 3 separate experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. the indicated group.

play a critical role in intracellular redox adaptation and participate in a variety of pathways related to metabolism, cell cycle progression, and antioxidative effects [19]. Ciamporcero et al. observed that the expression level of the antioxidant glutathione was higher in the cisplatin-resistant human BC cells than in the cisplatin-sensitive strains [7]. Our study indicated that the cisplatin administration to human BC cells resulted in an increased generation of ROS and the upregulated expression of antioxidant genes, Nestin1, and Nrf2. Further mechanistic evaluation revealed that the Nestin1 expression was positively associated with the Nrf2 protein level and thereby regulated the expression of the antioxidant genes (**Figure 7**). Additionally, our *in vivo* study demonstrated that the overexpression of Nestin1 could partially abolish the antitumor activity of cisplatin.

It has been established that Nrf2 plays a critical role in the adaptive response of cells to oxidative stress [20]. The discovery of new Nrf2 activators has gained attention because of their therapeutic potential against chronic inflammation and cancer. Classic Nrf2 activation occurred along with the modification of the Keap1 cysteine residue, which protected Nrf2 from ubiquitination following its accumulation in the cytoplasm and subsequent translocation to the nucleus. Eventually, Nrf2 bound with its target antioxidant response elements (AREs) and exerted cell protective effects [21, 22]. Therefore, Nrf2 has the ability to influence the expression levels of a variety of antioxidant enzymes, such as glutathione *S*-transferase, GCLM, HMOX1, and NQO1 [23, 24]. We found that cisplatin improved the Nrf2 protein levels, the ROS levels, and the GCLM, HMOX1, and NQO1 mRNA levels in BC cells. These findings are consistent with those of a previous study [25]. Wang et al. [16] reported the significance of Nestin1 in the Keap1/Nrf2/AREs signaling axis, showing that it binds competitively to the Kelch domain to shield Nrf2 from Keap1-mediated degradation, which in turn improves the expression levels of the antioxidant enzymes. Nestin1 can link to both Keap1 and Nrf2 and can upregulate the expression of the latter to modulate the oxidative equilibrium in lung cancer [16]. Previous studies have provided a molecular mechanism for the function of Nestin1 in tumors that progress internally as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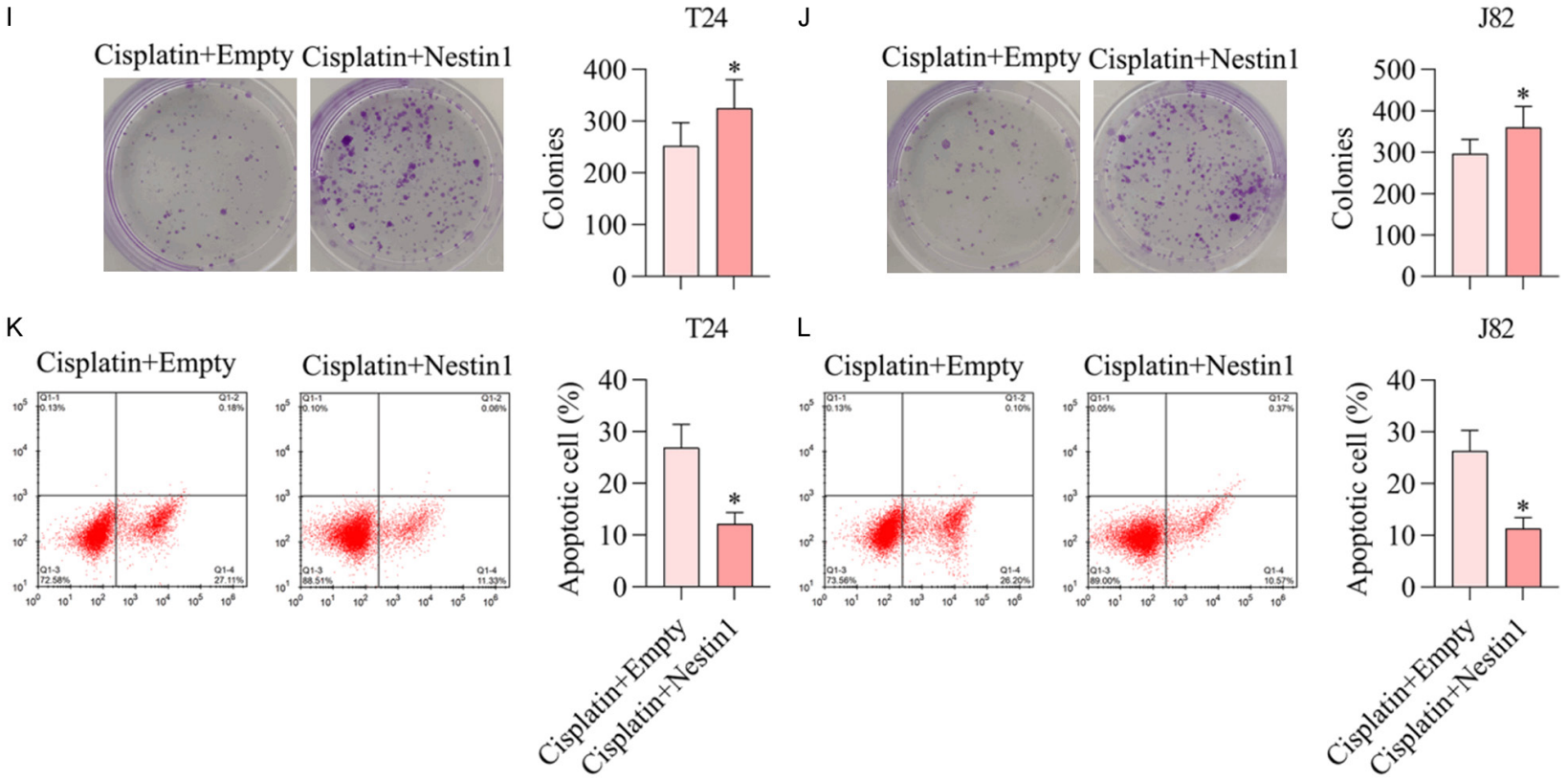
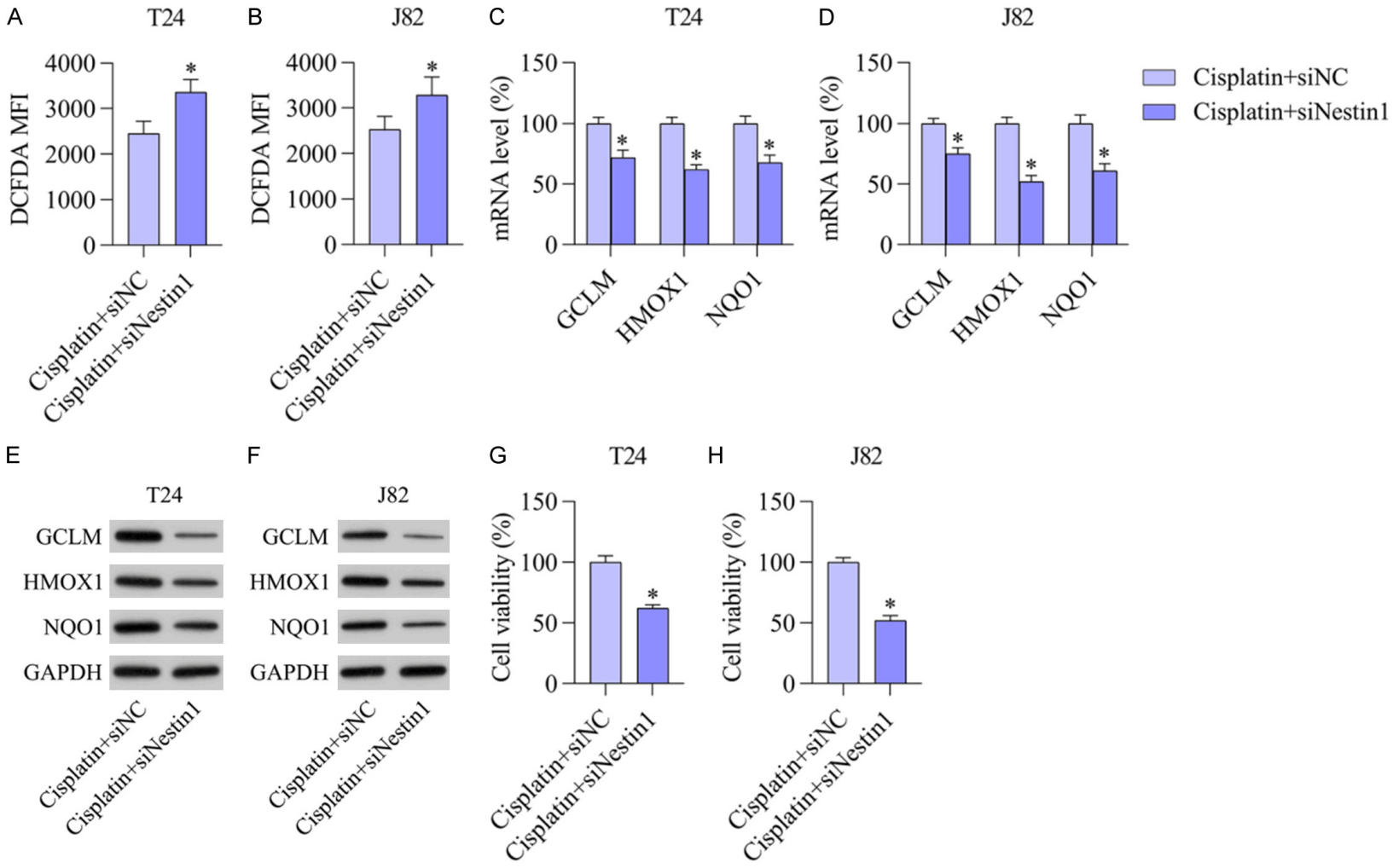


Figure 4. The effect of Nestin1 overexpression on the cisplatin-treated bladder cancer (BC) cells. T24 and J82 cells were transfected with pcDNA3.1 or pcDNA3.1-Nestin1 and then treated with 20 mg/L cisplatin for 60 h (A, B). The cells were stained using a ROS detection probe, and the production of ROS was analyzed using flow cytometry (C-F). The RT-qPCR and WB revealed the effect of the cisplatin on the expression levels of the antioxidant-related genes *GCLM*, *HMOX1*, and *NQO1* in the BC cells (G, H). CCK-8 assays were used to determine the cell viability (I, J). Colony formation of the cell lines treated with 20 mg/L cisplatin for 96 h (K, L). Cisplatin (20 mg/L) was added to the cells for 24 h, following which the cell apoptosis was measured using annexin V/PI staining and analyzed using flow cytometry. The results are expressed as a percentage of the control values and are presented as the mean ± SD of 3 separate experiments. $^{*}P \leq 0.05$ vs. Cisplatin + Empty.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Cisplatin+siNestin1

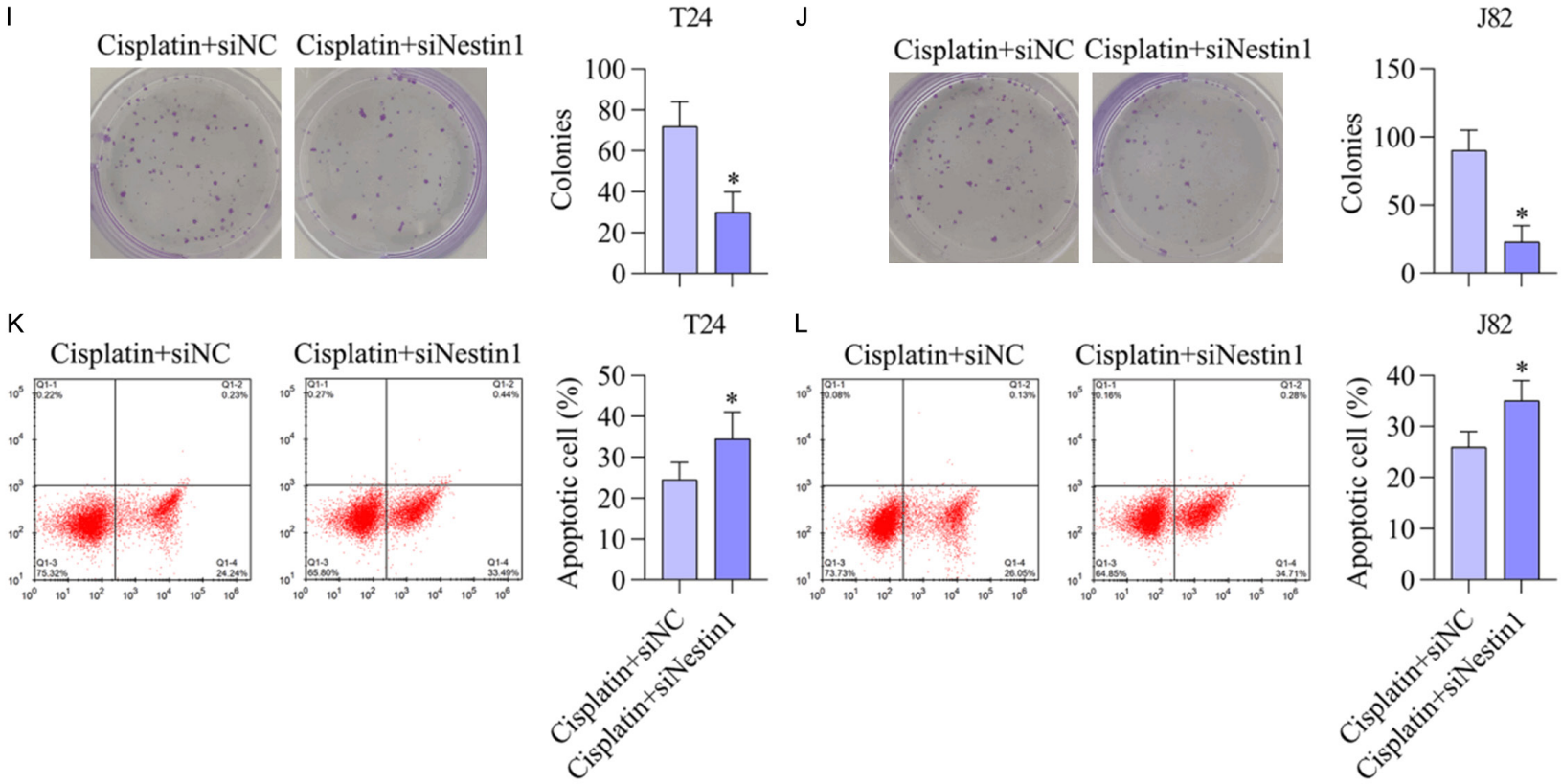

Figure 5. The effect of the Nestin1 silencing on the cisplatin-treated bladder cancer (BC) cells. T24 and J82 cells were transfected with normal control siRNA (siNC) or Nestin1 siRNA (siNestin1) and then treated with 20 mg/L cisplatin for 60 h (A, B). The cell lines were stained using a ROS detection probe, and the production of ROS was analyzed by flow cytometry (C-F). RT-qPCR and WB revealed the effect of cisplatin on the expression levels of the antioxidant-related genes *GCLM*, *HMOX1*, and *NQO1* in the BC cells (G, H). The CCK-8 assay was used to determine the cell viability (I, J). The colony formation of the cell lines was treated with 20 mg/L cisplatin for 96 h (K, L). Cisplatin (20 mg/L) was added to the cells for 24 h, following which the cell apoptosis was measured using annexin V/PI staining and analyzed using flow cytometry. The results are expressed as a percentage of the control values and are presented as the mean ± SD of three separate experiments. $^{*}P \leq 0.05$ vs. Cisplatin + siNC.

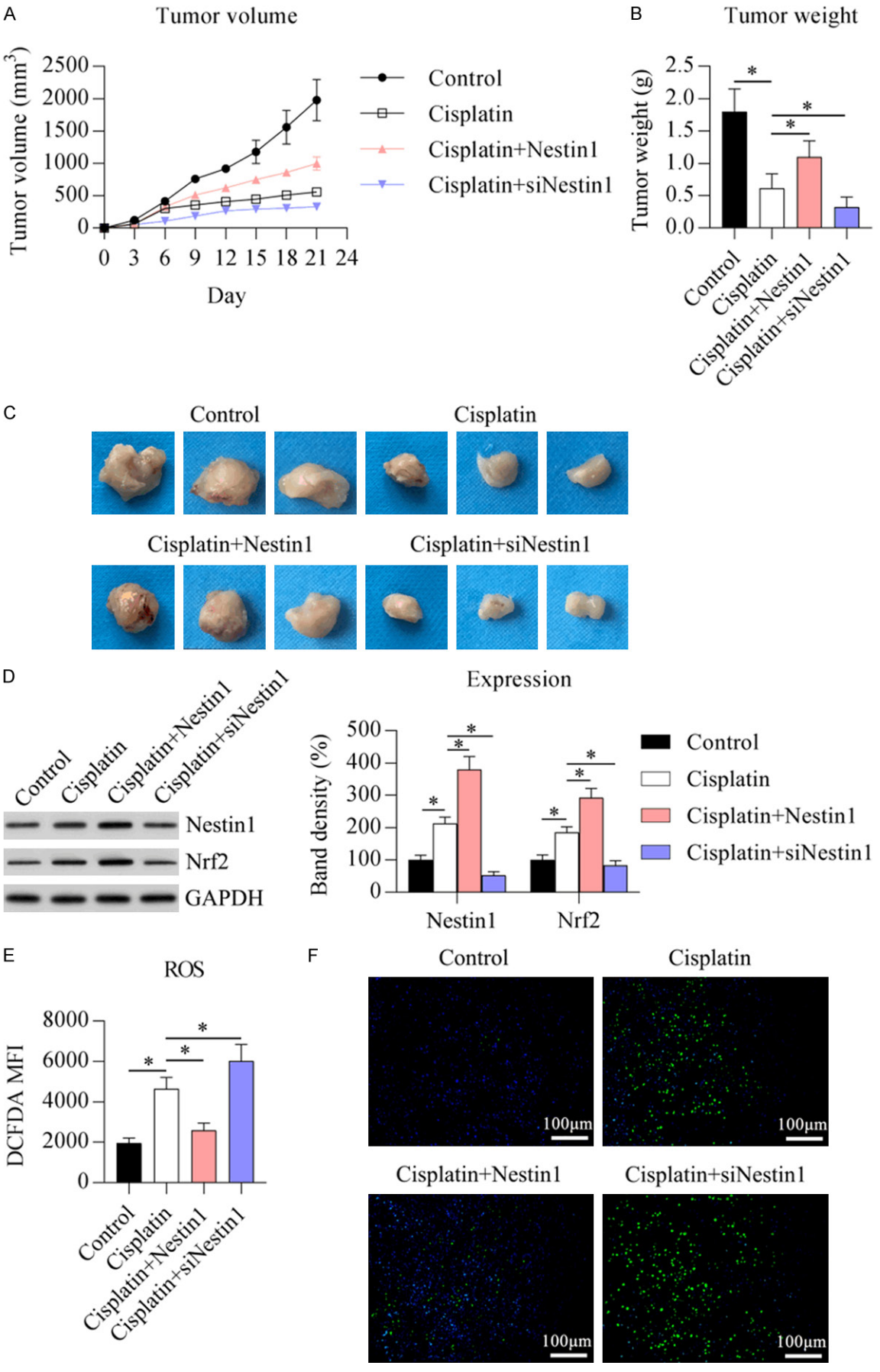
A
Tumor volume
Tumor volume (mm3)
2500
2000
1500
1000
500
0
0 3 6 9 12 15 18 21 24
Day
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
B
Tumor weight
Tumor weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
C
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
D
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
Nestin1
Nrf2
GAPDH
Expression
Band density (%)
500
400
300
200
100
0
Nestin1
Nrf2
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
E
ROS
DCFDA MFI
8000
6000
4000
2000
0
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
F
Control
Cisplatin
Cisplatin+Nestin1
Cisplatin+siNestin1
100μm

Figure 6. The effect of Nestin1 on tumor growth in a mouse model of cisplatin-treated xenograft bladder cancer. A. Nude mice were injected subcutaneously with 4 × 10^6 Nestin1-overexpressing or -silenced T24 cells. After 1 week, the mice were treated with 5 mg/L cisplatin for 10 continuous days. The tumor sizes were measured at specific time points after the treatment and calculated and plotted (n = 6/group). B. Tumor weights after the treatment. C. Representative tumors at the end of the study period. D. A Western blot analysis was carried out to examine the protein expression levels of Nestin1 and Nrf2 in the typical tumors. E. The cell lines were stained using an ROS detection probe, and the production of ROS was analyzed using flow cytometry. F. Sections from the paraffin-embedded tumor tissues were selected for analysis using TUNEL staining. Representative TUNEL staining pictures are shown. The results are expressed as a percentage of the control values and are presented as the mean ± SD of 3 separate experiments. $^*P \leq 0.05$ vs. indicated group.

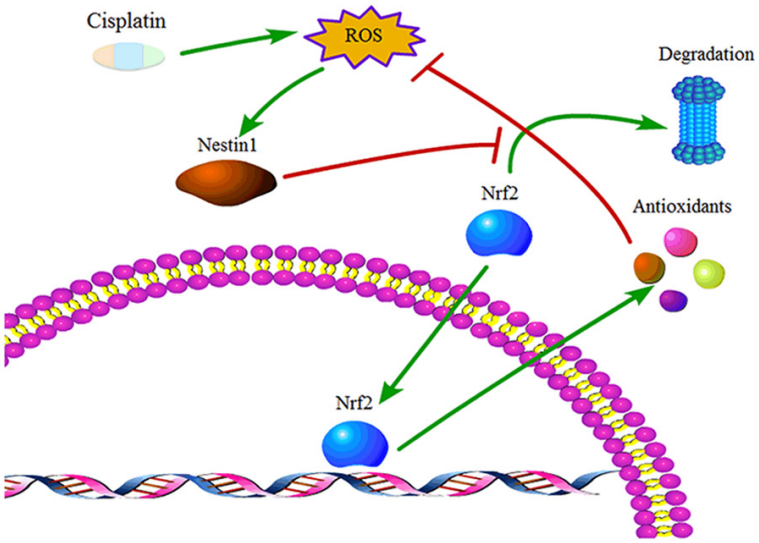

Figure 7. A schematic diagram illustrating the mechanism of Nestin1 induction using cisplatin treatment in bladder cancer cells. Treatment of the cells with cisplatin causes increases in the ROS levels and in the Nestin1 expression. This increase in the Nestin1 level subsequently protects Nrf2 from degradation, thereby allowing it to exert its function in triggering the transcription of the antioxidant genes. These antioxidants can then ameliorate the harmful effects of the ROS on the BC cells.

well as externally. Nestin1-positive progenitor cells within the cerebellum displayed more severe genomic instability and efficient tumor cell transformation than the granule neuron precursors [26]. Nestin1-expressing progenitor cell-like cells that had dedifferentiated from mature hepatocytes might develop into hepatocellular carcinomas and cholangiocarcinomas [27]. The use of a nestin1-targeting siRNA revealed that tumors could be inhibited internally through the suppression of tumor angiogenesis [28]. Our results demonstrated that cisplatin induced the expression of Nestin1 protein in BC cells and that there was a positive correlation between the Nestin1 and Nrf2 protein levels. Through loss- and gain-of-function assays in T24 and J82 cells, we determined that Nestin1 overexpression ameliorated the ROS generation induced by cisplatin and further upregulated antioxidant gene expression, thereby recovering the viability and growth of the BC cells and downregulating their apoptosis. By contrast, Nestin1 depletion had the opposite effects and resulted in more severe cell injury than that observed in the siNC-transfected group. Additionally, Nestin1 overexpression partially abolished the cisplatin-mediated suppression of the tumor growth *in vivo*, but the Nestin1 silencing enhanced the chemosensitivity of the BC xenograft. On the basis of these observations, we suggest that Nestin1 may enhance the chemoresistance of BC tumors to cisplatin *in vivo* by activating the Nrf2-mediated antioxidative pathway.

Our data showed that Nestin1 and Nrf2 participated in the maintenance of the antioxidative potential of BC cells and the crosstalk between the two critical pathways. Additionally, Nestin1 depletion was observed to significantly increase the cisplatin sensitivity by mediating ROS generation, antioxidative damage, and cell death in the T24 and J82 cells. However, one limitation of this study is the lack of more *in vivo* confirmation experiments, including the use of Nestin1-knockout/knockdown mice for the xenograft tumor experiment. Therefore, more animal experiments will be performed in our future studies to verify the function of Nestin1 in cisplatin-treated BC tumors.

In conclusion, the results of this study provide a theoretical basis for targeting Nrf2 transcriptional regulators, which may be applied as

effective and safe drugs for the chemoprophylaxis of BC and other tumors as well as a variety of other illnesses [29].

Disclosure of conflict of interest

None.

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