Antitumor effect of dsRNA-induced p21$^{WAF1/CIP1}$ gene activation in human bladder cancer cells

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Abstract

We recently reported that synthetic dsRNAs targeting promoter regions can induce gene expression in a phenomenon referred to as dsRNA-induced gene activation/RNA activation (RNAa) [Li et al. Proc Natl Acad Sci U S A 2006;103:17337–42]. The present study investigates the *in vitro* antitumor activity RNAa can elicit through triggering the expression of cell cycle repressor protein p21$^{WAF1/CIP1}$ (p21) in human bladder cancer cells. Transfection of a 21-nucleotide dsRNA targeting the p21 promoter (dsP21) was used to induce p21 expression in T24 and J82 bladder cancer cell lines. Reverse transcription-PCR and Western blot analysis accessed the increase p21 mRNA and protein levels, respectively, in transfected cells. In association to p21 induction, dsP21 transfection significantly inhibited bladder cancer cell proliferation and clonogenicity. Further analysis of cell viability and cell cycle distribution revealed that dsP21 transfection also enhanced apoptotic cell death and caused an accumulation in the G_1 phase in both cell lines. In conclusion, p21 activation by RNAa has antitumor activity *in vitro* in bladder cancer cells. These results suggest that RNAa could be used for cancer treatment by targeted activation of tumor suppressor genes. [Mol Cancer Ther 2008;7(3):698–703]

Introduction

Small dsRNAs are known to be the trigger of an evolutionally conserved mechanism known as RNA interference. RNA interference uses small dsRNAs to silence gene expression by degrading homologous mRNA molecules or inhibiting protein translation. Recently, new evidence has emerged that small dsRNAs may also function to induce gene expression in a phenomenon referred to as dsRNA-induced gene expression/RNA activation (RNAa; refs. 1, 2). By targeting gene promoter regions, we have shown that dsRNAs can be used to specifically activate the expression of p21, E-cadherin, and vascular endothelial growth factor (VEGF) expression in human cancer cell lines (1). Other have since reported supporting data by showing induction of progesterone receptor and the major vault protein gene by small dsRNA molecules (2).

RNA interference has been rapidly developing into a promising new approach for battling cancer and other diseases. With this method, it is possible to block the production of mutant genes including dominant-negative proteins, aberrant splicing isoforms, or overexpressed genes that have gain-of-function effects (3). However, RNA interference can only offer antagonism of specific molecular targets for disease treatment. Strategies that can provide agonism of specific gene targets, such as tumor suppressor genes, are equally crucial for the reversal of the tumor phenotype. Although traditional gene therapy methods have the capacity of correcting an abnormal copy of an endogenous gene or augmenting the expression of a normal gene, it has its inherent drawbacks including a tedious construction process and detrimental, sometimes even fatal, effects on the host genome.

Similar to RNA interference, RNAa offers a fast, simple, and cost-effective approach to alter gene expression. Using dsRNAs, RNAa can activate silenced genes or augment the expression of less active genes. Known and putative tumor suppressor genes that negatively control cell cycle, promote cell death, and/or inhibit invasion and metastasis are ideal targets for RNAa. In addition, gene induction by RNAa lasts much longer than gene silencing by RNA interference most likely due to the epigenetic changes associated with targeted promoters, which are presumably inheritable across cell divisions (1, 2). Such features make RNAa an ideal therapeutic strategy for cancer.

An important characteristic of tumor cells is their increased proliferative capability, which is often caused by impaired regulation of the cell cycle. The cell cycle has several checkpoints that are controlled by several complex modulation systems including the retinoblastoma (RB1) protein, cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (4). Cyclin-dependent kinase inhibitors are negative regulators of cell cycle progression and considered to be potential tumor suppressor genes.

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The $p21^{WAF1/CIP1}$ (p21) gene is a broad-acting cyclin-dependent kinase inhibitor. Ectopic expression of p21 by adenoviral vectors has been shown to inhibit tumor growth both *in vitro* and *in vivo* (5–7) as well as induce apoptosis (5, 6). The p21 gene is therefore an ideal target for gain-of-function manipulation to inhibit cancer cell growth and reverse tumor phenotypes.

In the present study, we investigate the antitumor effects of p21 activation via RNAa in human bladder cancer cells. Our results show that the dsRNA-induced activation of p21 significantly inhibits bladder cancer cell proliferation and survival and enhances apoptotic cell death.

Materials and Methods

dsRNAs

dsRNAs were designed and synthesized as described previously (1). A dsRNA targeting the p21 promoter at position -322 relative to the transcription start site (dsP21) was used to activate p21 expression. A dsRNA lacking significant homology to all known human sequences (dsControl) was used as a nonspecific control.

Cell Culture and Transfection

Bladder cancer cell line T24 (American Type Culture Collection) was grown in RPMI 1640 containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). Bladder cancer cell line J82 (American Type Culture Collection) was grown in McCoy's 5A medium supplemented with 10% FBS, penicillin, and streptomycin. Cells were incubated at 37°C in a humidified incubator under an atmosphere of 5% CO_2. The day before transfection, cells were plated in growth medium without antibiotics at a density of 50% to 60%. dsRNA was transfected at a concentration of 50 nmol/L using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

RNA Isolation and Reverse Transcription-PCR

Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA (1 μg) was used for cDNA synthesis using the ThermoScript reverse transcription-PCR system (Invitrogen). The resulting cDNA was amplified by PCR using gene-specific primers. PCR primers for p21 were GCCCAGTGGACAGCGAGCAG (sense) and GCCGGC-GTTTGGAGTGGTAGA (antisense). As a loading control, 18S rRNA was also amplified using primers GTAACC-CGTTGAACCCCATT (sense) and CCATCCAATCGG-TAGTAGCG (antisense). PCR amplification consisted of an initial denaturation step (94°C for 2 min), 30 cycles of denaturation (94°C for 20 s), annealing (60°C for 20 s), and extension (72°C for 30 s) followed by a final incubation at 72°C for 5 min.

Protein Isolation and Western Blotting Analysis

Cells were washed twice with cold PBS solution and lysed with M-PER extraction buffer (Pierce Biotechnology) containing protease inhibitors. The resulting cell lysates were collected and centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentrations were determined in the supernatant fraction by the Bradford assay. Equivalent quantities of protein were separated by electrophoresis on 15% SDS-polyacrylamide gels with prestained protein molecular weight standards. The separated proteins were then electroblotted on 0.45 μm nitrocellulose membranes (Bio-Rad). The resulting blots were blocked with 5% nonfat dry milk and probed with anti-p21 (Upstate) or anti–glyceraldehyde-3-phosphate dehydrogenase (Chemicon) primary antibodies. Immunodetection was followed by incubation with an anti-mouse horseradish peroxidase–linked IgG secondary antibody (Cell Signaling). Antigen-antibody complexes were visualized by chemiluminescence (Santa Cruz Biotechnology).

Cell Proliferation Assay

Cell proliferation was investigated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). Transfection of dsRNA was carried out using LipofectAMINE 2000 (Invitrogen) by following the reverse transfection protocol provided with the product. Briefly, transfection mixtures were prepared and added to each well of a 96-well plate. Cells were then subcultured into

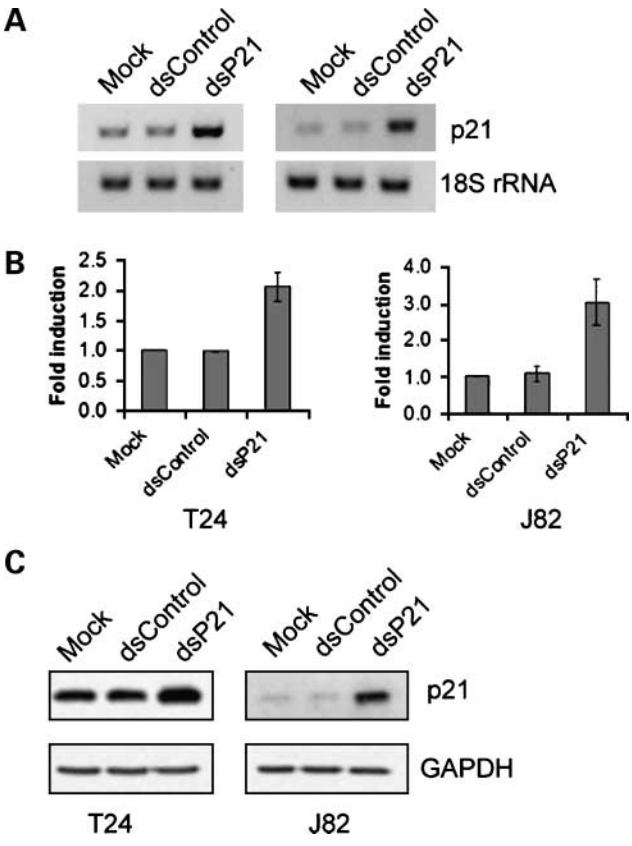

Figure 1. Induction of p21 expression by promoter-targeting dsRNA in bladder cancer cells. T24 and J82 cells were transfected with 50 nmol/L of the indicated dsRNAs for 72 h. Mock samples were transfected in the absence of dsRNA. **A,** expression of p21 and 18S rRNA levels was assessed by standard reverse transcription-PCR. 18S rRNA served as a loading control. **B,** p21 mRNA expression levels were normalized to 18S rRNA levels. Mean ± SE of two independent experiments. **C,** induction of p21 protein expression was detected by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase levels were also detected and served as a loading control.

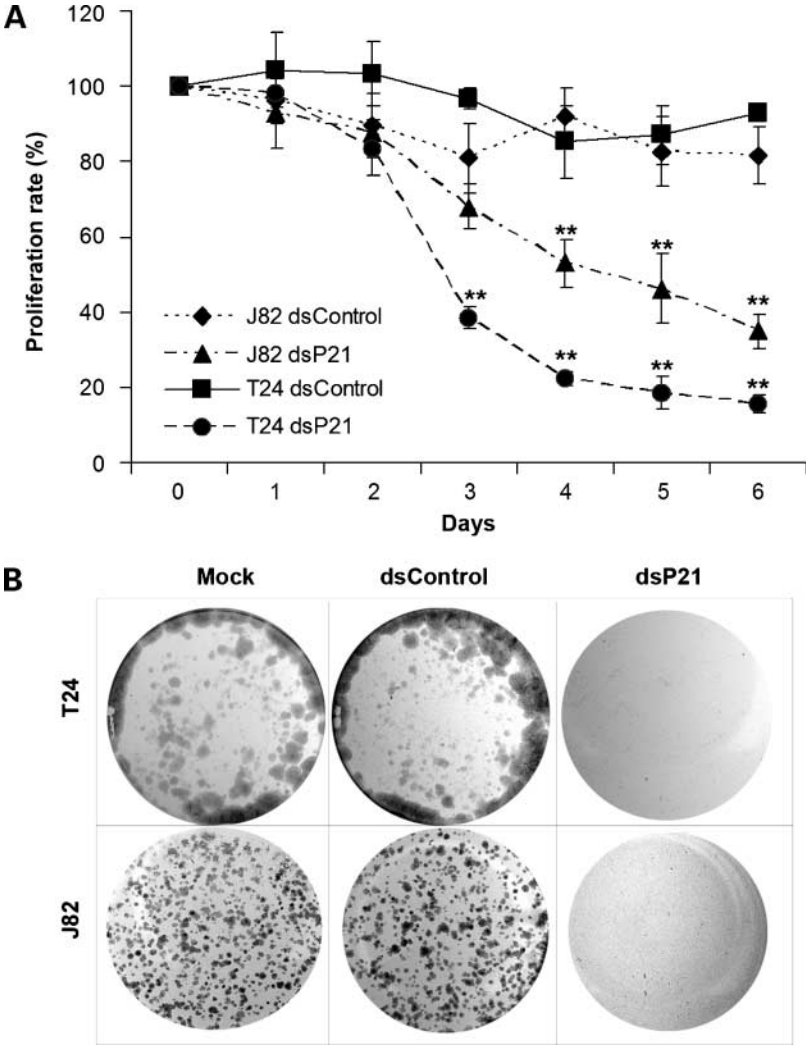

Figure 2. dsP21 inhibits cell proliferation and survival. **A,** cells were plated in 96-well plates and transfected with 50 nmol/L of the indicated dsRNAs. Cell proliferation was assessed on days 1 to 6 following treatments. Mean ± SE of two independent experiments relative to dsControl transfections. **, $P < 0.001$. **B,** 2,500 cells per well were plated in six-well plates and transfected with 50 nmol/L dsRNA. On day 12, cell colonies were stained with crystal violet and photographed. Note that no colonies were detected in dsP21-transfected T24 or J82 cells.

the wells containing the transfection mixtures at a density of 2×10^4/mL in complete medium without antibiotics. The plates were then incubated for 1 to 6 days. At end of the incubation period, 20 μL CellTiter 96 AQueous One Solution was added to each well. After 1 h, absorbance was measured on an ELISA plate reader (Bio-Tek Instruments) at 490 nm.

Analysis of Cell Cycle Distribution by Flow Cytometry

Transfected T24 and J82 cells (1×10^6/mL) were trypsinized and centrifuged at $2500 \times g$ for 5 min at 4°C and washed twice in cold PBS buffer. The pellet was gently resuspended in 500 μL cold saline GM solution (6.1 mmol/L glucose, 1.5 mmol/L NaCl, 5.4 mmol/L KCl, 1.5 mmol/L Na_2HPO_4, 0.9 mmol/L KH_2PO_4, 0.5 mmol/L EDTA). The resuspended cells were fixed in 3 mL cold 100% ethanol overnight at 4°C. The following day, cells were washed twice with cold PBS and resuspended in 1 mL propidium iodide staining solution (50 μg/mL propidium iodide, 10 mg/mL RNase A, 0.1% Triton X-100, 0.1% sodium citrate in PBS) and set at room temperature for 1 h. The stained cells were immediately analyzed on a FACScan flow cytometer for relative DNA content. The resulting data were analyzed using FlowJo software to determine cell cycle distribution (Tree Star).

Clonogenic Survival Assay

Exponentially growing cells were plated at very low cell densities (2,500 per well in six-well plates). After 24 h, dsRNA was transfected into each well using Lipofect-AMINE 2000 (Invitrogen). The following day, culture medium was replaced and subsequently changed every 3 days. Colony formation was analyzed at day 12 by staining the cells with crystal violet.

Terminal Deoxynucleotidyl Transferase – Mediated dUTP Nick End Labeling Apoptosis Assay

Apoptotic cell death was assessed by using the ApopTag Peroxidase kit (Millipore) according to the manufacturer's protocol. Briefly, cells were seeded on coverslips in six-well plates and transfected with dsRNA the following day. After 72 h, cells were fixed in 1% paraformaldehyde and permeabilized using ethanol/acetic acid (2:1) for 10 min at

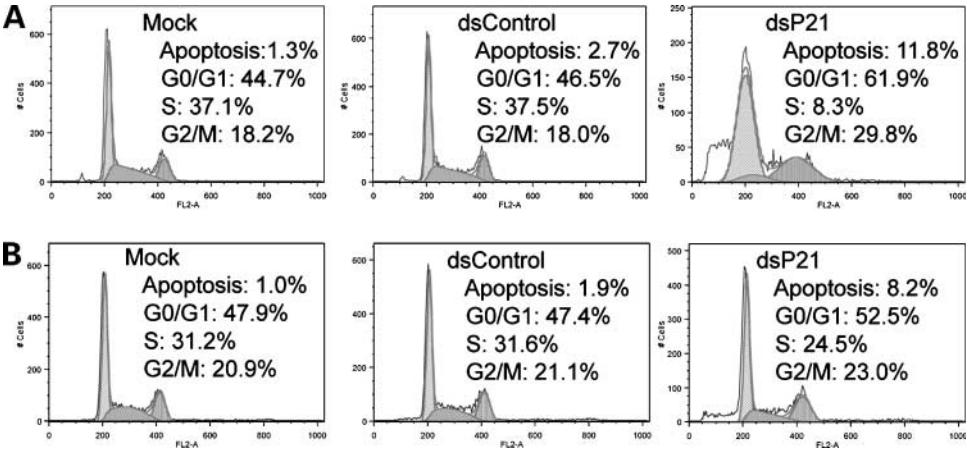

Figure 3. dsP21 induces cell cycle arrest in bladder cancer cells. T24 (**A**) and J82 (**B**) cells were transfected with mock, dsControl, or dsP21 for 72 h. Cells were stained with propidium iodide and DNA content was determined by flow cytometry. Percentages of sub-G_0-G_1 (apoptosis) cells were calculated from the entire gated whole-cell population. The percentages of cells in G_0-G_1, S, and G_2-M phases were calculated by the FlowJo program using the Dean-Jett-Fox model and excluded the sub-G_0-G_1 cell fraction. Representative example of two independent experiments.

room temperature. The reaction was quenched with 3% hydrogen peroxide for 5 min and the fixed cells were subsequently washed with PBS. Working strength terminal deoxynucleotidyl transferase enzyme was then applied to the coverslips followed by the addition of anti-digoxigenin peroxidase-conjugated antibody. Color detection was achieved by adding peroxidase substrate to the coverslips. Phase-contrast images of cells were taken under an inverted microscope. Positively staining cells having brown nuclei were recorded by counting 10 randomly selected fields under ×200 magnification for each treatment.

Results and Discussion

A dsRNA Targeting the Promoter of p21 Induces Gene Expression in Bladder Cancer Cells

A dsRNA targeting the p21 gene promoter at position -322 relative to the transcription start site (dsP21) was used to activate p21 expression as described previously (1). dsP21 and a nonspecific control dsRNA (dsControl) were transfected into T24 and J82 bladder cancer cells and p21 expression levels were evaluated 72 h later. As shown in Fig. 1A and B, dsP21 caused a ~2- and 3-fold induction in p21 mRNA levels compared with mock and dsControl transfections in both T24 and J82 cells, respectively (Fig. 1A and B). Induction of p21 was also confirmed by Western blot analysis (Fig. 1C). Elevated levels of p21 protein strongly correlated to the increase in p21 mRNA expression in both cell lines.

We have shown previously that dsP21 activates p21 expression in a variety of different types of cancer cells (1). Although the exact mechanism for RNAa remains unclear, small dsRNA-guided activation of tumor suppressor genes (e.g., p21) still has potential application in the treatment of cancer. Because the expression of p21 is frequently impaired in bladder cancer (8, 9), induction of p21 by RNAa in bladder cancer cells could have potent antitumor capabilities.

dsP21 Inhibits Bladder Cancer Cell Proliferation and Survival

Ectopic expression of p21 and induction of p21 by various agents (e.g., histone deacetylase inhibitors) have been shown to inhibit cell proliferation (10–16). To examine whether induction of p21 by RNAa had an effect on bladder cancer cell line growth rate, we measured cell proliferation in dsP21-transfected T24 and J82 cells. After 2 days, dsP21- transfected cells displayed phenotypic changes associated growth arrest; they had an enlarged and flattened cellular morphology and decreased cell density (Supplementary Fig. S1).[4] As shown in Fig. 2A, a slower growth rate was detectable as early as 48 h following dsP21 transfection in both T24 and J82 cells. The time required for the growth-inhibitory effect strongly correlates with the kinetics of RNAa, which usually manifests 48 h following transfection (1, 2). By day 6, dsP21 inhibited T24 and J82 growth by 84.4% and 65.1%, respectively, compared with dsControl transfections (Fig. 2A).

To further assess bladder cancer cell survival, we did clonogenicity assays in dsP21-transfected T24 and J82 cell lines. As shown in Fig. 2B, mock and dsControl-transfected cells formed numerous colonies after 12 days of incubation; however, no colonies were observed in either dsP21-transfected T24 or J82 cells. These results indicate that dsP21 induction by RNAa completely abolished bladder cancer cell line clonogenicity. It has been reported previously that overexpression of p21 by conventional means inhibits colony formation as a result of abnormal mitosis and growth arrest (17). Our results showing that dsP21 completely abolished clonogenicity may reflect a persistent induction of p21 by RNAa because high sustained p21 levels are required to deplete the cellular pool of mitosis control proteins (17) and thus offer a novel way to induce p21 expression and suppress bladder cancer cell division and survival.

dsP21 Induces Cell Cycle Arrest and Apoptosis in Bladder Cancer Cells

p21 is a well-known cyclin-dependent kinase inhibitor (18) that suppresses cell proliferation by interfering with cyclin/cdk2 activity (19) and blocks DNA replication by binding to proliferating cell nuclear antigen (20). To

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

examine the effect of p21 induction by RNAa on cell cycle progression, we analyzed the DNA content of propidium iodide–stained cells using flow cytometry analysis to measure cell cycle distribution. As shown in Fig. 3, flow cytometry analysis revealed a significant increase in the G_0-G_1 population of dsP21-transfected cells compared with mock and dsControl treatments. We also detected an increase in the G_2-M-phase populations and a corresponding decrease the S-phase populations (Fig. 3). These results are in agreement with the function of p21 as a negative regulator of cell cycle progression, which can cause G_1 and/or G_2 arrest (18, 19, 21). Interestingly, analysis of the entire gated whole-cell population revealed a significant increase in the sub-G_0-G_1 population of dsP21-treated T24 (11.8%) and J82 (8.2%) cells (Fig. 3). Because DNA fragmentation is associated with apoptosis (22), the sub-G_0-G_1 peak suggests p21 induction by RNAa also enhanced apoptotic cell death in bladder cancer cells.

To further analyze if p21 induction by dsP21 causes apoptosis in bladder cancer cells, we carried out terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays in dsP21-transfected T24 and J82 cells. As shown in Fig. 4, few to no apoptotic cells were observed in mock and dsControl-treated cells; however, 45.3% of T24 and 26.6% of J82 cells stained positively for DNA fragmentation/apoptosis (Fig. 4). This result is consistent with previous studies in which activation of p21 expression leads to enhanced apoptosis in bladder cancer cell lines (11, 16).

In summary, we show that induction of p21 by RNAa has antitumor activity *in vitro* in bladder cancer cells by promoting cell cycle arrest and enhancing apoptotic cell death. These observations suggest that activation of gene expression by RNAa may have significant therapeutic potential for the treatment of bladder and other cancer types. Furthermore, this method offers a practical and cost-effective approach to activate gene expression. To further evaluate the use of RNAa in disease treatment, studies in animal tumor models using promoter-targeting dsRNA designed to activate p21 or other tumor suppressor genes are warranted.

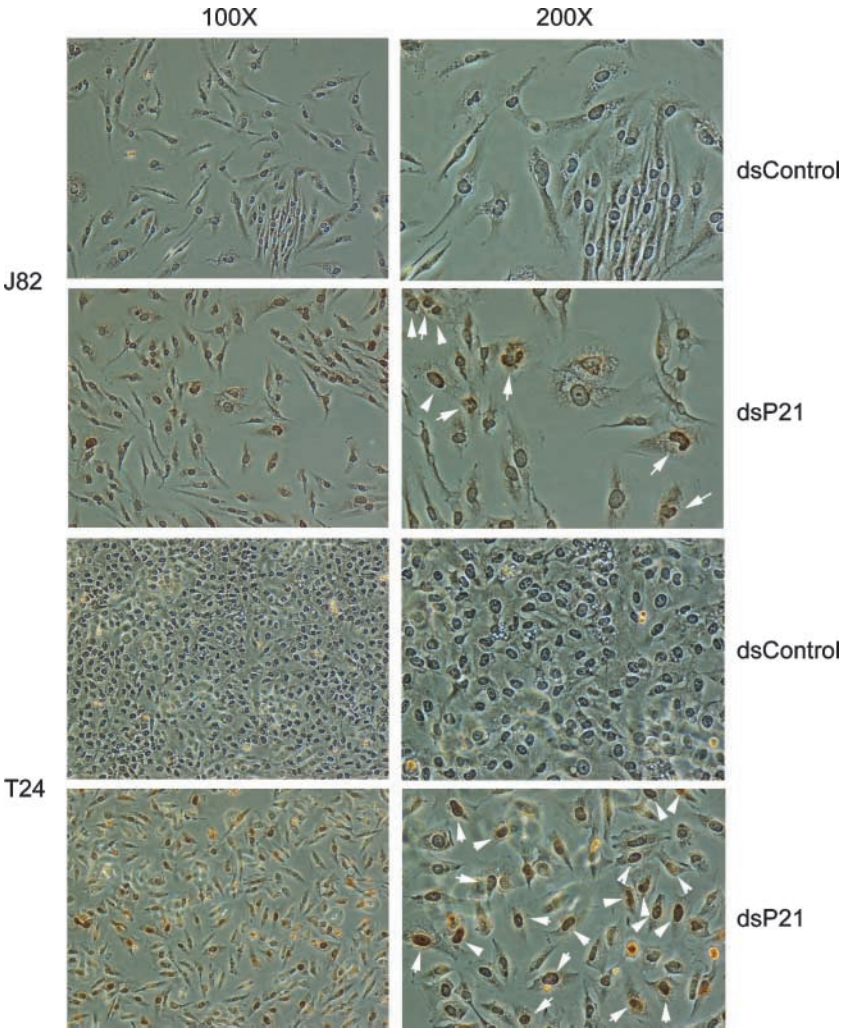

Figure 4. dsP21 induces apoptosis in bladder cancer cells. Cells were plated on coverslips and transfected with 50 nmol/L dsRNA. Apoptotic cells were stained by the terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assay and images were taken using phase-contrast microscopy. Cells positive for DNA fragmentation had nuclei that stained brown in color. Representative images at ×100 and ×200 magnifications. White arrows, apoptotic cells at ×200 magnification.

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