

Inhibiting Proliferation and Enhancing Chemosensitivity to Taxanes in Osteosarcoma Cells by RNA Interference-Mediated Downregulation of *Stathmin* Expression

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Stathmin (Oncoprotein18), a signal transduction regulatory factor, plays an important role in cell division and malignant tumor development. *Stathmin* is a ubiquitous intracellular phosphoprotein that is overexpressed in a variety of human malignancies, including osteosarcoma. To investigate the potential use of *stathmin* as a therapeutic target for human osteosarcomas, we employed RNA interference (small interfering RNA (siRNA)) to reduce *stathmin* expression in human osteosarcoma cell lines and analyzed their phenotypic changes. Results showed that the downregulation of *stathmin* expression in human osteosarcoma cells significantly inhibited cell proliferation in vitro and tumorigenicity in vivo. The specific downregulation induced cell arrest in the G₂/M phase of cell cycle and eventually apoptotic cell death. Taxanes are a group of effective chemotherapeutic agents whose activity is mediated through stabilization of the microtubules of the mitotic spindle. In the present study, we also observed a synergistic enhancement of the cytotoxicity effect by combination use of taxanes and RNA interference-mediated *stathmin* downregulation. All these experimental data indicate that *stathmin* downregulation can lead to potent antitumor activity and chemosensitizing activity to taxanes in human osteosarcomas.

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INTRODUCTION

Osteosarcomas, malignant tumors derived from bone tissues, are the most frequently occurring type of bone tumor and are characterized by aggressive invasion, early metastasis, and resistance to existing chemotherapeutic agents (1). To improve the formidable prognosis of osteosarcoma, it is essential to find novel approaches for both diagnosis and treatment that are far more efficient than currently available methods (2). Osteosarcoma employs complex signal transduction systems, some of which remain unclear. *Stathmin* family phosphoproteins (*stathmin*, SCG10, SCLIP, RB3/RB3'/RB3'') are important signal transduction molecules and regulators of mi-

cro-tubule dynamics (3). Recent studies have shown that *stathmin* is expressed at high levels in human osteosarcoma (4). Overexpression of this gene occurs in a number of other human malignancies such as leukemia and lymphoma, prostate carcinoma, ovarian carcinoma, Wilms tumor, breast carcinoma, and adenoid cystic carcinoma of the salivary glands, suggesting the existence of an important oncogene that plays a critical role in maintenance of malignant phenotypes in various human cancers (5-7).

Stathmin was first identified as a 17-kDa cytosolic protein that was rapidly phosphorylated when HL60 leukemic cells underwent terminal differentiation and ceased to proliferate (8). Also known

as oncoprotein 18, prosolin, p19, 19K, p18, and op18, *stathmin* is a conserved cytosolic protein that plays a critical role in mitosis and possibly other cellular processes (9,10). *Stathmin* has been shown to regulate the dynamics of the microtubules that make up the mitotic spindle, a role associated with carcinogenesis that indicates *stathmin* is one of the fundamental cancer-associated genes and a potential target for diagnosis and treatment (11,12). Previous studies have shown that manipulations which increase or decrease the level of *stathmin* expression can interfere with the orderly progression of leukemic cells through the cell cycle (13). Recent studies also demonstrated that *stathmin* inhibition decreases the rate of proliferation of tumor cells, accumulation in the G₂/M phase of the cell cycle, and synergistic interaction with Taxol (14-16). In a previous study, we found that RNA interference (RNAi)-mediated *stathmin* inhibition could reverse malignant phenotypes of tumor cells (17). In addition, *stathmin* inhibition has shown tumor-suppressor function in

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breast cancer cell lines (18) and therapeutic interactions with chemotherapeutic agents in prostate cancer (19). Thus, we believe that *stathmin* may provide an attractive molecular target for disrupting the mitotic apparatus and arresting the proliferation of malignant cells. In the present study, we employed small interfering RNA targeting *stathmin* to explore the potential of new therapeutic targets in the treatment of human osteosarcomas. Moreover, we analyzed the synergistic effects on both in vitro and in vivo proliferation of osteosarcoma cells by combined use of taxanes and RNAi-mediated *stathmin* downregulation. Such synergistic interaction might be relevant for the treatment of osteosarcoma because taxanes and RNAi targeting *stathmin* are two of the most effective treatment methods for this disease. Thus, this combination may provide a novel strategy of osteosarcoma therapy that avoids toxicities associated with the use of multiple chemotherapeutic agents at full therapeutic doses.

MATERIALS AND METHODS

Maintenance of Cell Lines

Two human osteosarcoma cell lines (Saos-2 and MG63) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were maintained in an atmosphere of 5% CO₂ with humidity at 37 °C.

siRNA Synthesis

We designed and synthesized double-strand siRNAs in vitro for the *stathmin* gene with two thymidine residues (dTdT) at the 3' end of the sequence extending between amino acid residues 398 and 417 (sense, 5'-GAAACGAGAGCACGAGAAA-3') (15) (AuGCT Biotechnology, Beijing, PRC). We also synthesized a nonspecific siRNA as a control (sense, 5'-GCAAAAGAGC-GAAAAG-3'). According to a BLAST database search, the scrambled sequences did not show significant homology to

any human mRNA. These RNAs were dissolved in Tris-Cl (pH 8.0) and 1 mM EDTA as 200 µM solutions. Double-strand siRNAs were generated by mixing the corresponding pair of sense and antisense RNA oligonucleotides at a concentration of 20 µM in 5 × annealing buffer [30 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2 mM MgCl₂, and 50 mM NH₄AC]. The reaction mixture was heated to 90 °C for 1 min, gradually cooled down to 37 °C for 60 min, and then aliquoted and stored at -20 °C.

Transient Transfection of siRNA

Two human osteosarcoma cell lines (Saos-2 and MG63) were plated in 10-cm tissue culture plates at approximately 1.0 × 10⁶ cells per well, respectively, and cultured overnight to give 50%-70% confluence prior to transfection. In vitro transfection of RNA oligonucleotides resulting in various RNA concentrations was performed using Lipofectamine2000 transfection reagent (Invitrogen). The cells were harvested at different time points for RT-PCR and Western blot analyses.

Quantitative Real-Time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen). RNA of 2 µL (1 µg/µL) was used to synthesize cDNA using a Superscript First-Strand Synthesis Kit (Promega, Madison, Wisconsin, USA) following the manufacturer protocols. Expression of *stathmin* mRNA was detected with the ABI 7700 Sequence Detection System (PE Applied-Biosystems) using specific primers: sense 5'-ATGGCTTCTCT-GATATCCAG-3' and antisense 5'-TTAGTCAGCTTCAGTCTCGTC-3'. Relative gene expression was quantified, using β-actin as an internal standard.

Western Blot Analysis

A total of 2.0 × 10⁶ cells were harvested and rinsed three times with phosphate-buffered saline (PBS). Cell extracts were prepared with lysis buffer (1 mM dithiothreitol, 0.125 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL pepstatin,

1 µg/mL aprotinin, and 1% Triton X-100 in 12.5 mM Tris-HCl buffer, pH7.0) at 4°C for about 30 min. An equal volume of lysate was electrophoresed with SDS polyacrylamide gel electrophoresis (12%). The separated proteins in the gel were transferred to the nitrocellulose membrane. The membranes were blocked for one hour with Tris-buffered saline plus 0.1% Tween-20 (TTBS) containing 5% nonfat milk and then incubated for two hours with the appropriate primary antibodies [anti-*stathmin* (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-β-actin (Sigma, St Louis, MO, USA)] in TTBS containing 5% nonfat milk, followed by incubation for 45 min with horseradish peroxidase-conjugated antimouse immunoglobulin antibodies (Sigma). The immunocomplexes were visualized by enhanced chemoluminescence using an ECL™ kit (Amersham, Piscataway, NJ, USA), followed by exposure to x-ray films.

Cell Proliferation Assay

A total of approximately 6.0 × 10³ osteosarcoma cells in 100 µL of the medium were plated in 96-well plates and allowed to attach for 48 h, then treated daily for two days with indicated concentrations of siRNA. After 48 hours of incubation, the medium was replaced with 100 µL of 0.05% 3-[4,5-dimethyl-2-thiazolyl]-2, then 5-diphenyl-2H-tetrazolium bromide (5 mg/mL) was added to each well and the plate was incubated for four hours at 37 °C. After the incubation, the reaction was stopped by the addition of 150 µL/well of DMSO for 10 min. The absorbance was measured using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) with a test wavelength of 570 nm and a reference wavelength of 630 nm; all experiments were performed in triplicate. The cell proliferation curve was plotted using the absorbance at each time point.

Soft Agar Colony Assays

Forty-eight hours after siRNA transfection, the cells were mixed with cell cul-

ture medium containing 0.6% agar to a final concentration of 0.4%. We immediately plated 1 mL of this cell suspension in 6-well plates coated with 0.6% agar (1 mL per well) in cell culture medium. The colonies were counted in triplicate 15 d after plating, and the number of colonies per 10^3 cells was calculated.

Plasmid Constructions

The DNA oligonucleotides coding for the short hairpin short hairpin (sh)*stathmin* and a nonspecific shRNA negative control (shControl) were designed and synthesized as follows: *shstathmin*: (5'-GATCCGAAACGAGAGCACGAGAAA-TTCAAGAGATTTCTCGTGCTCTCGTTT-CAGA-3') and *shControl*: (5'-GATCCGCAAAGAGCG-AAAAGTTCAAGAGATTTT-CGCTCTTTT-GCAGA-3'). All these sequences were inserted between *Bam*HI and *Hind*III restriction sites of pSilencer4.1-CMVneo (pSC) vectors (Ambion, Austin, TX, USA). Name designation of the recombinant plasmid was pSC-*shstathmin* and pSC-shControl. The recombinant vectors were confirmed by the digestion analysis of restriction endonuclease and all inserted sequences were verified by DNA sequencing by TaKaRa Biotech Company using ABI PRISM SigDyeTM Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer, Waltham, MA, USA).

Stable Transfection of Plasmids and Selection

Human osteosarcoma cell lines (Saos-2 and MG63) were seeded in 6-well plates at 2.0×10^4 cells/well, respectively, and cultured overnight to about 90% confluence prior to transfection. Then, transfection was performed using Lipofectamine2000 transfection reagent (Invitrogen) following the manufacturer's specifications. Forty-eight hours after transfection, stable cell lines were selected with G418 (800 μ g/mL). Three stably transfected osteosarcoma cell lines (transfected with pSC-*shstathmin*, pSC-shControl, or pSC-CMVneo parental vector) were established.

Table 1. Thrice weekly in vivo chemotherapy regimen (n = 10/group).

| Group | Treatment |
|-------|--|
| 1. | Control-siRNA(100 μ g/kg) |
| 2. | <i>stathmin</i> -siRNA(100 μ g/kg) |
| 3. | Taxol (10 mg/kg) or Docetaxel (8.0 mg/kg) |
| 4. | Control-siRNA(100 μ g/kg) + Taxol (10 mg/kg) or Docetaxel (8.0mg/kg) |
| 5. | <i>stathmin</i> -siRNA(100 μ g/kg) + Taxol (10 mg/kg) or Docetaxel (8.0 mg/kg) |

Flow Cytometry Analysis of Cell Cycle and Apoptosis

The cells were harvested with trypsinization, fixed with cold 70% ethanol, and stored at 4°C until analyzed. The fixed cells were pelleted and resuspended in PBS containing 20 μ g/mL propidium iodide (PI) and RNase A and then analyzed for PI fluorescence intensity by flow cytometry to assess cellular DNA content. The relative proportions of cells in the G₁, S, and G₂/M phases of the cell cycle were determined from the flow cytometry data. The percentage of apoptotic cells was determined by the sub-G₁ proportion. Apoptosis of stable transfectants was also measured with an annexin V-fluorescein isothiocyanate apoptosis detection kit (Zymed; Invitrogen) that was used to detect the cell apoptosis of stable transfectants.

Tumor Formation Assay in Nude Mice

Mouse studies were performed according to institutional guidelines and a protocol improved by the animal research committee. The effect of *stathmin* downregulation on the tumorigenic capacity was assessed by subcutaneous injection of pSC-*shstathmin*, pSC-shControl, or pSC-neo parental vector-transfected and untransfected Saos-2 cells into athymic nude mice. Approximately 1.0×10^6 stably transfected cells were injected into eight-week-old female BALB/c nude mice, which were maintained under pathogen-free conditions. The inoculations were performed in 10 mice for one group. Tumor growth from days 7 to 28 after inoculation was monitored, and tumor diameters were measured with a caliper. Tumor volumes (mm^3) were calculated by the following formula: $V = 1/2 \times L^2 \times W$ (L, tumor length; W, tumor

width). At 28 d after inoculation, all mice were killed, and subcutaneous tumors were resected. Survival tests were made using groups of mice (n = 10) treated as above and monitored daily until all the mice died.

Chemosensitivity Assay

First, cell chemosensitivity in vitro to taxanes (Taxol and Docetaxel; Sigma) was evaluated by MTT analysis; then, 72 hours after transfection, the cells (Saos-2 and MG63) in the 96 wells were treated with various concentrations of Taxol (at 0, 5, 15, 20, and 25 nM) and Docetaxel (at 0, 3, 6, 9, 12, and 15 nM). Cell viability was detected 48 hours later, as described above, and the in vivo tumor sensitivity to taxanes was evaluated in the mouse model. Mice were implanted with 1.0×10^6 Saos-2 cells. Two weeks after implantation, subcutaneous tumors were formed, mice were allocated to 5 groups (10 mice/group), and mice in each group were treated as described in Table 1 (siRNA in 60 μ L PBS by tail vein injection and taxanes in 100 μ L PBS by intraperitoneal injection thrice weekly). After 10 weeks of treatment, all mice were killed, and subcutaneous tumors were resected. Tumor growth was evaluated by the average volume and the average weight of tumors. The tumor volume was calculated as already described and tumors were weighed. All the animal experiments were performed in accordance with institutional guidelines of the Fourth Military Medical University.

Statistical Analysis

All experiments were performed at least in triplicate and all statistical analyses were performed with SPSS10.0. Com-

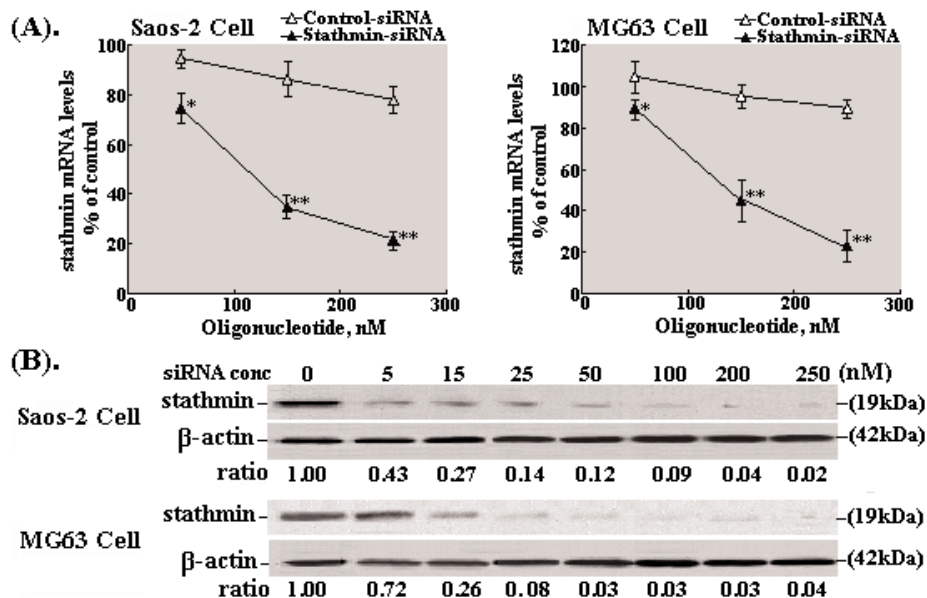


Figure 1. Specific downregulation of *stathmin* mRNA and protein expression by *stathmin*-siRNA. (A) Cells were transfected daily with 50, 150, or 250 nM Control-siRNA or *stathmin*-siRNA for 48 hours, and mRNA levels were quantified by real-time PCR analysis. Data were normalized by using β -actin as an internal standard. * $P < 0.05$ and ** $P < 0.01$ vs. Control-siRNA. (B) Cells were treated daily with various concentrations ranging from 0 to 250 nM for 48 hours. Cells were harvested, and *stathmin* protein levels were detected by Western blot analysis. β -Actin expression was monitored as the control. The ratio of *stathmin*/ β -actin was calculated by using densitometry, and values were normalized by dividing by the ratio at no treatment (0 nmol/L). * $P < 0.05$ vs. control-siRNA.

parisons among all groups were performed with the one-way analysis of variance (ANOVA) test and Student Newman Keuls method. Values of $P < 0.05$ were considered significant.

RESULTS

Downregulation of *Stathmin* Expression by Transient siRNA in Osteosarcoma Cells

We in vitro synthesized 21-mer oligonucleotide targeting *stathmin* (nucleotide 398-417) and transiently transfected two osteosarcoma cells (Saos-2 and MG63). Real-time RT-PCR (Figure 1A) and Western blot analyses (Figure 1B) were used to determine the effect of treatment with siRNA on *stathmin* expression at the mRNA and protein levels in osteosarcoma cell lines. As shown in Figure 1A, daily treatment of osteosarcoma cells with 50, 150, and 250 nM

stathmin-siRNA for two days reduced *stathmin* mRNA levels by 16.2%, 53.6%, or 64.3% in Saos-2 cells and by 15.0%, 50.2%, or 78.2% in MG63 cells. As shown in Figure 1B, *stathmin* protein levels were reduced in a dose-dependent manner, and partial to complete inhibitions were observed with increasing concentrations of the siRNA oligonucleotides.

Effects of *Stathmin*-siRNA on Cell Proliferation and Colony Formation In Vitro

To analyze phenotypic changes, we first investigated the effects of transient *stathmin*-siRNA on cellular proliferation of osteosarcoma cells. Cell proliferation was evaluated by MTT assay daily for various concentrations of *stathmin*-siRNA. As shown in Figure 2A, treatment of Saos-2 and MG63 cells with *stathmin*-siRNA resulted in a dose-dependent inhibition of the proliferation of two

osteosarcoma cells and reduced cell proliferation by 51.2% and 61.0% at 250 nM, respectively. Next, we explored whether *stathmin*-siRNA affected colony formation of osteosarcoma cells in soft agar assays. Cells were transfected with *stathmin*-siRNA or control-siRNA, and at 48 hours after transfection, the cells were plated into medium with soft agar. Colony formation was assayed at 15 days. As shown in Figure 2B, colony formation was significantly inhibited in the presence of *stathmin*-siRNA compared with that seen with untreated or Control-siRNA treatment. All these results showed that RNAi-mediated transient *stathmin* downregulation resulted in marked inhibition of osteosarcoma cell proliferation in vitro.

Effects of Vector-Mediated shRNA on *Stathmin* Expression and Cell Proliferation

First, we established stable transfectants of Saos-2 cells (transfected with pSC-sh*stathmin*, pSC-shControl, or pSC-neo parental vector). In those stable transfectants expressing sh*stathmin*, RT-PCR and Western blot analysis results showed that the levels of *stathmin* mRNA and protein expression were reduced by 64.7% and 75.5%, respectively (Figure 3A). We then tested the in vitro proliferation of these stably transfected cells by MTT assay daily for seven days. The cell proliferation curve showed that the stably transfected Saos-2 cells expressing sh*stathmin* showed incomplete inhibition but moderate proliferation retardation, and the highest inhibitory rate was $38.3 \pm 1.46\%$ on day seven ($P < 0.05$, Figure 3B).

Effects of RNA Interference Targeting *Stathmin* on Cell Cycle and Apoptosis

The proliferation inhibition of osteosarcoma cells by knockdown of *stathmin* expression was caused by disrupting the cell cycle and affecting microtubule assembly shown in other types of mammalian cells (16,17). To reveal the mechanisms underlying RNAi-mediated proliferation inhibition, we used flow cytometric analysis to detect changes in the

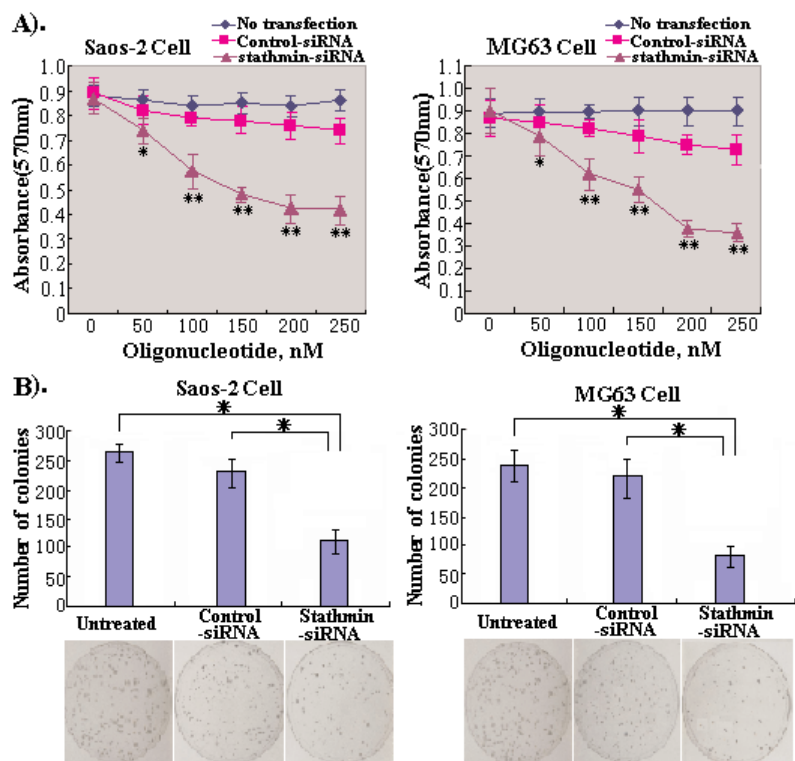


Figure 2. Effects of *stathmin*-siRNA on the cell proliferation and colony formation in vitro. (A) Cell proliferation assay. Human osteosarcoma cells (Saos-2 and MG63) were transfected with Control-siRNA or *stathmin*-siRNA (250 nM) for 24 h. Cell viability was evaluated by MTT assay. The data in each time point are averaged values from four replicates. These experiments were performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs. Control-siRNA. (B) Colony formation assay. Compared with that seen in the untransfected group, the number of colonies was significantly reduced in the *stathmin*-siRNA-transfected group but not in the Control-siRNA-transfected group. These experiments were performed in triplicate. * $P < 0.05$.

cell cycle and quantify apoptotic rates in osteosarcoma cells. We first analyzed the DNA contents of cell populations after transient transfection of *stathmin*-siRNA at the concentration of 250 nM into Saos-2 and MG63 cells. In both Saos-2 and MG63 cells 72 hours after *stathmin*-siRNA treatment, the population of G_2/M phase was significantly increased and the population of G_1/G_0 phase was obviously decreased ($P < 0.05$), and a marked increase in the sub- G_1 population was also detected (Figure 4, Table 2).

Next, an annexin V-fluorescein isothiocyanate apoptosis detection kit (Zymed) was used to detect cell apoptosis of stably transfected Saos-2 cells. Cell apoptosis analysis by flow cytometry showed

that compared with untransfected Saos-2 cells, the apoptosis rate of pSC-sh*stathmin* transfected Saos-2 cells significantly increased by $22.6\% \pm 2.1\%$ ($P < 0.05$, Figure 5), whereas there were no obvious changes among other stably transfected Saos-2 cells (transfected with pSC-shControl or pSC-neo vector). All above results suggested that RNAi-mediated transient or stable downregulation of *stathmin* expression in osteosarcoma cells could induce cell accumulation in the G_2/M phase and final apoptosis.

Inhibition of In Vivo Tumor Growth by *Stathmin* Downregulation

With the above findings of the effects of *stathmin*-siRNA in vitro, we next ex-

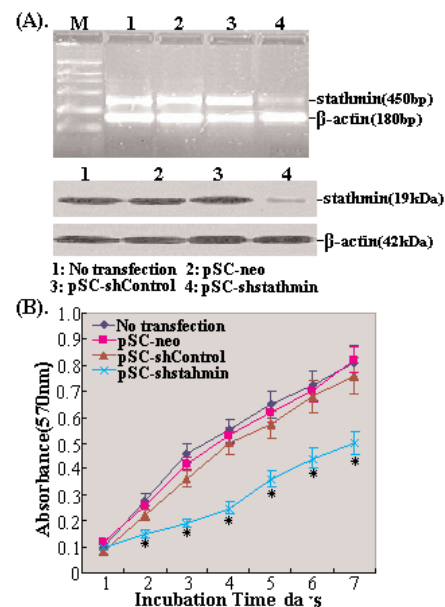


Figure 3. Vector-mediated *shstathmin* inhibited its expression and induced proliferation retardation. (A) The levels of *stathmin* mRNA and protein expression were detected in the stably transfected Saos-2 cells. Compared with untransfected cells, the levels of *stathmin* mRNA and protein expression in pSC-sh*stathmin* transfected Saos-2 cells were reduced by 64.7% and 75.5%, respectively ($P < 0.05$), but there were no obvious changes among other transfected Saos-2 cells ($P > 0.05$). (B) Stably transfected Saos-2 cell proliferation in vitro evaluated by MTT assay. These experiments were performed in triplicate. * $P < 0.05$ (significant differences among no transfection, pSC-neo, pSC-shControl, and pSC-sh*stathmin* transfectants).

plure whether *stathmin* plays a critical role in tumor formation in vivo, and whether it can be used in clinical gene therapy. We subcutaneously injected aliquots of 1.0×10^7 stably transfected Saos-2 cells into 4 groups of mice and monitored tumor growth. As shown in Figure 6A, the growth of tumors formed from the pSC-sh*stathmin* xenografts was significantly inhibited compared with tumors formed from other control xenografts. At 28 days after inoculation, the average tumor volume and tumor weight of the mice was decreased by 55.1% and 52.0% in pSC-sh*stathmin*

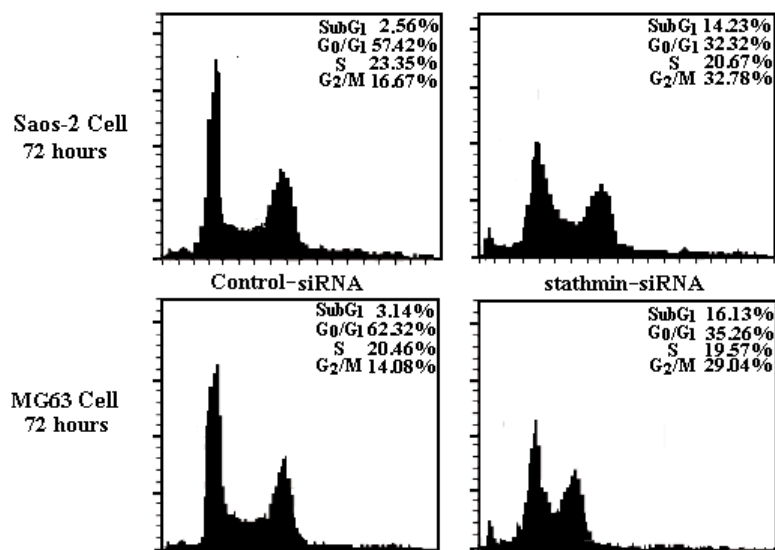


Figure 4. The changes of cell cycle detected by flow cytometry. Cells were harvested 72 hours after siRNA transfection at 250 nM and subsequently analyzed for their DNA content by flow cytometry. Representative results are shown. These experiments were performed in triplicate. **P* < 0.05.

xenografts compared with untreated xenografts. (*P* < 0.01, Figure 6B and Figure 6C). Figure 6D shows the survival time of the mice. *Stathmin* downregulation prolonged the lifespan of mice bearing Saos-2 tumor cells (*P* = .00352). These results indicated that RNAi-mediated *stathmin* downregulation exerted a strong growth-suppressive effect on osteosarcoma in vivo.

Stathmin-siRNA Synergistically Enhances Taxane-Induced Cytotoxicity In Vitro and In Vivo

Stathmin can disrupt the microtubule structure and destabilize microtubules through its phosphorylation. Taxanes, a family of chemotherapeutic agents, impair disassembly of microtubules that is essential for the normal segregation of chromosomes during mitosis of eukaryotic cells. Thus, we conclude that RNAi-mediated *stathmin* downregulation may synergistically enhance the cytotoxicity of taxanes, because taxanes can accumulate cells in the G₂/M phase where *stathmin* plays important roles. To determine whether *stathmin*-siRNA enhanced the

cytotoxicity of taxanes (Taxol and Docetaxel), osteosarcoma cells (Saos-2 and MG63) were treated with 100 nM Control-siRNA or *stathmin*-siRNA daily for three days, and incubated with indicated concentrations of each chemotherapeutic agent for two days. As shown in Figure 7, *stathmin*-siRNA significantly enhanced the chemosensitivity of these agents, decreasing the 50% inhibitory concentration of Taxol by 54.2 (or 47.3%) and Docetaxel by 78.5% (or 53.6%). Considering these findings in vitro, we next investigated the efficacy of *stathmin*-siRNA as an in vivo chemosensitizing strategy in a nude mouse xenograft model. After mice were treated with Control-siRNA and *stathmin*-

siRNA, alone and in combination with Taxol or Docetaxel for 10 weeks, the average tumor volume (mm³) and tumor mass (mg) were detected. The average tumor volume and average tumor mass of mice treated with Taxol or Docetaxel in combination with *stathmin*-siRNA were 87.5 mm³ and 64 mg, respectively. Compared with other groups of mice, the tumor growth in this group of mice treated with Taxol or Docetaxel in combination with *stathmin*-siRNA was significantly inhibited (*P* < 0.05, Figure 8). All above results showed that the downregulation of *stathmin* expression mediated by siRNA can synergistically enhance the chemosensitivity of human osteosarcoma cells to taxanes both in vivo and in vitro.

DISCUSSION

Stathmin (Op18), a cytosolic phosphoprotein, is the founding member of a family of microtubule-destabilizing proteins that regulate the dynamics of microtubule polymerization and depolymerization (20,21). *Stathmin* is overexpressed in various types of human cancers, including osteosarcoma, and its high expression levels could affect the distribution of cells throughout the cell cycle (22,23). To explore the possibility of *stathmin* as an effective therapeutic target, we employed an RNA interference technique to silence endogenous *stathmin* expression in osteosarcoma cells and analyzed phenotypic changes of transiently or stably transfected osteosarcoma cells. In our study, we achieved almost complete downregulation of *stathmin* expression by using an siRNA treatment strategy in osteosarcoma cell lines. Experimental data showed that transient *stathmin* downregulation led to

Table 2. Changes of population in cell cycle of transfected osteosarcoma cells.

| Cell phase | Saos-2 cell (72h after treatment) | | MG63 cell (72 h after treatment) | |
|--------------------------------|-----------------------------------|--------------------|----------------------------------|--------------------|
| | Control-siRNA (%) | stathmin-siRNA (%) | Control-siRNA (%) | stathmin-siRNA (%) |
| SubG ₁ | 2.56 ± 0.35 | *14.23 ± 0.88 | 3.14 ± 0.74 | *16.13 ± 1.35 |
| G ₀ /G ₁ | 57.42 ± 3.16 | *32.32 ± 2.03 | 62.32 ± 2.64 | *35.26 ± 2.83 |
| S | 23.35 ± 1.14 | 20.67 ± 1.55 | 20.46 ± 2.13 | 19.57 ± 1.46 |
| G ₂ /M | 16.67 ± 1.76 | *32.78 ± 2.43 | 14.08 ± 1.17 | *29.04 ± 2.23 |

* *P* < 0.05 compared with Control-siRNA.

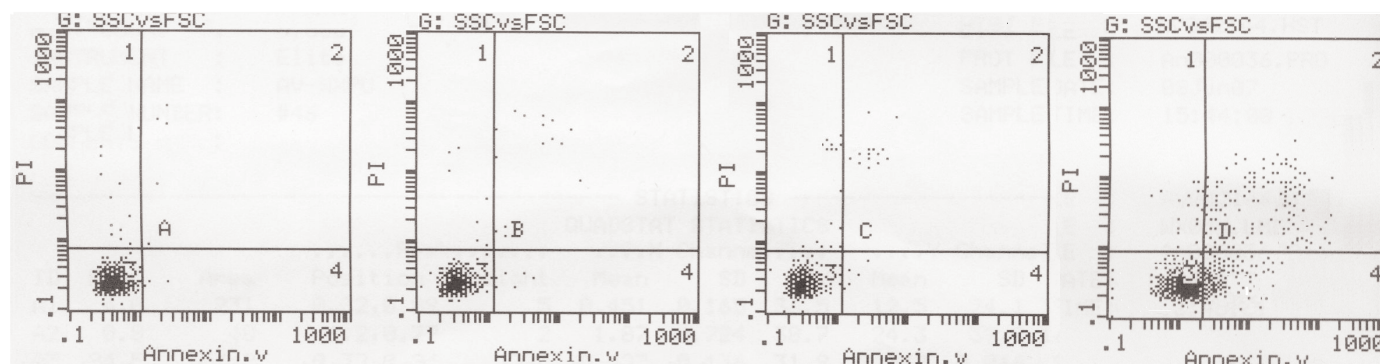


Figure 5. Cell apoptosis of stable transfectants detected by flow cytometry. The apoptotic rate of pSC-shstathmin transfected Saos-2 cells obviously increased by $22.6 \pm 2.1\%$ ($P < 0.05$), whereas there were no significant differences in cell apoptosis among untransfected, pSC-neo, or pSC-shControl transfected Saos-2 cells ($P > 0.05$).

significant inhibition of in vitro proliferation and colony formation, accumulation of G₂/M phase, and final apoptosis of osteosarcoma cells. We established stably transfected osteosarcoma cells expressing shstathmin and observed that stable stathmin downregulation in osteosarcoma cells induced incomplete proliferation inhibition but moderate proliferation retardation. We also detected increased apoptosis rates of pSC-shstathmin transfectants, and the stable transfectants almost completely suppressed tumorigenicity in a mouse xenograft model. All these results suggest that stathmin is an important molecule of proliferation of tumor cells and a potential target for suppressing proliferation and triggering apoptosis, which can be explained by its key roles in mitosis. Thus, we have reasons to believe that stathmin may provide an excellent molecular target for osteosarcoma therapy.

Taxanes, a family of chemotherapeutic drugs that exert antitumor effects by interacting with microtubules and interfering with their dynamic equilibrium, induce cell accumulation in the G₂/M phase of the cell cycle (24). The antimetabolic effects of taxanes are exerted by promoting microtubulins to stable microtubules and suppressing their breakdown by combining with free microtubules, which are essential for the segregation of chromosomes during mitosis of eukaryotic cells. This action interferes with the normal regulation of the mitotic spindle and leads to

mitotic arrest (25,26). After exposure to taxanes, some of the mitotically arrested cells undergo apoptosis, whereas others complete the division cycle, producing

aneuploid cells that undergo apoptosis during subsequent cell cycles (27). Although taxanes significantly inhibit proliferation and induce apoptosis of os-

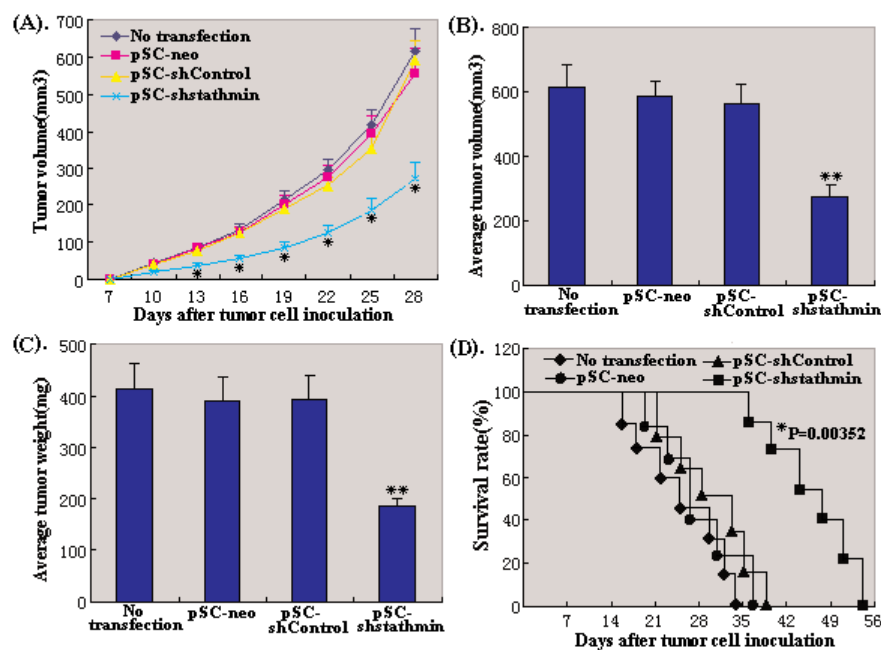


Figure 6. Effects of stathmin downregulation on tumor growth in vivo. (A) The growth in mice of tumors developed from untransfected and stably transfected Saos-2 cells (transfected with pSC-neo, pSC-shControl or pSC-shstathmin vector). The inoculation was performed in four groups ($n = 10$). (B) The average tumor volume at day 28 after inoculation of the above cells; $**P < 0.01$. (C) The average tumor weight at day 28 after inoculation of the above cells; $**P < 0.01$. (D) Survival curve of mice ($n = 10$) inoculated with untransfected and stably transfected Saos-2 cells (transfected with pSC-neo, pSC-shControl, or pSC-shstathmin vector). Results were evaluated by the Kaplan-Meier method. The differences between pSC-shstathmin treatment group and other control groups were statistically significant, $*P < 0.01$.

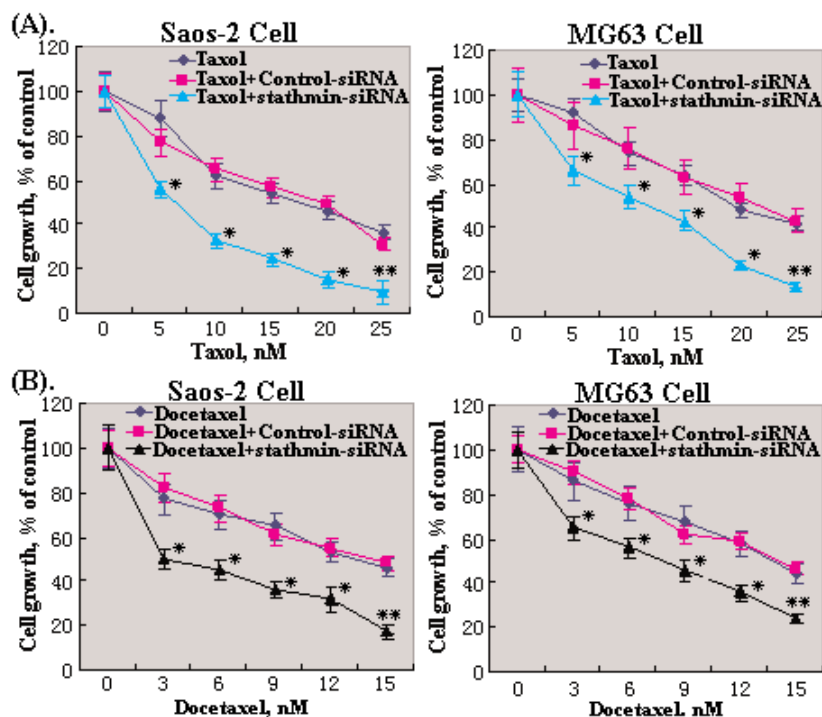


Figure 7. *Stathmin*-siRNA synergistically enhanced the cytotoxicity effect of taxanes in vitro. Saos-2 or MG63 Cells were treated daily with 100 nM *stathmin*-siRNA or Control-siRNA for three days, followed by the indicated concentrations of (A) Taxol and (B) Docetaxel. After 72 hours of incubation, cell growth was evaluated by MTT assay. **P* < 0.05 and ***P* < 0.01 vs. Control-siRNA.

teosarcoma cells in vitro (28-30), their clinical uses as single chemotherapeutic agents are very limited because of the toxic effects related to long-term administration at high doses. Thus strategies to target mitosis will help to enhance the cytotoxicity effects of taxanes while reducing toxicity (or side effects) caused by high doses.

In the present study, we observed that RNAi-mediated *stathmin* downregulation in combination with taxanes could synergistically enhance the cytotoxicity effects both in vitro and in vivo. Taxanes and *stathmin* downregulation both interfere with the regulation of microtubules that make up the mitotic spindle, so it is not surprising that the combined use of two interventions would induce a synergistic interaction. In fact, RNAi-mediated *stathmin* inhibition and taxane exposure target different steps in the same mitotic pathway, so the simultaneous effects of both of

these methods will increase tumor cell cytotoxicity. These results are consistent with other studies that showed synergistic inhibition of tumor cell proliferation associated with *stathmin* inhibition and taxol exposure (31,32). Much evidence has demonstrated that *stathmin* deficiency can decrease the rate of destruction and sequestration of tubulin molecules, thereby disrupting the equilibrium between polymerized and unpolymerized tubulin in favor of polymerized tubulin (33,34). Taxanes, on the other hand, make microtubules stable by binding to polymerized tubulin (35). Thus, once the *stathmin*-downregulated cells are exposed to taxanes, cells affected by *stathmin* deficiency will fail to depolymerize the microtubules, and the polymerized microtubules will be further stabilized by taxane binding. Therefore, the combine use of *stathmin* downregulation and taxanes will lead to stronger impairment of G₂/M phase progression and synergistic induction of apoptosis. This result may be attributable, at least in part, to the mechanism by which RNAi-mediated *stathmin* downregulation synergizes with taxane exposure to exert more potent antiproliferative and an-

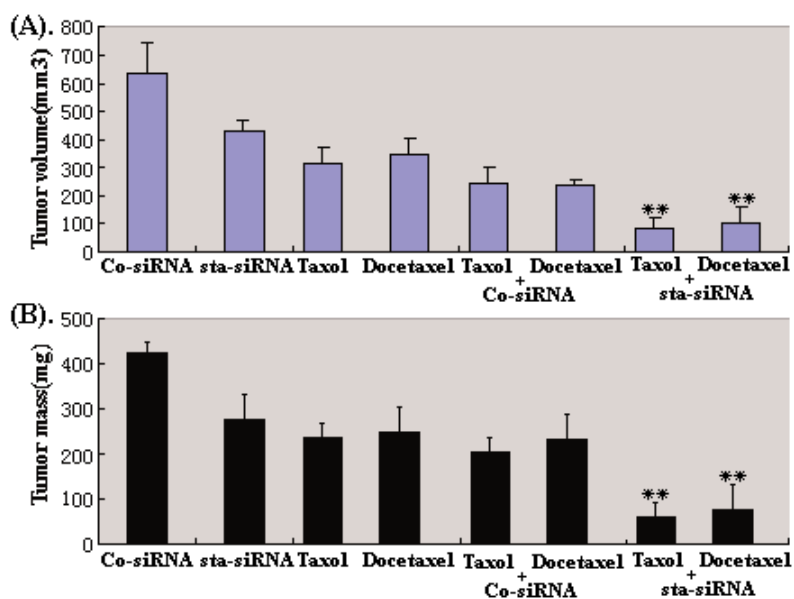


Figure 8. *Stathmin*-siRNA synergistically enhanced the cytotoxicity effect of taxanes in vivo. (A) Tumor mass, (B) tumor volume following treatment with Taxol or Docetaxel, alone and in combination with Control-siRNA or with *stathmin*-siRNA. ***P* < 0.01 vs. Control-siRNA.

titumor effects. These results are also consistent with our previous reports and those of others that the overexpression of *stathmin* increased resistance to chemotherapy by decreasing microtubule polymer mass (36,37). The exact molecular mechanism that accounts for the observed synergistic interaction between *stathmin* inhibition and taxanes appears complex and remains to be further clarified.

RNA interference is characterized by high efficiency, high specificity, and low toxicity (38,39). This novel technology is becoming a conventional application for in vivo cancer therapy (40-42). In our study, we presented a proof of principle for the use of novel RNA interference technology to target candidate oncogenes in osteosarcoma cells and thereby exert antitumor effects at multiple levels. RNAi-mediated *stathmin* downregulation effectively inhibited cell proliferation in vitro and tumorigenicity in vivo, induced cell accumulation in the G₂/M phase, led to apoptotic cell death, and enhanced chemosensitivity in human osteosarcoma cells. All these findings suggest that *stathmin* may be a pivotal determinant for tumorigenesis and chemosensitivity, so it is expected to be a potential therapeutic target for the treatment of osteosarcomas.

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