

Effects of *Dermatopontin* gene silencing on apoptosis and proliferation of osteosarcoma MG-63 cells

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Abstract. The present study aimed to investigate the effect of *Dermatopontin* (*DPT*) gene silencing on the apoptosis and proliferation of osteosarcoma MG-63 cells. Three eukaryotic expression vectors of short hairpin (sh)RNA fragments targeting different loci of *DPT* were designed and transfected into an osteosarcoma cell line MG-63. The cells were assigned to a blank, shRNA-control, *DPT*-shRNA-a, *DPT*-shRNA-b or *DPT*-shRNA-c group. The shRNA with the highest silencing efficiency was screened using reverse transcription-quantitative polymerase chain reaction and western blotting. The screened shRNA was transfected into MG-63 cells. The proliferation, cell cycle and apoptosis of MG-63 cells were measured using a Cell Counting Kit-8 assay, flow cytometry and Annexin V-fluorescein isothiocyanate assay. The recombinant plasmids containing *DPT* shRNA were successfully constructed. *DPT* gene silencing was able to significantly reduce the proliferation rate of MG-63 cells ($P<0.05$). The proportion of cells in the G₀/G₁ phase and in the G₂/M phase increased significantly (both $P<0.05$), while the proportion of cells in the S phase decreased ($P<0.05$). Furthermore, the cell apoptosis rate increased significantly ($P<0.05$). These results demonstrate that *DPT* gene silencing is able to reduce the proliferation of MG-63 cells, slow down cell cycle progression and promote apoptosis, hence may become a novel target for the treatment of osteosarcoma.

Introduction

Osteosarcoma is a relatively common primary bone malignancy and tends to occur in young people (1). The incidences of osteosarcoma in children and adolescents are high and

accounts for approximately 20% of all primary bone cancers; the incidence of osteosarcoma is around 0.2-3/100,000 per year, which is even higher in the age group of 15-19 years (0.8-11/100,000 per year) (2). Despite the fact that several improvements have been made in the curative protocols, osteosarcoma remains a devastating disease with poor early diagnosis as well as a low long-term survival rate (3), and the 5-year survival rate obtained by traditional chemotherapy in combination with surgical resection is still below 70% (4), mainly due to the resistance to chemotherapy and resulting failure of osteosarcoma treatment (5). Therefore, a deeper understanding of osteosarcoma development on the molecular level may pave the way for future therapy development. In addition, the process of tumorigenesis, i.e., a cumulative occurrence of gene mutations that influence the expression of both oncogenes and tumor suppressor genes, may also affect osteosarcoma development, where the osteoprogenitor cells acquire the ability of uncontrolled proliferation and bone formation (6). Therefore, using genome-wide sequencing, many studies have been carried out to identify genes responsible for the development of osteosarcoma (7-9).

Dermatopontin (*DPT*), initially found by purification of dercoin in calf skin, is an extracellular matrix (ECM) protein with a tyrosine residue and a molecular weight of 22 kDa, and can promote cell adhesion and ECM assembly by closely interacting with other ECM proteins (10). Since ECM degradation is an important prerequisite for tumor metastasis (11,12), *DPT* can affect tumor prognosis by regulating tumor invasion and metastasis (13,14). The expression of *DPT* is decreased in systemic sclerosis and hypertrophic scarring (15) as well as uterine leiomyomas and keloids (16) and may play an important role in wound healing (17). Recently, it has been shown that *DPT* can mediate *in vivo* prostate cell growth (18) and inhibit the metastasis of human oral cancer (13). Unfortunately, the *DPT* expression in osteosarcoma and its functional characteristics have not yet been thoroughly studied. Therefore, in this paper, we will analyze the effect of *DPT* during apoptosis and proliferation of osteosarcoma MG-63 cells, thus providing a potential therapeutic target and new ideas for chemotherapy and gene therapy of osteosarcoma.

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Materials and methods

Cell culture and treatment. The osteosarcoma MG-63 cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in 1640 media supplemented with 10% inactivated fetal calf serum plus 100 units/ml penicillin and 100 mg/ml streptomycin (all from Gibco, Thermo Fisher, Waltham, MA, USA) at 37°C and 5% CO₂. When the cells grew to 80% confluency, they were digested using 0.25% trypsin and passaged.

Establishment of DPT gene silencing model. According to the design principle of shRNA, the sequence of the DPT gene was obtained from GenBank (NM_001937.4), and RNA interference sites provided by Invitrogen on-line (Thermo Fisher) were used to design 3 interference sequences, i.e., shRNA-a, shRNA-b and shRNA-c. Blast software was used for comparison on the human genome, and the sequence of DPT gene was identified as unique. In the same way, a non-specific shRNA-control was designed as a negative sequence without any matching sequences in the human genome. Four shRNA sequences were synthesized by Sangon (Shanghai, China): ShRNA-control: 5'-ACTACCGTTGTTATAGGTG-3'; DPT-shRNA-a: 5'-TGGCGGGAAGTGTGAAGCG-3'; DPT-shRNA-b: 5'-CATCAACGAGTGCTCCAGC-3'; DPT-shRNA-c: 5'-GGTGTCTTCCAGATCCTGA-3'. BamHI and HindIII restriction enzyme cutting sites (TaKaRa Bio, Tokyo, Japan) were added to both ends of the sequences, and each designed oligonucleotide sequence was inserted into a pSIREN-RetroQ-TetH vector (TaKaRa Bio). The recombinant plasmids were amplified by Escherichia coli (*E. coli*) DH5α (Tiangen, Beijing, China) and cultured overnight in LB medium containing Ampicillin. The plasmids were extracted using a plasmid miniprep kit (Beyotime, Shanghai, China) and were sequenced by Invitrogen (Invitrogen, Thermo Fisher, Waltham, MA, USA) to check whether the base sequences of the shRNA fragments were correct.

Cell grouping and transfection. In the experiment, the cells were assigned to blank group, shRNA-control group, DPT-shRNA-a group, DPT-shRNA-b group and DPT-shRNA-c group. The blank group received no treatment. The shRNA-control group was transfected with shRNA-control plasmids. DPT-shRNA-a, DPT-shRNA-b and DPT-shRNA-c groups were transfected with DPT-shRNA-a, DPT-shRNA-b and DPT-shRNA-c plasmids, respectively. According to the instructions of Eugene transfection reagent (Invitrogen, Thermo Fisher), the MG-63 cells were seeded on 6-well plates the day prior to cell transfection and routinely incubated. The culture media were changed at 2 h before the transfection, followed by adding 2 ml of 1640 culture media into each well. For each well, 8 μl Eugene reagent and 3.2 μg of plasmids were mixed and incubated for 20 min at room temperature. The 1640 culture media in each well were aspirated and 200 μl of Opti-MEN media were added into each well, followed by 800 μl of Eugene/plasmid mixture. The plates were incubated at 37°C for 5 h. After the transfection, 2 ml of 1640 media were added into each well and the plates were incubated at 37°C. After 24, 36, 48 and 72 h, the transfection efficiency was observed by fluorescence microscopy.

Table I. Effects of DPT gene silencing on cell cycle of osteosarcoma MG-63 cells, as determined by flow cytometry.

Group	G0/G1	S	G2/M
Blank	61.8±3.1	26.3±0.7	11.9±3.6
shRNA-Control	62.3± 1.0	26.8±0.5	10.9±1.5
DPT-shRNA-a	68.7±3.8 ^a	14.2±0.2 ^a	17.1±3.9 ^a

^aP<0.05 compared with the blank group. shRNA, short hairpin RNA.

Quantitative real time polymerase chain reaction (qRT-PCR). After 36 h of transient shRNA transfection, the cells were collected in a 15 ml centrifuge tube and the total RNA of osteosarcoma MG-63 cells was extracted according to the instruction of a RNA extraction kit (Promega Corp., Madison, WI, USA). The optic density (OD) 260/280 ratio of the extracted RNA samples was analyzed by an ultraviolet spectrophotometer, and the RNA concentration was calculated. The RNA samples were then stored at -80°C for future use. PCR primers were designed using the Primer6.0 primer design software, according to the gene sequences in the Genbank database: β-actin: Forward (5'-3') TGGCATTGCCGACAG GATGCAGCAA; reverse (5'-3') CTCCTCATACTCCTG CTTGCTGAT; DPT: Forward (5'-3') GGTGGCTACGGG TACCCATA; reverse (5'-3') GTCAGAGCCTTCCTTCTTGC. The primers were synthesized by Sangon (Shanghai, China). Reverse transcription (RT) was performed according to the protocol provided with the RNA Reverse Transcription Kit (Promega Corp.). PCR was carried out in a two-step reaction: Pre-denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 30 sec. The qRT-PCR reaction system consisted of 12.5 μl of SYBR Green Mix, 1 μl of forward primer, 1 μl of reverse primer, 2 μl of DNA template, and 8.5 μl of ddH₂O (Promega Corp.). β-actin was used as an internal reference. The reliability of the PCR was evaluated by the dissolution curve and the cycle quantification (Cq) value was obtained. The relative expression of the target gene was calculated according to 2^{-ΔΔCq}, and each measurement was repeated three times.

Western blot analysis. The protein in each cell group was extracted at 36 h after transient shRNA transfection. The protein concentration was determined by according to the manual of a BCA kit (Boster, Wuhan, Hubei, China). The extracted protein was added to the loading buffer and then boiled at 95°C for 5 min. A total of 30 μg samples were added into each well of a 10% polyacrylamide gel (Boster, Wuhan, Hubei, China), and the protein was then separated by electrophoresis. The electrophoresis voltage was 80 V changed to 120 V with wet transfer. The membrane transfer voltage was 100 mV, and the duration was 45-70 min. After transfer using a polyvinylidene fluoride (PVDF) membrane, the membrane was mounted in 5% bovine serum albumin (BSA) for 1 h at room temperature, followed by addition of primary anti-DPT (1:1,000, Abcam, Cambridge, UK) antibody and incubation at 4°C overnight. TBST solution was used to rinse the membrane (3 times of 5 min each), and the membrane

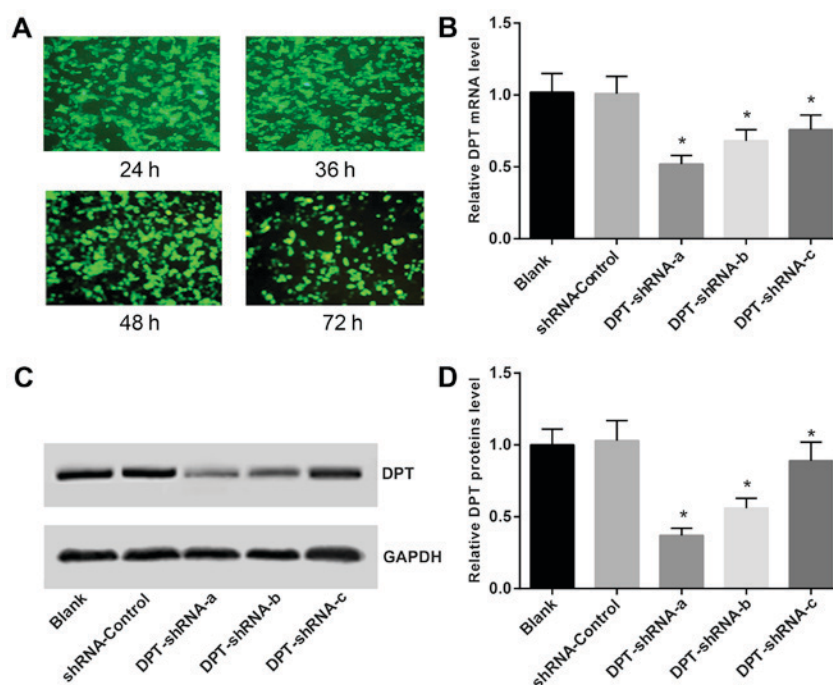


Figure 1. Comparisons of changes of *DPT* expression in MG-63 cells transfected with DPT-shRNA plasmids. (A) transfection efficiency in MG-63 cells transfected with DPT-shRNA plasmids; (B), the expression of *DPT* mRNA in MG-63 cells at 36 h after transfection with DPT-shRNA plasmid; (C), The expression of *DPT* protein in MG-63 cells at 36 h after transfection with DPT-shRNA plasmid; (D), the grey values of *DPT* protein in different groups of MG-63 cells were measured. * $P < 0.05$ compared with the blank group and the shRNA-control group; DPT, Dermatopontin.

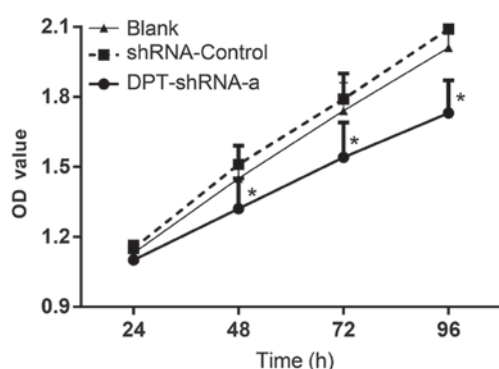


Figure 2. The inhibitory effect of *DPT* gene silencing on the proliferation of osteosarcoma MG-63 cells. * $P < 0.05$ compared with the blank group and the shRNA-control group; DPT, Dermatopontin.

was incubated with the corresponding secondary antibodies at room temperature for 1 h. After additional membrane washing (3 times of 5 min each), chemiluminescence reagent was added and the membrane was developed. β -actin was used as the internal reference (1:5,000, Kangchen, Shanghai, China). Each measurement was repeated 3 times. A Bio-rad Gel DOC EZ imager (Bio-Rad, Hercules, CA, USA) was used to image the membrane. The gray value analysis of the target band was conducted using the Image J software.

Cell Counting Kit-8 (CCK-8) assay. After the normal MG-63 cells and the cells transfected with DPT-shRNA-a and shRNA-control reached the phase of logarithmic growth, they were collected and seeded into 96-well plates at a concentration of 10^3 cells/well. In each well, 200 μ l of cell culture

media were added. Three duplicated wells were set up for each cell group, and the experiment was repeated 3 times. After culturing for 24, 48, 72, and 96 h, 20 μ l of CCK-8 reagent were added into each well of the 96-well plates, which were returned to an incubator for cell culture. Subsequently, the cells were removed from the incubator 4 h later and the absorbance (A) value at 490 nm was measured with a microplate reader. The cell growth curve was plotted using time as the X-axis and the value of A490 nm as the Y-axis.

Flow cytometry. Cells were harvested after 36 h of transfection and washed once with 1x PBS, and then resuspended in PBS containing 75% ethanol and 0.5 mmol/l EDTA. After fixing for 1 h at 4°C, the cells were centrifuged at 2000 rpm for 5 min, and the supernatant was discarded. The cells were washed once with 1x PBS and resuspended in 500 μ l of PBS containing 0.1% Triton X-100 and 50 μ g/ml RNase, and 90 μ l of 0.5 mg/ml propidium iodide (PI) were added into the suspension rapidly. The suspension was then mixed well using a pipette, and placed in the dark for 30 min of reaction at room temperature. The solution was then filtered with a nylon membrane and analyzed by an EPICS XL-4 flow cytometer (Beckman Coulter, Brea, CA, USA). Each measurement was repeated 3 times.

Annexin V-FITC was used to detect apoptosis. After 36 h of transfection, the cells were taken out from the incubator, washed twice with PBS, digested with 0.25% trypsin and collected by centrifugation at 1,000 rpm for 10 min. The cells were then washed 3 times with PBS and resuspended. The number of cells was counted and the cell concentration was adjusted to 5×10^5 cells/ml. A volume of 5 μ l Annexin V-FITC

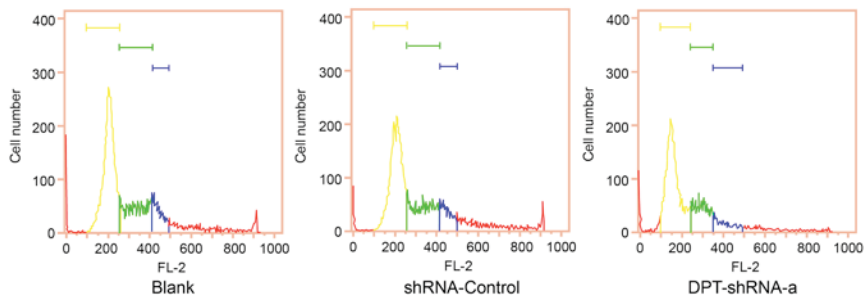


Figure 3. Effects of *DPT* gene silencing on cell cycle of osteosarcoma MG-63 cells, as determined by flow cytometry. *DPT*, Dermatopontin.

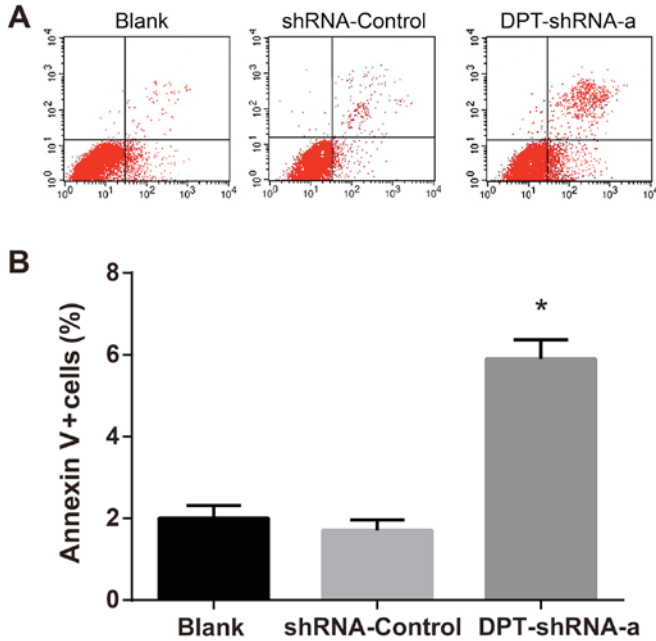


Figure 4. Effects of *DPT* gene silencing on apoptosis of osteosarcoma MG-63 cells. (A) The flow cytometry analysis; (B) the comparison of apoptotic rate. * $P < 0.05$ compared with the blank group and the shRNA-control group; *DPT*, Dermatopontin.

was added into 100 μ l cells, gently mixed, and incubated in the dark at room temperature for 10 min. The suspension was then centrifuged at 1000 rpm for 5 min and the supernatant was discarded. A volume of 10 μ l PI staining solution was added and gently mixed before analyzing the cells by flow cytometry. The percentage of Annexin V+/PI- cells in the total cell number was used as the apoptosis rate, and each measurement was repeated 3 times.

Statistical analysis. The data were analyzed by SPSS20.0 statistical software. The measurement data were expressed as mean \pm standard deviation (SD). The differences among multiple groups were analyzed by analysis of variance (ANOVA). The difference between two groups was verified using t test. A P -value of < 0.05 was considered statistically significant.

Results

***DPT* gene silencing in osteosarcoma MG-63 cells.** After transfection with *DPT*-shRNA, the percentage of MG-63 cells with green fluorescence was 91, 97, 92 and 87%, respectively,

at 24, 36, 48 and 72 h post transfection. At 36 h after transfection, the proportion of green fluorescent cells reached the highest level (Fig. 1A). The expression of *DPT* was detected by qRT-PCR. The results showed that the expression of *DPT* in *DPT*-shRNA-transfected cells was significantly lower than that in the shRNA-control group ($P < 0.05$), while the expression of *DPT* in the shRNA-control group was similar to that in the blank group ($P > 0.05$). This indicates that the *DPT*-shRNA recombinant plasmids can significantly inhibit the expression of *DPT* gene, whose expression was the lowest in the *DPT*-shRNA-a group (Fig. 1B). Western blotting showed that the expression of *DPT* protein in cells transfected with *DPT*-shRNA-a, b and c was significantly attenuated compared to that in the shRNA-control group (all $P < 0.05$), especially for the *DPT*-shRNA-a group. There was no significant difference between the *DPT*-shRNA-c group and the blank group (Figs. 1C and 1D). Therefore, *DPT*-shRNA-a recombinant plasmid was selected for subsequent experiments.

Effects of *DPT* gene silencing on the proliferation of MG-63 cells. MG-63 cells were transfected with *DPT*-shRNA-a plasmid and the cell proliferation was detected by the CCK-8 assay. The OD values of cells in the blank group were 1.13 ± 0.04 , 1.45 ± 0.07 , 1.74 ± 0.12 and 2.01 ± 0.18 , respectively, at 24, 48, 72 and 96 h post transfection. The OD values of cells in the shRNA-control group were 1.15 ± 0.03 , 1.51 ± 0.08 , 1.79 ± 0.11 and 2.09 ± 0.17 , respectively, at 24, 48, 72 and 96 h post transfection. The OD values of cells in the *DPT*-shRNA-a group were 1.10 ± 0.05 , 1.32 ± 0.13 , 1.54 ± 0.15 and 1.73 ± 0.14 , respectively, at 24, 48, 72 and 96 h post transfection. There were significant differences between cells in the *DPT*-shRNA-a group and cells in the shRNA-control, the blank groups at 48, 72 and 96 h (all $P < 0.05$), while the OD values in the shRNA-control and blank groups were not significantly different ($P > 0.05$) (Fig. 2). These results showed that *DPT* silencing could significantly inhibit the proliferation of MG-63 cells.

Effects of *DPT* gene silencing on cell cycle of MG-63 cells. Flow cytometry analysis showed that the proportion of cells at G0/G1 and G2/M phases in the *DPT*-shRNA-a group was significantly higher than that in the blank group (both $P < 0.05$), while the proportion of cells at S phase in the *DPT*-shRNA-a group was significantly lower than that in the blank group ($P < 0.05$), indicating that DNA replication in *DPT*-shRNA-a cells decreased and their cell cycle progression was slowed down. In addition, there was no significant difference between

the shRNA-control group and the blank group ($P>0.05$) (Fig. 3, Table I).

Effects of *DPT* gene silencing on apoptosis of MG-63 cells. After double staining with Annexin V and PI, flow cytometry measurement was carried out to detect the percentage of Annexin V+/PI- cells, which was used as the apoptotic rate and was compared among different groups. The results revealed that the apoptotic rate in the DPT-shRNA-a group was significantly higher than that in the blank group and the shRNA-control group (both $P<0.05$), but there was no significant difference between the shRNA-control group and the blank group ($P>0.05$) (Fig. 4).

Discussion

It has been shown that current treatment of osteosarcoma by surgical operation and adjuvant chemotherapy cannot achieve satisfactory outcomes (5,19,20). Multiple examples are given to demonstrate the potential positive role of gene therapy in the treatment of osteosarcoma (7,21,22), indicating that the search of osteosarcoma target on genetic level may be of great significance for a better treatment of osteosarcoma. In this study, the effect of *DPT* gene silencing on the apoptosis and proliferation of osteosarcoma MG-63 cells was studied, and the results may provide some insights for the development of new *DPT*-targeting inhibitors with clinical efficacy in the treatment of osteosarcoma.

Initially, in this study, *DPT* gene silencing was found to reduce the proliferation of MG-63 cells. *DPT* is an acidic protein that promotes the formation of collagen fibril and reduces the diameter of newly formed collagen fibril (23,24). It is reported that *DPT* in mammals may promote the adhesion of fibroblasts through an integrated receptor channel function as well as accelerating the synthesis of collagen fibrils (25). *DPT* binds to decorin, a small leucine-rich proteoglycan, which can be involved in tumor stroma formation, normal tissue development and differentiation (26). Besides, Decorin may mediate the function of proteins involved in extracellular-matrix formation (27,28). Study conducted by Catherino *et al* also suggested that altered *DPT* expression could interfere with decorin activity and cause abnormal extracellular-matrix formation (16). Furthermore, RNA interference, a well-known technique for silencing gene expression, can cause the inhibition of proliferation of tumor cells (29). We can therefore speculate that *DPT* gene silencing could reduce the proliferation of MG-63 cells. The further mechanism should be further investigated.

Besides, in this study, it was also shown that *DPT* gene silencing can slow down the cell cycle progression and promote apoptosis. The ability of cell adhesion and invasion is the key to cancer metastasis, and as a proteoglycan binding to other proteins, *DPT* can enhance the adhesion between fibroblasts and keratinocytes by accelerating the formation of collagen and fibronectin fibrils as well as by promoting the cell-matrix interaction (13,30). When *DPT* is active, it can improve wound healing by altering the extracellular environment; whereas when *DPT* is not active, it cannot induce early osteogenesis (30-32), suggesting that cell regeneration is relatively slow when *DPT* is inactive. *DPT* is a downstream target of vitamin

D receptor during the differentiation of pluripotent stromal cells into osteoblasts and may function as growth factors, i.e., *DPT* produced by tumor cells may up-regulate growth factor expression in stromal cells (18). Therefore, it can be deduced that *DPT* silencing will reduce the expression of cell growth factors, thereby slowing the cell cycle progression. Apoptosis is an important mechanism to eliminate malignant cells and cancer cells can block the apoptotic pathway, thus avoiding apoptosis (33). Takeuchi *et al* (18) showed that *DPT* receptors are located in the tumor matrix of the rats and will promote the occurrence and progression of certain cancers, suggesting that *DPT* is not conducive to apoptosis. Therefore, it can be postulated that *DPT* silencing may reinstate the apoptosis mechanism and promote cell death.

In conclusion, shRNA plasmids that can effectively silence the expression of *DPT* gene were successfully constructed in our study. The results showed that *DPT* gene silencing could effectively reduce the proliferation of MG-63 cells, slow down the cell cycle progression and promote cell apoptosis. This provides some insights for the development of new inhibitors targeting *DPT* in the treatment of osteosarcoma. However, the discovery reported in this paper is still preliminary. Therefore, further studies are required to investigate the potential application of relevant drugs as well as their role in the prognosis of osteosarcoma.

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