

# Downregulation of 14-3-3 $\beta$ inhibits proliferation and migration in osteosarcoma cells

QUANMING WU, JIANWEI ZHU, FAN LIU, JIN LIU and MINGPENG LI

Department of Orthopedics, The Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, P.R. China

Received February 7, 2016; Accepted February 9, 2017

DOI: 10.3892/mmr.2017.8144

**Abstract.** The 14-3-3 protein isoform  $\beta$  (14-3-3 $\beta$ ), which is an intracellular adaptor protein that exists in all eukaryotic organisms, is highly expressed in many cancer tissues, including glioma, lung carcinoma and breast cancer. However, 14-3-3 $\beta$  expression and function in osteosarcoma progression remain unknown. In the present study, the endogenous expression of 14-3-3 $\beta$  was assessed in osteosarcoma samples and the effect of 14-3-3 $\beta$  knockdown was examined in human osteosarcoma MG63 cells using small interfering RNA (siRNA). mRNA and protein expression levels for 14-3-3 $\beta$  were detected by reverse transcription-quantitative polymerase reaction and western blotting, respectively. The results demonstrated that endogenous 14-3-3 $\beta$  mRNA and protein were highly expressed in human osteosarcoma tissues and osteosarcoma cell lines (U2OS, MG63 and SaOs-2), but not in normal bone tissues or normal osteoblast hFOB1.19 cells. These data suggested that increased expression of 14-3-3 $\beta$  may be significantly associated with the development and progression of osteosarcoma. Therefore, the effect of 14-3-3 $\beta$  knockdown in MG63 cells was further examined *in vitro*. Knockdown of 14-3-3 $\beta$  by siRNA significantly decreased cell viability, and inhibited cell proliferation and invasion. In addition, 14-3-3 $\beta$  knockdown significantly decreased the protein expression levels of  $\beta$ -catenin, cyclin D1, v-myc avian myelocytomatosis viral oncogene homolog and matrix metalloproteinase 9 in osteosarcoma MG63 cells. These results suggested that the anticancer effects of 14-3-3 $\beta$  knockdown in MG63 cells might be mediated by the inhibition of the Wnt/ $\beta$ -catenin signaling pathway. In summary, 14-3-3 $\beta$  knockdown decreased proliferation and invasion in MG63 cells, which suggests a potential therapeutic application for 14-3-3 $\beta$  as a novel target for the treatment of osteosarcoma patients.

## Introduction

Osteosarcoma is a frequently observed primary malignant tumor that most commonly affects children, adolescents and young adults (1). Current therapy for osteosarcoma consists of comprehensive treatment. In ~20% of patients, osteosarcoma is resistant to available therapies, leading to recurrence and lung metastases (1-3). Treatment of metastatic osteosarcoma remains a challenge in oncology. Therefore, a better understanding of the pathogenesis and biology of osteosarcoma may provide a rational basis for improving treatment efficacy, particularly for metastatic disease (4).

The 14-3-3 proteins are a group of intracellular proteins that exists in all eukaryotic organisms. The 14-3-3 protein family has seven isoforms,  $\beta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\theta$ ,  $\gamma$  and  $\sigma$ , which serve as scaffolds to interact with various proteins, including transcription factors, signaling molecules, tumor suppressors, cytoskeletal proteins and apoptosis factors (5). Interaction with 14-3-3 can alter the localization, activity, stability, phosphorylation state and conformation of target proteins (6). More than 200 14-3-3 target proteins have been identified, including proteins involved in cell apoptosis, cell cycle progression, signal transduction, differentiation, senescence and DNA replication (7,8). Previous studies have indicated that abnormal expression of 14-3-3 is associated with the development and progression of various tumors (9). Each 14-3-3 isoform has distinct tissue localization and isoform-specific functions (10). The majority of 14-3-3 proteins are tumor promoters, whereas 14-3-3 $\sigma$  serves as a tumor suppressor (11). Loss of 14-3-3 $\sigma$  expression has been observed in several types of human cancers, including lung cancer, breast cancer and prostate cancer (12). Expression of 14-3-3 $\sigma$  is significantly downregulated in cancerous lung tissues and is associated with the differentiation grade and prognosis of the patient, whereas increased 14-3-3 $\sigma$  expression significantly suppresses the proliferation of lung squamous cell carcinoma cells (13). 14-3-3 $\gamma$  is frequently upregulated in lung cancer, accompanied by loss of functional p53, which suggests that the oncogenic activities of 14-3-3 $\gamma$  act synergistically with the loss of p53 to promote lung tumorigenesis (14). Mulvey *et al* demonstrated that 14-3-3 $\zeta$  is overexpressed in colorectal cancer, and 14-3-3 $\zeta$  knockdown reduces anchorage-independent growth of colorectal cancer cells (15), suggesting that 14-3-3 $\zeta$  may be a putative drug target for the treatment of colorectal cancer. 14-3-3 $\theta$  expression is markedly higher in breast cancer, and elevated 14-3-3 $\theta$  is correlated with poor prognosis (16).

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*Correspondence to:* Dr Jianwei Zhu, Department of Orthopedics, The Affiliated Hospital of Nantong University, 20 Xishi Road, Nantong, Jiangsu 226001, P.R. China  
E-mail: zhujianwei\_nt@163.com

**Key words:** osteosarcoma, 14-3-3 isoform  $\beta$ , proliferation, invasion

Additionally, 14-3-3 $\theta$  knockdown inhibits the growth and metastasis of breast cancer, indicating that 14-3-3 $\theta$  may serve as a candidate prognostic biomarker and target for new therapies in metastatic breast cancer (16). Accumulating evidence suggests that 14-3-3 $\beta$  is important in tumorigenesis and tumor progression. For example, 14-3-3 $\beta$  is expressed abundantly in a majority of primary tumors, and elevated 14-3-3 $\beta$  is associated with subsequent extrahepatic metastasis and decreased survival rates in patients with hepatocellular carcinoma (17,18). 14-3-3 $\beta$  is overexpressed in astrocytoma, and knockdown of 14-3-3 $\beta$  inhibits the proliferation of U87 human glioblastoma cells (19). Overexpression of 14-3-3 $\beta$  in NIH 3T3 fibroblast cells stimulates cell growth and promotes tumor formation in nude mice (20). High cytoplasmic levels of 14-3-3 $\beta$  independently correlate with poor disease-specific survival in vulvar squamous cell carcinoma (21). These data suggest a crucial role of 14-3-3 $\beta$  in the abnormal growth of tumor cells, and new therapeutic strategies or drugs aimed at 14-3-3 $\beta$  may have potential for the treatment of cancer. Nevertheless, there is a paucity of information regarding 14-3-3 $\beta$  expression and its exact role in osteosarcoma progression.

The present study demonstrated that 14-3-3 $\beta$  was highly expressed in human osteosarcoma tissues and osteosarcoma cell lines. These data indicated that increased 14-3-3 $\beta$  expression may be associated with the development and progression of osteosarcoma, and suggested that 14-3-3 $\beta$  may be a novel target in developing therapeutic applications for the treatment of osteosarcoma patients.

## Materials and methods

**Specimens.** The current study was reviewed and approved by the Clinical Research Ethics Committee of The Affiliated Hospital of Nantong University (Nantong, China). Fresh osteosarcoma tissues and matched normal tumor-adjacent tissues were collected from 16 patients (11-27 years-old, male 10, female 6) that underwent resection surgery between January 2009 and June 2015 at The Affiliated Hospital of Nantong University. All tissue specimens were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent experiments.

**Reagents.** All cell culture reagents were from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Human osteoblastic cell line (hFOB1.19) and osteosarcoma cell lines (U2OS, Saos-2 and MG63) were obtained from the American Type Culture Collection (Manassas, VA, USA). Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA, USA). The iScript cDNA Synthesis kit and the iTaq Fast SYBR Green Supermix were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Transwell invasion chambers were purchased from Costar (Thermo Fisher Scientific, Inc.). The protein extraction kit, propidium iodide (PI), Cell Counting kit-8 (CCK-8) and RNase A were purchased from Beyotime Institute of Biotechnology (Haimen, China). The enhanced chemiluminescence (ECL) kit was from Pierce (Thermo Fisher Scientific, Inc.). The primers were as follows: 14-3-3 $\beta$  (GenBank accession no. GI:197692220), forward 5'-atgggcaaa gactaccgtga-3', reverse 5'-tgtgtctccagatgccact-3'; and GAPDH (GenBank accession no. GI:182976), forward 5'-aggtcggagtcacggattt-3', reverse 5'-atctcgtcctctggaagatgg-3'. Primers were

synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). 14-3-3 $\beta$  small interfering (si)RNA (5'-AUUGGAUAC GCUGAAUGAAGA-3') and negative control siRNA (cat. no. siN05815122147-1-5) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Rabbit anti-14-3-3 $\beta$  (cat. no. ab97273) and anti- $\beta$ -catenin (cat. no. ab23512) polyclonal antibodies were purchased from Abcam (Cambridge, UK). Mouse anti-cyclin D1 (cat. no. 554180) monoclonal antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit anti- $\beta$ -actin (cat. no. 253613) and anti-v-myc avian myelocytomatosis viral oncogene homolog (c-myc; cat. no. 251334) polyclonal antibodies were purchased from Abbiotec, LLC (San Diego, CA, USA). Rabbit anti-matrix metalloproteinase 9 (MMP9; cat. no. ABIN1873732) polyclonal antibody was purchased from Abnova (Taipei, Taiwan). Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. 31460) and goat anti-mouse (cat. no. 31430) immunoglobulin (Ig)G polyclonal antibodies were purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

**Cell culture and transfection.** Human osteosarcoma U2OS, Saos-2 and MG63 cells were maintained in RPMI-1640 medium (cat. no. 11875127; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; cat. no. 10099141; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine and 100  $\mu\text{g}/\text{ml}$  penicillin/streptomycin at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator. Human osteoblast-like hFOB1.19 cells were cultured in Dulbecco's Modified Eagle's Medium (cat. no. 11965118; Thermo Fisher Scientific, Inc.)/Ham's F-12 nutrient mixture (cat. no. 11765054; v/v: 1:1; Thermo Fisher Scientific, Inc.) containing 10% FBS and Geneticin (cat. no. 10131027; 400  $\mu\text{g}/\text{ml}$ ; Thermo Fisher Scientific, Inc.) at  $34^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator.

Among the human osteosarcoma cell lines, MG63 cells were selected for siRNA experiments. MG63 cells were transfected with 50 nM 14-3-3 $\beta$  siRNA (knockdown group) or negative control siRNA (control group), using Lipofectamine<sup>®</sup> 2000 transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Untransfected MG63 cells served as the blank control group. At 48 h following transfection, MG63 cells were washed twice with cold phosphate-buffered saline (PBS), trypsinised, and collected by centrifugation at 1,200 g x 5 min for further experiments.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RNA was extracted from  $5 \times 10^7$  cells using 1 ml TRIzol<sup>™</sup> reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA synthesis and PCR were subsequently performed using the Bio-Rad PCR Detection System (Bio-Rad Laboratories, Inc.) as previously described (22). PCR was performed with an initial denaturation step at  $95^{\circ}\text{C}$  for 5 min, followed by 35 cycles of:  $95^{\circ}\text{C}$  for 3 sec;  $60^{\circ}\text{C}$  for 30 sec; and finally  $72^{\circ}\text{C}$  for 10 min. Relative mRNA expression was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method (23). All the experiments were repeated in triplicate.

**Western blotting.** Proteins were extracted from  $5 \times 10^7$  cells with radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology) and quantified by a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). A total of

40  $\mu\text{g}$  total proteins were separated on 12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skimmed milk in TBS for 1 h at ambient temperature and then incubated with primary antibodies (against the following antigens: 14-3-3 $\beta$ ,  $\beta$ -catenin, cyclin D1, c-myc, MMP9 and  $\beta$ -actin) at a 1:2,000 in 5% skimmed milk in TBS + 0.1% Tween-20 (TBST) at 4°C overnight. Membranes were washed with TBST, then probed with goat anti-rabbit immunoglobulin (Ig)G or goat anti-mouse IgG diluted at a 1:2,000 in 5% skimmed milk in TBST for 2 h at ambient temperature. Finally, the membranes were washed in TBST and developed using the ECL kit. Band intensities were quantified by densitometric analysis software Quantity-one v4.62 (Bio-Rad Laboratories, Inc.). Relative expression levels of target proteins were normalized to the  $\beta$ -actin loading control. All the experiments were repeated in triplicate.

**Cell viability and cell cycle analysis.** Cell viability was determined with CCK-8, according to the manufacturer's protocol. Briefly, MG63 cells were synchronized in G0 by serum deprivation for 24 h. MG63 cells were then collected by centrifugation at 1,200  $\times$  g for 5 min and seeded at a density of 3,000 cells/well in 96-well microplates. CCK-8 solution (10  $\mu\text{l}$ ) and RPMI-1640 (100  $\mu\text{l}$ ) were added to each well and incubated for 2 h at 30°C. The optical density was detected at a wavelength of 450 nm using a microplate reader. All the experiments were repeated in triplicate.

For cell cycle analysis, MG63 cells were synchronized in G0 by culturing in serum-free RPMI-1640 medium for 24 h, and then cultured in complete medium for an additional 24 h. Following incubation, MG63 cells were collected by centrifugation at 1,200  $\times$  g for 5 min and fixed in 70% cold ethanol (0.5 ml) at 4°C for 30 min. Fixed MG63 cells were washed twice in PBS, and 50  $\mu\text{l}$  a 100  $\mu\text{g}/\text{ml}$  stock of RNase was added to 200  $\mu\text{l}$  PI (50  $\mu\text{g}/\text{ml}$  stock) which was added to each cell suspension and incubated for 30 min at room temperature in the dark prior to analysis. Cell cycle phase distribution was detected by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest software (version 6.0; BD Biosciences) was used to analyze the results, as described previously (22). Data were expressed as the fraction of cells in the different cell cycle phases, and the experiment was repeated 3 times.

**Transwell invasion assay.** Cell invasion capacity was examined by Transwell invasion assay. Matrigel was diluted to 100  $\mu\text{g}/\text{ml}$  with serum-free, cold RPMI-1640 culture medium; 100  $\mu\text{l}$  of the diluted Matrigel was added to the upper chamber of a 24-well Transwell plate and incubated at 37°C for at least 4-5 h for the Matrigel to solidify. The solid Matrigel was washed with warmed serum-free culture media, and 200  $\mu\text{l}$  RPMI-1640 medium containing  $1 \times 10^3$  MG63 cells were added to the Matrigel-coated, upper chamber of each Transwell plate. The bottom chamber was filled with 600  $\mu\text{l}$  RPMI-1640 medium containing 10% FBS as a chemoattractant. The Transwell plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> and 95% air incubator for 48 h. Following incubation, the MG63 cells that remained on the upper chamber were removed using a cotton swab. The migrated cells at the bottom surface were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with a crystal violet solution for

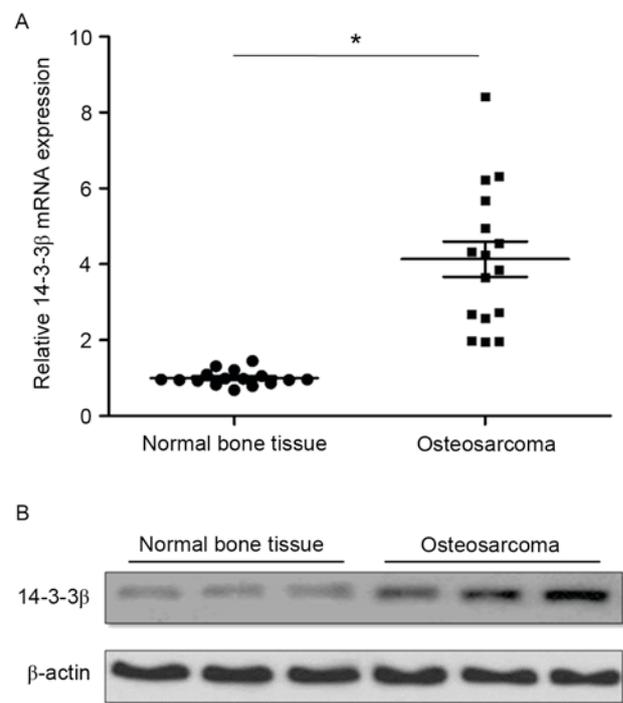


Figure 1. Endogenous 14-3-3 $\beta$  expression in osteosarcoma tissues and in matched normal tumor-adjacent bone tissues from patients. (A) 14-3-3 $\beta$  mRNA expression levels were assessed by reverse transcription-quantitative polymerase chain reaction. \*P<0.05. (B) 14-3-3 $\beta$  protein expression levels were assessed by western blotting. All data are presented as mean  $\pm$  standard deviation.

10 min at room temperature, followed by observation under a Leica DMI3000 B inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and an average number of cells was taken by counting six random fields of view per filter. Experiments were performed in triplicate.

**Statistical analysis.** SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze statistical significance of the data, by performing one-way analysis of variance and Bonferroni's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Upregulation of 14-3-3 $\beta$  in osteosarcoma.** 14-3-3 $\beta$  mRNA and protein expression levels were detected by RT-qPCR and western blotting, respectively. RT-qPCR analysis demonstrated that 14-3-3 $\beta$  mRNA was significantly overexpressed in osteosarcoma tissues compared with matched normal tumor-adjacent bone tissues (Fig. 1A). Western blotting results were similar to RT-qPCR results, demonstrating that the expression of 14-3-3 $\beta$  protein was also markedly upregulated in osteosarcoma tissues compared with matched normal tumor-adjacent bone tissues (Fig. 1B). The levels of 14-3-3 $\beta$  expression were also examined in three osteosarcoma cell lines: U2OS, Saos-2 and MG63. All 3 cell lines exhibited significantly higher 14-3-3 $\beta$  mRNA and protein expression compared with the normal osteoblast hFOB1.19 cells (Fig. 2A and B, respectively). The present results indicated that 14-3-3 $\beta$  upregulation may be important in the tumorigenesis and progression of osteosarcoma.

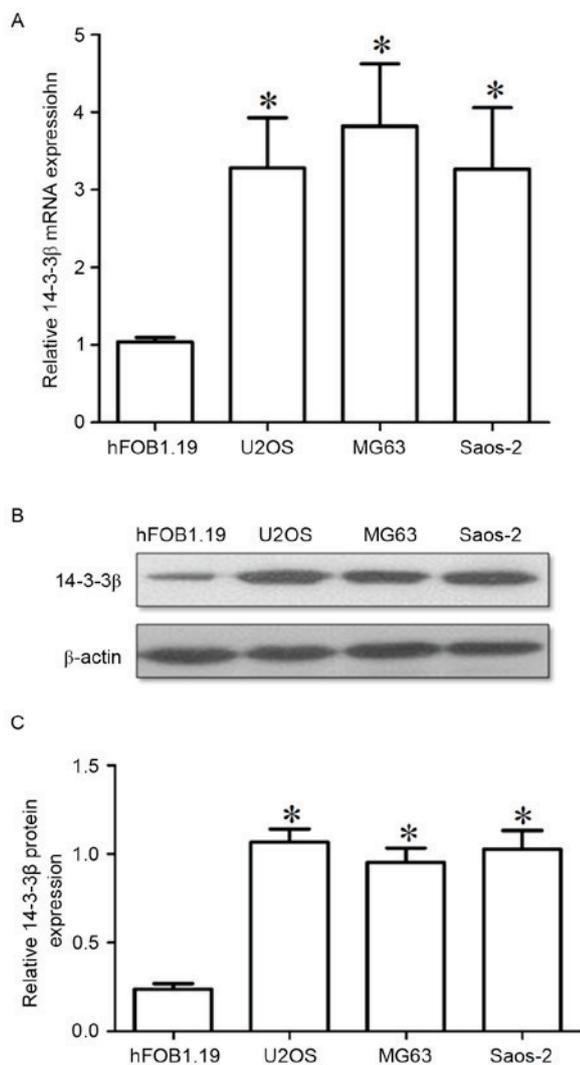


Figure 2. Endogenous 14-3-3 $\beta$  expression in osteosarcoma cell lines. Endogenous expression of 14-3-3 $\beta$  was assessed in the osteosarcoma cell lines U2OS, Saos-2 and MG63 and in the human normal osteoblast cell line hFOB1.19. (A) 14-3-3 $\beta$  mRNA expression levels were assessed by reverse transcription-quantitative polymerase chain reaction. (B) 14-3-3 $\beta$  protein expression levels were assessed by western blotting. (C) Quantification of protein expression relative to  $\beta$ -actin (loading control). All data are presented as mean  $\pm$  standard deviation. \* $P$ <0.05 vs. hFOB1.19.

#### 14-3-3 $\beta$ knockdown inhibits cell viability in MG63 cells.

To explore the effects of 14-3-3 $\beta$  on the biological behavior of osteosarcoma cells, the expression of 14-3-3 $\beta$  mRNA in MG63 cells was silenced by siRNA transfection. Results from RT-qPCR and western blotting demonstrated that 14-3-3 $\beta$  mRNA and protein expression levels were significantly down-regulated in the knockdown group compared with the control (negative control siRNA) and the blank (untransfected cells) groups (Fig. 3). The present data demonstrated that the expression of 14-3-3 $\beta$  had been effectively suppressed by siRNA in the MG63 osteosarcoma cells.

Subsequently, the viability of MG63 cells was assayed using the CCK-8 assay. The results demonstrated that MG63 cell viability in the 14-3-3 $\beta$  knockdown group was significantly decreased compared with the control and the blank group (Fig. 4). The data suggest that 14-3-3 $\beta$  may be important in MG63 cell viability.

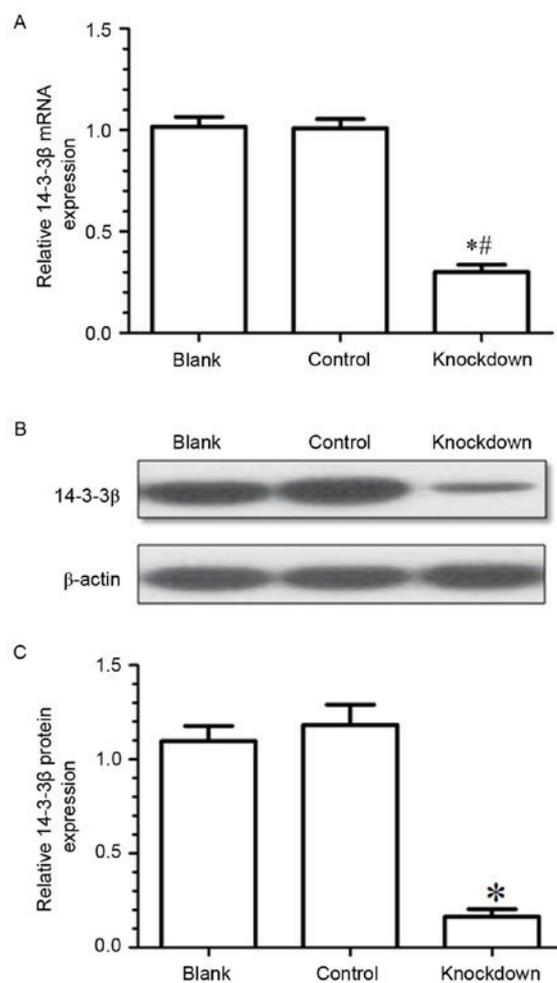


Figure 3. 14-3-3 $\beta$  knockdown in MG63 osteosarcoma cells. MG63 cells were transfected with either a 14-3-3 $\beta$ -specific siRNA (knockdown group) or a non-specific control siRNA (control group). Untransfected cells were used as the blank group. (A) 14-3-3 $\beta$  mRNA expression levels were assessed by reverse transcription-quantitative polymerase chain reaction. (B) 14-3-3 $\beta$  protein expression levels were assessed by western blotting. (C) Quantification of protein expression relative to  $\beta$ -actin (loading control). All experiments were repeated three times with three replicates each. All data are presented as mean  $\pm$  standard deviation. \* and # $P$ <0.05 knockdown group vs. blank group and control group, respectively.

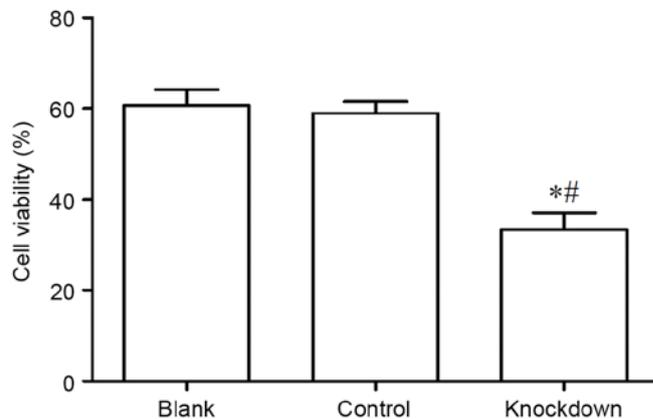


Figure 4. 14-3-3 $\beta$  knockdown reduces MG63 cell viability. MG63 cells were transfected with either a 14-3-3 $\beta$ -specific siRNA (knockdown) or a non-specific control siRNA (control), and cell viability was measured by Cell Counting Kit-8 assay. All experiments were repeated three times with three replicates each. All data are presented as mean  $\pm$  standard deviation. \* and # $P$ <0.05 knockdown group vs. blank group and control group, respectively.

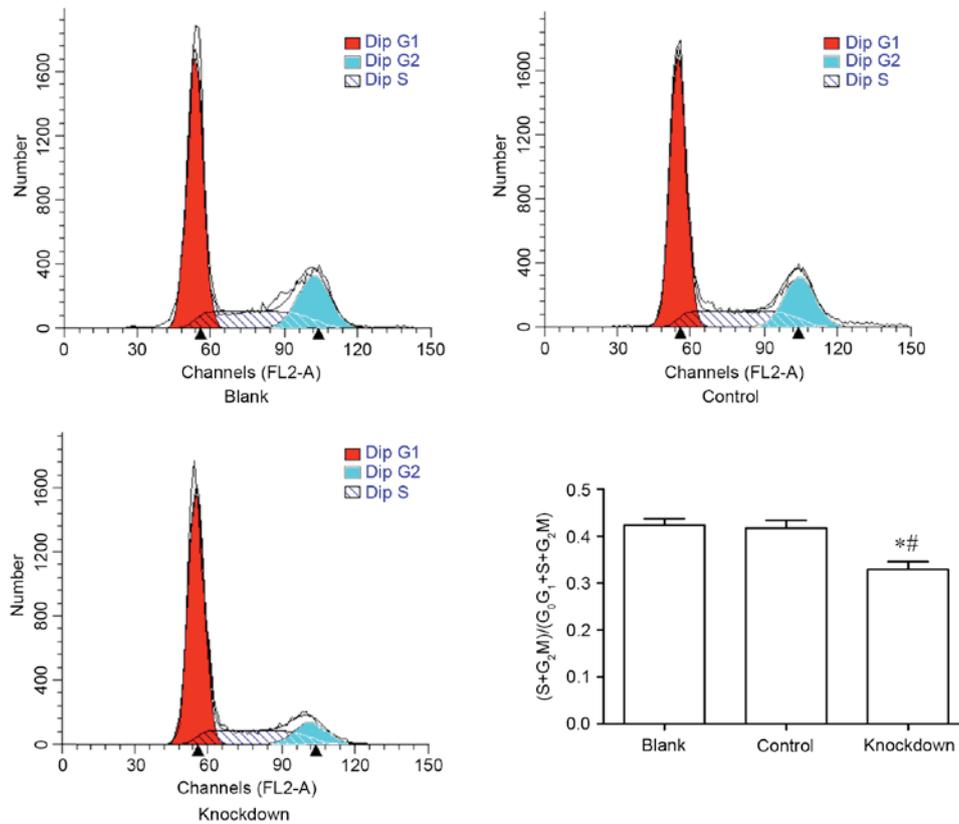


Figure 5. 14-3-3 $\beta$  knockdown suppresses MG63 cell cycle progression. MG63 cells were transfected with either a 14-3-3 $\beta$ -specific siRNA (knockdown) or a non-specific control siRNA (control), and cell cycle distribution was examined by flow cytometry. All experiments were repeated three times with three replicates each. Data are presented as the mean ratio of cells in the S and the G<sub>2</sub>-M (S+G<sub>2</sub>M) phases to the total number of cells (G<sub>0</sub>G<sub>1</sub>+S+G<sub>2</sub>M). All data are presented as mean  $\pm$  standard deviation. \*and #P<0.05 knockdown group vs. blank group and control group, respectively.

**14-3-3 $\beta$  knockdown suppresses cell cycle progression in MG63 cells.** The effect of 14-3-3 $\beta$  knockdown in cell cycle phase distribution was assessed by flow cytometry. The percent of MG63 cells at the S phase and the G<sub>2</sub>-M phase in the 14-3-3 $\beta$  knockdown group was significantly decreased compared with the control and the blank group (Fig. 5). By contrast, the percent of MG63 cells at the G<sub>0</sub>-G<sub>1</sub> phase in the 14-3-3 $\beta$  knockdown group was increased compared with the control and the blank group (Fig. 5). These findings demonstrated that 14-3-3 $\beta$  knockdown inhibited cell cycle progression in MG63 osteosarcoma cells.

**14-3-3 $\beta$  knockdown inhibits cell invasion in MG63 cells.** The Transwell invasion chamber assay was used to determine the invasive ability of MG63 osteosarcoma cells. The results demonstrated that the 14-3-3 $\beta$  knockdown group had significantly fewer MG63 cells invaded into the Matrigel membrane compared with the control and the blank group (Fig. 6). The number of invaded MG63 cells did not differ significantly between the control group and the blank group (Fig. 6). These data suggested that 14-3-3 $\beta$  downregulation suppressed the invasive ability of MG63 osteosarcoma cells.

**14-3-3 $\beta$  knockdown suppresses the expression of  $\beta$ -catenin, cyclinD1, c-myc and MMP9 in MG63 cells.** The present study demonstrated that 14-3-3 $\beta$  is upregulated in osteosarcoma tissues and cell lines, and that 14-3-3 $\beta$  knockdown inhibited osteosarcoma MG63 cell viability, proliferation and invasion.

Western blotting demonstrated that the level of  $\beta$ -catenin expression was significantly downregulated in the 14-3-3 $\beta$  knockdown group compared with the control and the blank group (Fig. 7), which suggests that 14-3-3 $\beta$  may regulate the expression of  $\beta$ -catenin.

$\beta$ -catenin is a positive regulator of the canonical Wnt signaling pathway, therefore, the hypothesis that 14-3-3 $\beta$  may act through the canonical Wnt pathway was tested. Protein expression levels of cyclin D1, c-myc and MMP9, which are known target genes of the canonical Wnt pathway, were determined by western blotting. The results demonstrated that expression levels of cyclin D1, c-myc and MMP9 were significantly decreased in the 14-3-3 $\beta$  knockdown group compared with the control and the blank group (Fig. 7). These data indicated that 14-3-3 $\beta$  may suppress proliferation and invasion of osteosarcoma cells through the inhibition of the canonical Wnt signaling pathway.

## Discussion

14-3-3 is a family of highly conserved proteins that are involved in a number of cellular processes (24). The majority of 14-3-3 proteins have been demonstrated to be essential in regulating apoptosis, proliferation and oncogenic transformation (11). 14-3-3 $\beta$  exhibits oncogenic potential, and increased expression of 14-3-3 $\beta$  is detected in multiple types of carcinomas (18). However, the expression pattern and the exact role of 14-3-3 $\beta$  in osteosarcoma remain unclear.

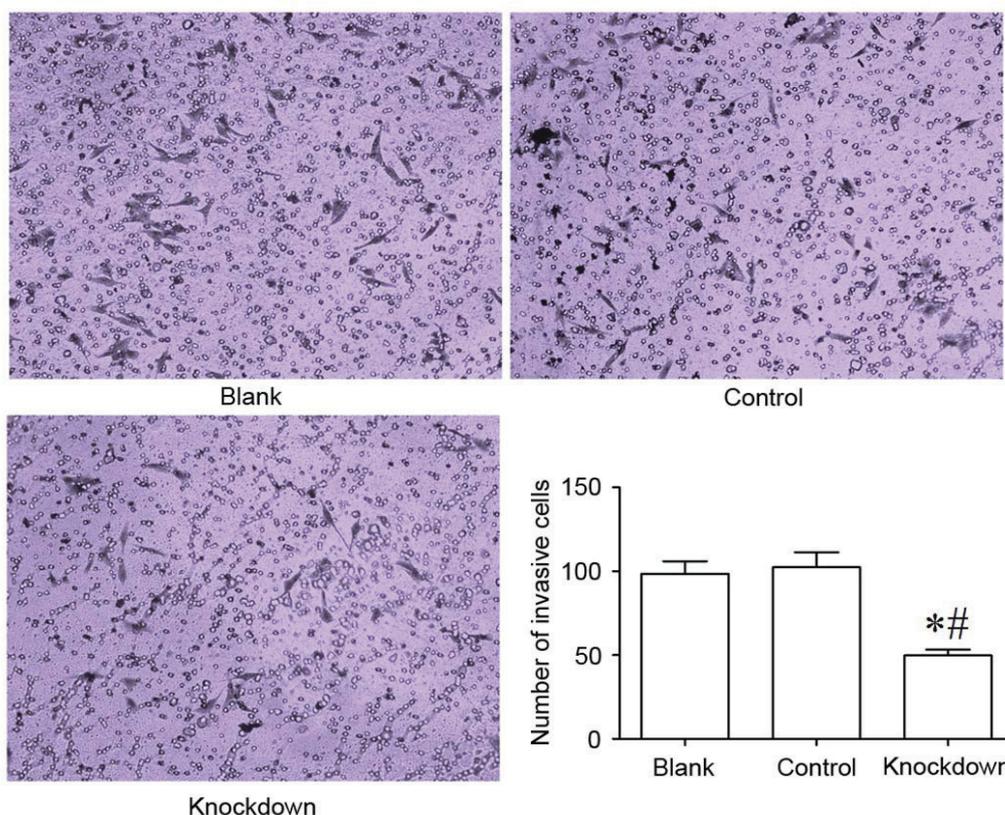


Figure 6. 14-3-3 $\beta$  knockdown inhibits MG63 cell invasion. MG63 cells were transfected with either a 14-3-3 $\beta$ -specific siRNA (knockdown) or a non-specific control siRNA (control), and cell invasion ability was determined by Transwell invasion chambers. Magnification, x40. Data are presented as the number of cells invading the bottom of the Transwell filter. All experiments were repeated three times with three replicates each. All data are presented as mean  $\pm$  standard deviation. \* and #P<0.05 knockdown group vs. blank group and control group, respectively.

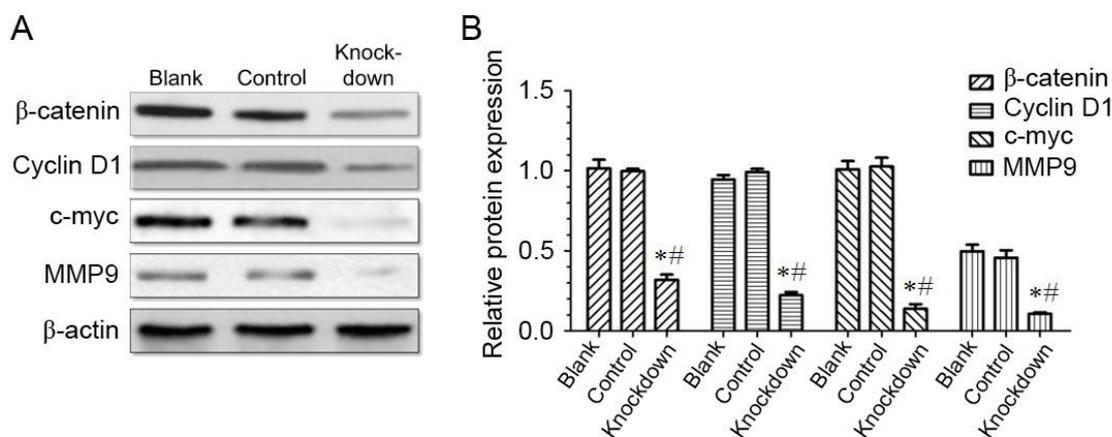


Figure 7. 14-3-3 $\beta$  knockdown suppresses the expression of  $\beta$ -catenin, cyclinD1, c-myc and MMP9 proteins in MG63 cells. MG63 cells were transfected with a 14-3-3 $\beta$ -specific siRNA (knockdown), a non-specific control siRNA (control) or were not transfected (blank). (A) Protein expression levels of  $\beta$ -catenin, cyclinD1, c-myc and MMP9 were assessed by western blotting. (B) Quantification of proteins relative to  $\beta$ -actin (loading control). All experiments were repeated three times with three replicates each. All data are presented as mean  $\pm$  standard deviation. \* and #P<0.05 knockdown group vs. blank group and control group, respectively. c-myc, v-myc avian myelocytomatosis viral oncogene homolog; MMP9, matrix metalloproteinase 9; siRNA, small interfering RNA.

In the present study, the expression of 14-3-3 $\beta$  was demonstrated to be significantly increased in osteosarcoma tissues and cell lines, compared with normal tissues and normal osteoblast cells, respectively. These findings are similar to the upregulated expression of 14-3-3 $\beta$  in hepatocellular carcinoma, astrocytoma, lung cancer, colorectal cancer,

gastric cancer and vulvar squamous cell carcinoma (17-19,21). These data indicate that the upregulation of 14-3-3 $\beta$  may be essential in tumorigenesis and progression of osteosarcoma. To further explore the effect of 14-3-3 $\beta$  on the biological behavior of osteosarcoma cells, 14-3-3 $\beta$  expression was silenced in MG63 osteosarcoma cells by siRNA. The results

demonstrated that cell viability was significantly inhibited following 14-3-3 $\beta$  knockdown in MG63 cells. The number of MG63 cells at the S and G<sub>2</sub>-M phases of the cell cycle was lower in the 14-3-3 $\beta$  knockdown cell group compared with the control group, which suggests that 14-3-3 $\beta$  silencing inhibited MG63 cell proliferation. Therefore, 14-3-3 $\beta$  overexpression may be responsible for cell cycle deregulation, a key feature of carcinogenesis. The present data are similar to previous findings for other types of cancer, which have demonstrated that 14-3-3 $\beta$  knockdown inhibits proliferation of hepatocellular carcinoma cells and astrocytoma cells (19,25). Activation of the Wnt/ $\beta$ -catenin signaling pathway promotes cellular proliferation, whereas Wnt inhibition reduces proliferation (26,27). Downregulation of 14-3-3 $\beta$  significantly decreases nuclear localization of  $\beta$ -catenin, followed by a decrease in the activity of Wnt/ $\beta$ -catenin signaling (28). Therefore,  $\beta$ -catenin nuclear translocation, induced by overexpression of 14-3-3 $\beta$ , activates the transcription of oncogenes, including cyclin D1 and c-myc (19,29). In the present study,  $\beta$ -catenin expression was significantly downregulated following 14-3-3 $\beta$  knockdown in osteosarcoma cells, which suggests that 14-3-3 $\beta$  may regulate expression of  $\beta$ -catenin. Since  $\beta$ -catenin is a positive regulator of the canonical Wnt signaling pathway, it was hypothesized that 14-3-3 $\beta$  may inhibit proliferation of osteosarcoma cells through Wnt. To test this hypothesis, expression of cyclin D1 and c-myc, which are known targets downstream of the canonical Wnt signaling pathway, were determined. The results demonstrated that expression of cyclin D1 and c-myc was significantly inhibited following 14-3-3 $\beta$  knockdown, suggesting that 14-3-3 $\beta$  may regulate growth and proliferation in osteosarcoma cells through Wnt/ $\beta$ -catenin signaling.

Previous studies have demonstrated that overexpression of 14-3-3 $\beta$  is significantly correlated with metastasis, increased invasive ability and poor prognosis in many types of cancer, including hepatocellular carcinoma and gastric cancer (18,30). Tang *et al* (18) demonstrated that elevated expression of 14-3-3 $\beta$  in hepatocellular carcinoma cell lines led to enhanced cell migration and invasion, as well as upregulation of MMP2 and MMP9 expression. In the current study, 14-3-3 $\beta$  knockdown suppressed MG63 osteosarcoma cell invasion, suggesting that 14-3-3 $\beta$  overexpression may enhance osteosarcoma cell invasion. This is in agreement with a previous study, which demonstrated that targeted depletion of 14-3-3 $\beta$  reduces lung cancer cell migration and invasion (31). Activation of Wnt/ $\beta$ -catenin signaling promotes the invasive activities of several types of cancer cells (32,33), while suppression of Wnt/ $\beta$ -catenin signaling inhibits cancer cell invasion (34,35). In the present study, 14-3-3 $\beta$  inhibited  $\beta$ -catenin expression, as well as osteosarcoma cell invasion, suggesting that 14-3-3 $\beta$  may regulate cell invasion through the canonical Wnt signaling pathway. The expression of MMP9, which is a target gene of the canonical Wnt pathway and a promoter of cell invasion, was further explored. The results demonstrated that MMP9 expression levels were significantly decreased following 14-3-3 $\beta$  knockdown, compared with the control siRNA-transfected cells.

In conclusion, the present study demonstrated that 14-3-3 $\beta$  silencing was able to suppress proliferation and migration of osteosarcoma cells, possibly through the inactivation of the canonical Wnt/ $\beta$ -catenin signaling pathway. These findings

suggest that the increased expression of endogenous 14-3-3 $\beta$  observed in osteosarcoma cells compared with normal tissues, may be important in regulating proliferation and migration of osteosarcoma cells, and that 14-3-3 $\beta$  may be valuable as a prognostic marker and/or therapeutic target for osteosarcoma.

### Acknowledgements

The present study was supported by the Natural Science Foundation of Jiangsu Province (grant no. BK20131199), the fifty-fifth batch funding of the China Postdoctoral Science Foundation (grant no. 2014M551640), Nantong Science and Technology Innovation Program (grant no. MS22016008) and by the Project of Jiangsu provincial Health and Family Planning Commission (grant no. H201524).

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