Effects of siRNA-mediated Cdc2 silencing on MG63 cell proliferation and apoptosis

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Abstract. The present study aimed to determine the effect of small interfering RNA (siRNA)-induced inhibition of cyclin-dependent kinase 2 (Cdc2) expression on osteosarcoma MG63 cell proliferation and apoptosis. An siRNA expression plasmid, psilencer 2.1-U6/Cdc2, targeting the Cdc2 gene, and a control psilencer 2.1-U6/Scramble plasmid were constructed and transfected into MG63 cells using liposomes. Cdc2 expression in the MG63 cells was investigated by western blot analysis and real-time polymerase chain reaction. Cell morphology was also examined. The effects of psilencer 2.1-U6/Cdc2 on MG63 cell proliferation and the cell cycle were detected via MTT and flow cytometry, respectively. Expression levels of apoptosis-related molecules, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (Bax) were determined by western blot analysis. MG63 cells stably transfected with the psilencer 2.1-U6/Cdc2 plasmid (MG63-siRNA/Cdc2) and negative control cells, MG63-siRNA/Scramble, were successfully obtained. The silencing efficiencies of the Cdc2-expressing mRNA and protein in MG63-siRNA/Cdc2 were 86 and 89% of that of the control MG63-siRNA/Scramble cells, respectively. Interference of Cdc2 expression inhibited MG63 cell proliferation and was demonstrated to significantly increase and decrease cells in the G_2/M and S phases, respectively. Cdc2 expression silencing had negligible effects on Bcl-2 and Bax expression in MG63 cells. In conclusion, silencing of Cdc2 expression suppresses proliferation of osteosarcoma MG63 cells but has negligible effects on apoptosis.

Introduction

Osteosarcoma is one of the most common primary malignant tumour observed in children and adolescents, with a current annual incidence rate of 3/1,000,000 (1). At present, combined neoadjuvant chemotherapy and surgery are mainly used to treat osteosarcoma, however, patient survival rates remain low (2). Studies of potential gene therapy candidates for the treatment of osteosarcoma have increased with the development of molecular biology and genomic sciences (3,4). In previous studies (5,6), we utilised a limiting dilution to obtain an osteosarcoma MG63 monoclonal cell substrain. Cell electrophoresis and invasion assays were then used to identify MG63 monoclonal cell substrains with variations in transfer and malignant characteristics. A cell substrain with high transfer and malignant characteristics (M8) and another with low transfer and malignant characteristics (M6) were verified by tumour transfer experiments *in vitro*. A gene chip was then used to scan and compare M8 with M6. Cyclin-dependent kinase 2 (Cdc2) expression in M8 was high at 2.67. Following this, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was utilised to verify the increase in gene expression of Cdc2 in M8 compared with M6 cells (5,6) and Cdc2 was identified as a key gene for the proliferation and apoptosis of osteosarcoma MG63 cells. In addition, silencing of Cdc2 expression was demonstrated to inhibit proliferation and promote apoptosis of osteosarcoma MG63 cells. To verify these findings, a small interfering RNA (siRNA) expression plasmid targeting the Cdc2 gene was constructed in the current study and the effect of siRNA interference of Cdc2 expression on the proliferation and apoptosis of osteosarcoma MG63 cells was investigated. The results obtained may serve as an experimental basis for gene therapy of osteosarcoma.

Materials and methods

Cell culture. MG63 cells were grown in RPMI-1640 medium supplemented with 10% calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified 5% CO_2 and 95% air incubator at 37°C.

Plasmid construction. The genetic code sequence for Cdc2 was obtained from GenBank (NM_001170406) in accordance with the principles of siRNA design. One siRNA-2 interference sequence was designed by Invitrogen Life Technologies (Carlsbad, CA, USA). An additional siRNA-1 interference sequence that was previously reported to be more effective (7) than that in the human genome was identified only as a Cdc2

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Table I. Template sequence of Cdc2 siRNA.

siRNA	Sequence (5'-3')
Negative siRNA-Scramble	
Forward	GATCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTGGAAA
Reverse	AGCTTTTCCAAAAAACTACCGTTGTTATAGGTGTCTCTTGAACACCTATAACAACGGTAGG
Cdc2 siRNA-1	
Forward	GATCCGGGGGTTCCTAGTACTGCAATTCAAGAGATTGCAGTACTAGGAACCCCTTTTTTGGAAA
Reverse	AGCTTTTCCAAAAAAGGGGGTTCCTAGTACTGCAATCTCTTGAATTGCAGTACTAGGAACCCCG
Cdc2 siRNA-2	
Forward	GATCCGTTGATCAACTCTTCAGGATTTCAAGAGAATCCTGAAGAGTTGATCAATTTTTTGGAAA
Reverse	AGCTTTTCCAAAAAATTGATCAACTCTTCAGGATTCTCTTGAAATCCTGAAGAGTTGATCAACG

Cdc2, cyclin-dependent kinase 2; siRNA, small interfering RNA.

gene interference sequence. A non-specific siRNA-scrambled (siRNA-Scramble) sequence, designed in a similar manner to serve as the negative control, was obtained from the Institute of Molecular Biology of (Three Gorges University; Table I). The three synthesised siRNA sequences were annealed using sticky ends and *Bam*HI and *Hind*III digestion and then connected to a psilencer 2.1-U6 that had been cut by the same enzyme. The three restructured psilencer 2.1/siRNA plasmids were then transformed into *Escherichia coli* DH5, screened for ampicillin resistance on an agar plate and then amplified in culture. Following this, plasmids were selected and sequenced.

Transient transfection. One day prior to transient transfection, the cells were plated in six-well plates at a concentration of $2x10^5$ cells/well. When the cell density reached ~80%, transfection was performed using Lipofectamine™ 2000 in accordance with the manufacturer's instructions. Following transfection (~24 h), the proteins were extracted from the cells. Western blot analysis was then performed to determine the siRNA silencing efficiency.

Stable transfection. MG63 cells were plated in six-well plates at a concentration of $2x10^5$ cells/well and then cultured in a conventional culture medium, as previously described. Cells were transfected the following day according to the manufacturer's instructions. In brief, diluted DNA and Lipofectamine™ 2000 complexes (total volume, 25 ml) were prepared and added to each MG63 cell-containing well. Cells were then incubated for 4-6 h. Post-transfection (~48 h), cells were incubated with 100 μg/ml hygromycin in RPMI-1640 medium with 10% calf serum for three weeks. Single clones with low Cdc2 expression were then identified via RT-PCR, western blot analysis and cell immunofluorescence.

Quantitative PCR. RNA from the cell lines was extracted using TRIzol reagent. cDNA was synthesised using SuperScript™ II Reverse Transcriptase according to the manufacturer's instructions. The primers used for amplification were as follows: Cdc2, 5'-TACCTATGGAGTTGTGTATAA-3' and 5'-ATTCCA CTTCTGGCCACACTT-3'; β-actin, 5'-CCACAGCTGAG

AGGGAAATC-3' and 5'-ATCCTCTTCCTCCCTGGAGA-3'. PCR cycling conditions were as follows: 94°C for 5 min, 25 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and 78°C for 1 sec for plate reading and 72°C for 5 min. PCR products were separated via electrophoresis at 100 V for 30 min on a 1% agarose gel and then detected using ethidium bromide staining. The expected sizes of specific PCR products were verified using a 1-kb DNA reference ladder.

Western blot analysis. Cell lysates were prepared from six cloning strains. Normal MG63 and negative control cells were cultured for 24 h. Protein samples were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes. Following blocking with 5% non-fat dry milk in a Tris-buffered saline Tween-20 buffer for 1 h, the samples were probed with a primary antibody against Cdc2 and a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein bands were detected using an enhanced chemiluminescence detection system and X-ray film exposure (Kodak, Rochester, NY, USA). β-actin was used as a loading control.

Cell immunofluorescence. MG63 and MG63-siRNA-Cdc2 cells were seeded into 24-well culture plates. When the growth density reached 60%, cells were fixed with ice-cold 4% paraformaldehyde and incubated with 10% (v/v) goat serum as a blocking background. Following overnight incubation with the primary antibody against Cdc2, the cells were washed three times with phosphate-buffered saline (PBS) and then incubated with rhodamine-123-labeled anti-rabbit IgG for 1 h at 37°C. The cells were washed three times with PBS and then examined via fluorescence/phase-contrast microscopy (TE2000S; Nikon, Tokyo, Japan).

Assay of cellular proliferation. MG63, MG63-siRNA/Scramble and MG63-siRNA/Cdc2 cells were seeded into 96-well culture plates (2,000 cells/well in 100 μl RPMI-1640 medium). Culture medium was removed following culture for 0, 24, 48, 72 and 96 h. A 200 μl MTT solution (0.25 g/l in RPMI-1640 medium)
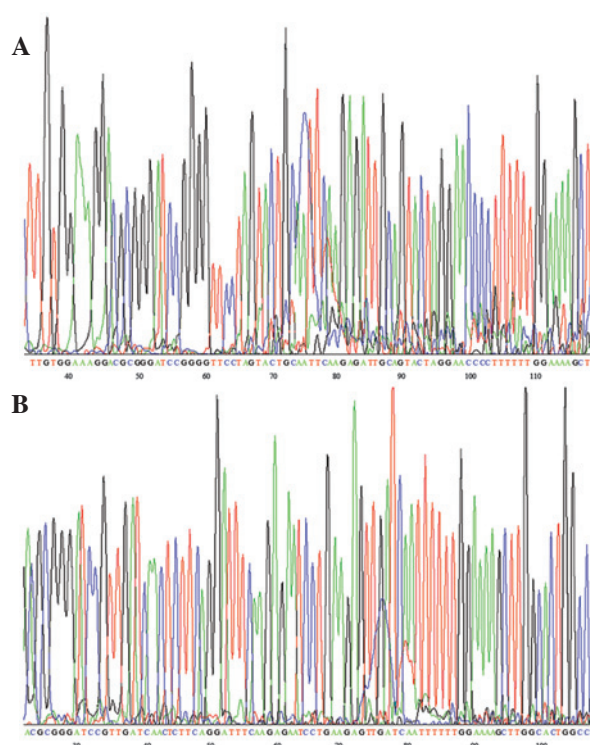
A

B

Figure 1. DNA sequencing assay of siRNA plasmids. (A) Insert DNA fragment in plasmid psilencer 2.1/siRNA-1. (B) Insert DNA fragment in plasmid psilencer2.1/siRNA-2. siRNA, small interfering RNA.
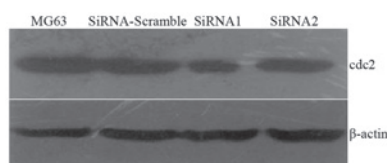

Figure 2. Western blot analysis of three siRNAs designed against Cdc2 to determine silencing efficiency following transient transfection. Cdc2, cyclin-dependent kinase 2; siRNA, small interfering RNA.

was added to each well and the cells were incubated at 37°C for 4 h. The medium was removed and 200 μl dimethyl sulfoxide was added to each well. Absorbance (A) at 570 nm was recorded following 30 min incubation at room temperature. The cell survival rate was calculated as follows: Cell survival rate = $A_{drug}/A_{control}$ x 100.

Apoptosis protein detection via western blot analysis. Experiments were conducted as described for Cdc2. Primary monoclonal antibodies against B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (Bax) were utilised. Secondary antibodies were mouse and goat anti-rabbit IgG (both 1:2,000).

Flow cytometry. MG63, MG63-siRNA/Scramble and MG63-siRNA/Cdc2 were collected, washed twice with ice-cold PBS and then fixed in 75% ethanol overnight at 4°C. Following centrifugation at 2,000 rpm for 5 min, cell pellets were resuspended in PBS containing 0.1% Triton X-100 and 50 μg/ml RNase A. The cell samples were then stained with 50 mg/l propidium iodide for 30 min at 37°C in the dark prior to flow cytometry.
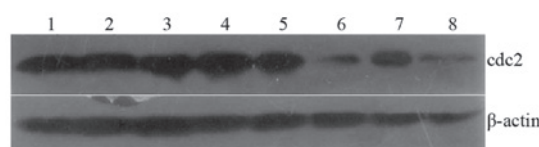

Figure 3. Cdc2 expression in six cloned strains was detected by western blot analysis. siRNA1-5 demonstrated the best effect in Cdc2 silencing. Lane 1, MG63; 2, siRNA-Scramble; 3, siRNA1-1; 4, siRNA1-2; 5, siRNA1-3; 6, siRNA1-5; 7, siRNA1-7; 8, siRNA1-8. Cdc2, cyclin-dependent kinase 2; siRNA, small interfering RNA.
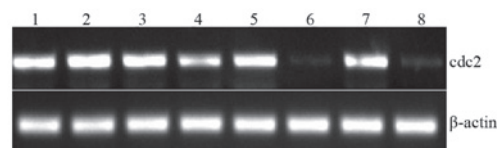

Figure 4. Cdc2 expression in six cloned strains was analysed by RT-PCR. siRNA1-5 was identified to have the best silencing effect on Cdc2. Lane 1, MG63; 2, siRNA-Scramble; 3, siRNA1-1; 4, siRNA1-2; 5, siRNA1-3; 6, siRNA1-5; 7, siRNA1-7; 8, siRNA1-8. Cdc2, cyclin-dependent kinase 2; siRNA, small interfering RNA; RT-PCR, reverse-transcription polymerase chain reaction.

Statistical analysis. Data are expressed as mean ± SD. Differences between two groups were analysed using the Student's t-test, whereas differences between three or more groups were analysed using ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

Plasmid construction. The siRNA-restructured plasmid was connected to psilencer 2.1-U6. The identity of the restructured plasmid psilencer 2.1/siRNA was confirmed via DNA sequencing (Fig. 1).

Cdc2 low-expression cell line. siRNA was transiently transfected into MG63 cells. Western blot analysis was then performed to determine the siRNA silencing efficiency (Fig. 2). siRNA-Scramble had no effect on Cdc2 expression and siRNA-1 exhibited higher silencing efficiency than siRNA-2. Therefore, siRNA-1 was used in the stable transfection. Western blot analysis and RT-PCR detected Cdc2 expression in six clones (Figs. 3 and 4). The siRNA1-5 clone exhibited the highest silencing efficiencies: 89 and 86% at the protein and mRNA levels, respectively. Immunofluorescence results demonstrated that, compared with normal MG63, MG63-siRNA/Cdc2 cells shrank and their structures changed into short, irregular, oblate spheroids. In addition, a reduced number of MG63-siRNA/Cdc2 cells were identified to express Cdc2 protein. No Cdc2 expression was observed in MG63-siRNA/Cdc2 cells.

MTT assay. The MTT assay detected MG63 cell proliferation following Cdc2 expression silencing (Fig. 5). Following culture for 24 h, the growth rate of the MG63-siRNA/Cdc2 was identified as significantly lower than those of the MG63-siRNA/scramble and MG6 cells (P<0.01). These observations indicate that Cdc2 expression silencing inhibits cell proliferation.

Table II. Effect of Cdc2-siRNA on the cell cycle in MG63 cells (n=3).

	G_0/G_1	S	G_2/M
MG63	63.1±0.8	25.0±0.7	11.9±0.2
MG63-siRNA/Scramble	62.8±0.9	26.7±0.6	10.5±0.2
MG63-siRNA/Cdc2	66.8±0.7[a]	11.8±0.4[b]	21.4±0.3[b]

[a]P<0.05 and [b]P<0.01, MG63-SiRNA/Cdc2 vs. MG63-SiRNA/Scramble. MG63 vs. MG63-SiRNA/Scramble was not identified to be statistically significant. Data are presented as the mean ± SD. Cdc2, cyclin-dependent kinase 2; siRNA, small interfering RNA.
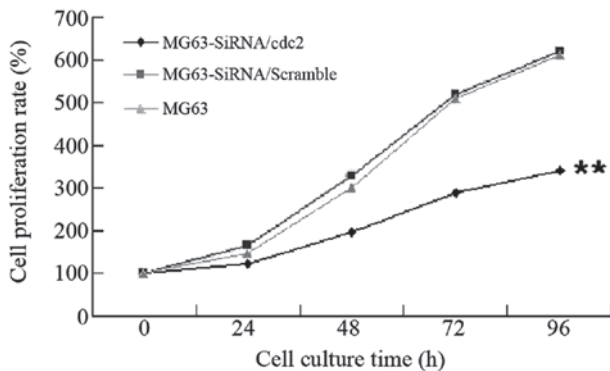

Figure 5. Effect of Cdc2 on proliferation of MG63 cells. Cdc2, cyclin-dependent kinase 2; siRNA, small interfering RNA.
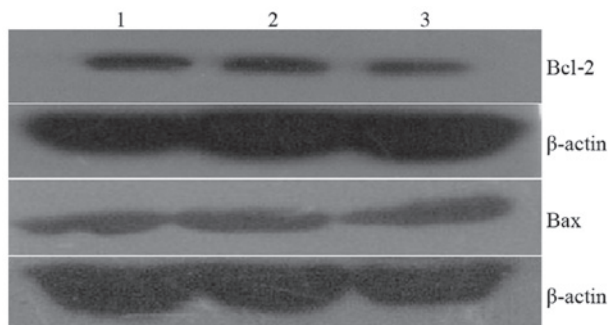

Figure 6. Expression of Bcl-2 and Bax in various MG63 cell groups (n=3). Lane 1, MG63; 2, MG63-siRNA/Scramble; 3, MG63-siRNA/Cdc2. Cdc2, cyclin-dependent kinase 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; siRNA, small interfering RNA.

Bcl-2, Bax gene expression. Bcl-2 expression in the MG63-siRNA/Cdc2 was not revealed to decrease significantly compared with the MG63-siRNA/scramble and MG63 cells (126±16 vs. 148±18, 151±13, respectively; P>0.05). In addition, Bcl-2 expression in the MG63-siRNA/Cdc2 cells was not identified to increase significantly (198±21 vs. 186±26, 178±25, respectively; P>0.05; Fig. 6).

Cell cycle. Following Cdc2 expression silencing, MG63-siRNA/Cdc2 accumulated at the G_2/M and G_0/G_1 phases compared with MG63-siRNA/scramble and MG63 cells. However, the number of MG63-siRNA/Cdc2 cells in the S phase was observed to be significantly reduced (P<0.05; Table II).

Discussion

Osteosarcoma is the most common highly malignant tumour of the bone, often manifesting during the second or third decade of life. It accounts for ~60% of all malignant bone tumours that occur during the first 20 years of life (8). The development of neoadjuvant chemotherapy has increased long-term survival rates from 10-20 to 60-70%. However, although adjuvant chemotherapy effectively improves patient survival, as well as the treatment of primary tumours (9), patients who present with metastatic disease and/or tumour relapse remain associated with a poor prognosis. The estimated five-year survival rate following relapse is only 25% (10). To date, gene therapy studies of osteosarcoma pathogenesis and progression, have investigated p53, ezrin and surivin (11-16). However, few studies have focused on the molecular mechanism of Cdc2 in osteosarcoma cells.

In the present study, siRNA interference technology was used to obtain a low-Cdc2 expression cell line. The cell line was then used to study the effects of siRNA-induced interference of Cdc2 expression on the proliferation and apoptosis of osteosarcoma MG63 cells. Normal MG63 cells exhibit long shuttle-like or triangular structures. Following Cdc2 expression silencing, cells exhibited short, irregular, oblate shapes which accumulated easily. MTT results demonstrate that Cdc2 expression silencing inhibits cell proliferation by blocking the cell cycle at the G_2/M phase. Expression of the apoptosis genes, Bcl-2 and Bax, was also analysed to determine the possible effects of Cdc2 expression silencing on cell apoptosis. Cdc2 expression silencing had no effect on Bcl-2 and Bax, indicating that Cdc2 silencing does not promote MG63 cell apoptosis. Results of the present study indicate that Cdc2 expression silencing in MG63 cells inhibits cell proliferation but does not promote cell apoptosis. However, we previously hypothesised that disruption of Cdc2 expression inhibits osteosarcoma MG63 cell proliferation and promotes apoptosis. The absence of an observable effect of Cdc2 expression silencing on the apoptosis of MG63 cells may be due to the large number of genes associated with the regulation of apoptosis of MG63 cells. Although Cdc2 expression in MG63 cells, itself a regulation mechanism, is silenced, additional genes may be activated to offset low Cdc2 expression. Therefore, cells do not undergo apoptosis when Cdc2 alone is silenced. Inhibition of cell proliferation by Cdc2 expression silencing is attributed to the association between Cdc2 and the eukaryotic cell division cycle gene CDC2, which codes for a serine/threonine protein kinase with a molecular weight of 3,400 Da.

Cdc2 enzyme activation is an indicator of cell division. Cdc2 is vital to cell cycle regulation, inducing DNA replication and cell mitosis (17-19). Overexpression of Cdc2 is associated with cell cycle progression disorders, including abnormal growth and cell differentiation, as well as malignant cell proliferation and tumour formation (20). A number of previous studies have reported overexpression of Cdc2 in breast cancer (21,22), B-cell lymphoma (23), colon carcinoma (24), ovarian cancer (25) and glioma (7) cells, as well as a marked correlation with tumour stage, grade, relapse and prognosis. Results of the current study are consistent with these observations.

In the present study, the expression levels of Bax and Bcl-2 only were analysed to detect cell apoptosis. Therefore, additional data are required to verify these results. Future studies must include analyses using an anticancer drug. The effect of Cdc2 expression silencing on the strength of the anticancer drug activity may then be determined. Three cell lines will be implanted in mice to investigate the effect of Cdc2 expression silencing on tumour growth, transfer and prognosis *in vivo*.

Acknowledgements

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