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Suppression of CDK2 expression by siRNA induces cell cycle arrest and cell proliferation inhibition in human cancer cells

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Cyclin-dependent kinase 2 (CDK2) is a member of serine/threonine protein kinases, which initiates the principal transitions of the eukaryotic cell cycle and is a promising target for cancer therapy. The present study was designed to inhibit *cdk2* gene expression to induce cell cycle arrest and cell proliferation suppression. Here, we constructed a series of RNA interference (RNAi) plasmids which can successfully express small interference RNA (siRNA) in the transfected human cells. The results showed that the RNAi plasmids containing the coding sequences for siRNAs down-regulated the *cdk2* gene expression in human cancer cells at the mRNA and the protein levels. Furthermore, we found that the cell cycle was arrested at G0G1 phases and the cell proliferation was inhibited by different siRNAs. These results demonstrate that suppression of CDK2 activity by RNAi may be an effective strategy for gene therapy in human cancers. [BMB reports 2010; 43(4): 291-296]

INTRODUCTION

Cyclin-dependent kinases (CDKs), including CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, CDK8, *et al*, play crucial roles in cell cycle (1-5). Among them, CDK2 is critical for cell proliferation through regulating the DNA synthesis at the beginning of cell cycle and switching the cell cycle from G1 phase to S phase (6-10). Since cancer cells often contain high levels of CDK2 activity, to inhibit *cdk2* gene expression may be a useful therapeutic strategy in cancer treatment.

Cervical cancer is a common malignant tumor of women and it occupies the first place in the female reproductive organs cancer lump. Moreover, osteosarcoma is the most common malignant tumor in the skeletal system. Although the surgery, radiotherapy and chemotherapy are widely used in the treatments of these two cancers, the clinical effects do not meet expectations for lower side effect and less pain. Recently gene therapy becomes a new method for the clinical treatment of these two cancers (11-14). And the Hela and U-2 OS cells are the typical cell lines of cervical cancer and Osteosarcoma respectively. They are commonly used to study the molecular mechanism of gene therapy at the cell level.

RNA interference (RNAi) is a gene-silencing method, triggered by double-stranded RNA (dsRNA) molecules (15, 16). Initially, the dsRNA is recognized by ribonuclease III enzyme Dicer, and processed into small interference RNA (siRNA). Following that the siRNAs are bound by a multi-protein complex that is RNA-induced silencing complex (RISC), which induces the target mRNA to degradation (17, 18). Presently, RNAi is a technique widely used to down-regulate the specific gene expression for gene function study and used as a therapeutic approach for the treatment of diseases such as autoimmune diseases, dominant genetic disorders and viral infections (19, 20).

RNAi is usually induced by siRNA or small hairpin RNA (shRNA). SiRNAs are a large class of about 21 bp-long dsRNA molecules, which can be recognized by the enzymatic machinery of RNAi, and eventually leads to homology-dependent degradation of the target mRNA (21). In mammalian cells, siRNAs are produced from cleavage of longer dsRNA precursors by Dicer. They can be also synthesized by chemical or biochemical methods. ShRNA consists of two short inverted repeats linked by a stem-loop sequence and expresses to form a hairpin structure ended in a run of U residues. Since exogenous siRNAs is easily subjected to degradation by endogenous enzymes, and is too large to cross cellular membranes, shRNA has advantages of long-term silencing, optional delivery and low cost (22-24). Though the viral expression vectors have been widely used to drive the expression of shRNAs, they are limited by their toxicity in some human cells, while the plasmid system has the advantages of low toxicity and easy transfection (25). Recently both siRNAs and shRNAs are used to suppress gene expression and investigate gene function, such as *bcl2*, *cdk2*, *hTERT*, *et al* (26, 27).

In the plasmid system, shRNAs are generated by RNA polymerase II or III-based vectors under the control of U6 or H1 promoter. To suppress endogenous gene expression through RNAi has been reported, but we know little about the effect of RNAi on cell cycle and proliferation in human cancer cells. In present study, we evaluated the effect of inhibiting endoge-

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nous *cdk2* expression on the characteristic of cell growth by using RNA ploymerase III-based plasmids with U6 promoter in Hela and U-2 OS cells.

RESULTS

Construction of RNAi plasmid for shRNA-*cdk2*

According to the available methods for shRNA construction, four *cdk2* shRNA expressing plasmids were constructed using the parent plasmid BS/U6 (Suppl. Fig. 1A). The target sequences were selected in the coding region of *cdk2* gene (Suppl. Table 1). In order to construct the plasmids, four pairs of oligonucleotides were successfully synthesized. The oligonucleotides were annealed and were used as the templates for the synthesis of RNAs under the control of the U6 promoter. The small RNAs were composed of two identical target sequence motifs in an inverted orientation, separated by a 6-bp spacer. And five Ts were added at the 3′end of the repeat. The small RNAs were predicted to fold back to form a hairpin-like dsRNA with a 3′overhang of several Ts (Suppl. Fig. 1B). These four RNAi plasmids for shRNA-*cdk2* were successfully generated after the precise sequences of them were confirmed by sequencing.

Suppression of endogenous *cdk2* gene by siRNA synthesized from DNA templates

The successful post-transcriptional silencing of the *cdk2* by the selected siRNA nucleotide sequence was demonstrated by the highly significant decrease in the level of CDK2 protein. In order to examine the effects of the shRNA plasmids on inhibiting the expression of CDK2 protein, western blot was employed. The results showed the expression levels of CDK2 both in Hela and in U-2 OS cells transfected with the four shRNA plasmids were significantly different from the cells transfected with the control plasmid at 1, 2 and 3 days after transfection (Fig. 1A, B). Compared to the cells transfected with the control vector after one day post-transfection, the CDK2 protein levels were decreased by 68.2%, 72.7%, 77.3% and 41% in Hela cells; and the one were decreased by 37.5%, 53.4%, 57.3%, and 33.3% in U-2 OS cells respectively. In contrast, β-actin expression was unchanged both in Hela cells and U-2 OS cells. The results indicated that the highest inhibition ratio was 77.3% and 57.3% induced by shRNA-834 in Hela and U-2 OS cells respectively (Fig. 1C).

To determine whether the shRNA plasmids could suppress the expression of endogenous *cdk2* mRNA, RT-PCR was applied. We evaluated the changes of *cdk2* expression both in Hela and U-2 OS cells at 1, 2 and 3 d after transfection. Comparing with the cells transfected by the control plasmid, the *cdk2* expression levels in the cells transfected with the RNAi plasmids were decreased significantly both in Hela and in U-2 OS cells (Fig. 2). Compared to the cells transfected with the control vector after three days post-transfection, the *cdk2* mRNA levels were decreased by 73.1%, 86.1%, 88.4% and 77.1% in Hela cells; and the one were decreased by 76.8%, 84.3%, 84.6%, and 75.8% in U-2 OS cells respectively. The results indicated that the highest inhibition ratio was 88.4% and 84.6% induced by shRNA-834 in Hela and U-2 OS cells respectively after three days post-transfection. Together, these results demonstrated that the shRNA plasmids could suppress

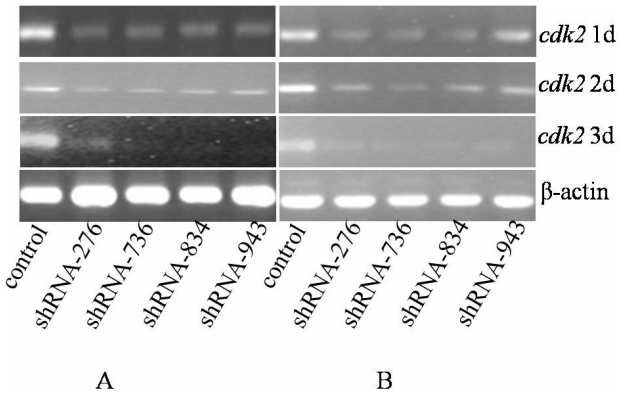

Fig. 2. RT-PCR analysis of the *cdk2* mRNA expression in Hela (A) and U-2 OS (B) cells.

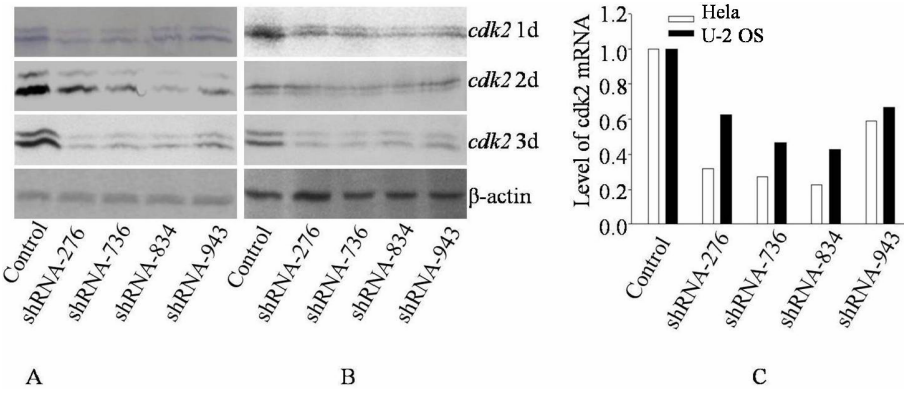

Fig. 1. Effect of RNAi on *cdk2* protein expression by western blot analysis. At1, 2 and 3 d after transfection with the four shRNA plasmids and the control plasmid both in the Hela (A) and U-2 OS (B) cells were analysized. The relative amount of *cdk2* protein at 1 d after transfection was evaluated by the density of the line with the Quantity One analysis software (C). The *cdk2* protein expression in the cells transfected with the control plasmid was assigned a value of 1.0.

the expression of *cdk2* mRNA successfully.

Inhibition of cell proliferation through suppression of cdk2 expression

The effects of these shRNA plasmids on cell proliferation were determined by MTT assay with the shRNA plasmids and BS/U6 plasmid transfected Hela cell and U-2 OS cells. The cell proliferation was dramatically suppressed by the shRNA plasmids. Compared with the cells transfected with the control plasmid (0.496 ± 0.013), the shRNA plasmids showed significantly reduced at 3 d after transfection both in Hela (0.222 ± 0.012; 0.187 ± 0.005; 0.189 ± 0.004; 0.269 ± 0.011) and U-2 OS (0.205 ± 0.009; 0.201 ± 0.007; 0.203 ± 0.008; 0.222 ± 0.006) cells (Suppl. Fig. 2, all the values of $P < 0.001$).

Cell cycle arrest by suppression of cdk2 expression

To examine the effects of RNAi induced by shRNA plasmids on cell cycle, flow cytometry assay was used. The DNA content analysis by flow cytometry revealed that transfection of shRNA plasmids induced a significant increase in the percentage of cells at the G1 phase of the cell cycle. In detail, the percentage of cell at G1 phase increased by 6.88%, 7.99%, 9.17%, 6.25% in the Hela cells transfected with the four plasmids respectively while the percentage of cells at S phase decreased by 3.65%, 8.57%, 13.16%, 5.12% in them respectively (Fig. 3B-E). Meanwhile, the percentage of cells at G1 phase increased by 2.5%, 8.21%, 4.59%, 2.48% respectively and the percentage of cells in S phase in U-2 OS cells decreased by 2.12%, 9.46%, 6.44%, 2.11% respectively (Fig. 4B-E). The cells transfected with the control plasmids were set as the control group. Taken together, we found that cell proliferation suppression induced by the shRNA plasmid was caused by induction of G1 arrest. These results demonstrated that *cdk2* commands the cell cycle from G1 phase to S both in Hela and U-2 OS cells.

DISCUSSION

Cervical cancer is one of the most common cancers in women worldwide with about 493,000 new cases each year resulting in 274,000 deaths globally (28). In 2006, cervical cancer remains the second most common cancer in women (29). Osteosarcoma, the most common bone malignant tumor, it had risen rapidly in recent years (30). The incidence rate occupies the first place in the bone primary stage malignant tumor. At present the treatment of the cervical cancer and osteosarcoma is mainly surgery and chemotherapy, but the prognosis misses extremely. Inhibiting specific gene expression by RNAi has become an important method of cancer treatment (31-34).

Inhibiting *cdk2* expression by RNAi is a promising method to the therapy of cancer as cancer cells often contain high levels of *cdk2* activity. In this study, we constructed shRNA-based DNA vectors which can inhibit the expression of *cdk2* both in Hela and U-2 OS cells. Comparing with control group, the mRNA and protein expressions were decreased significantly in

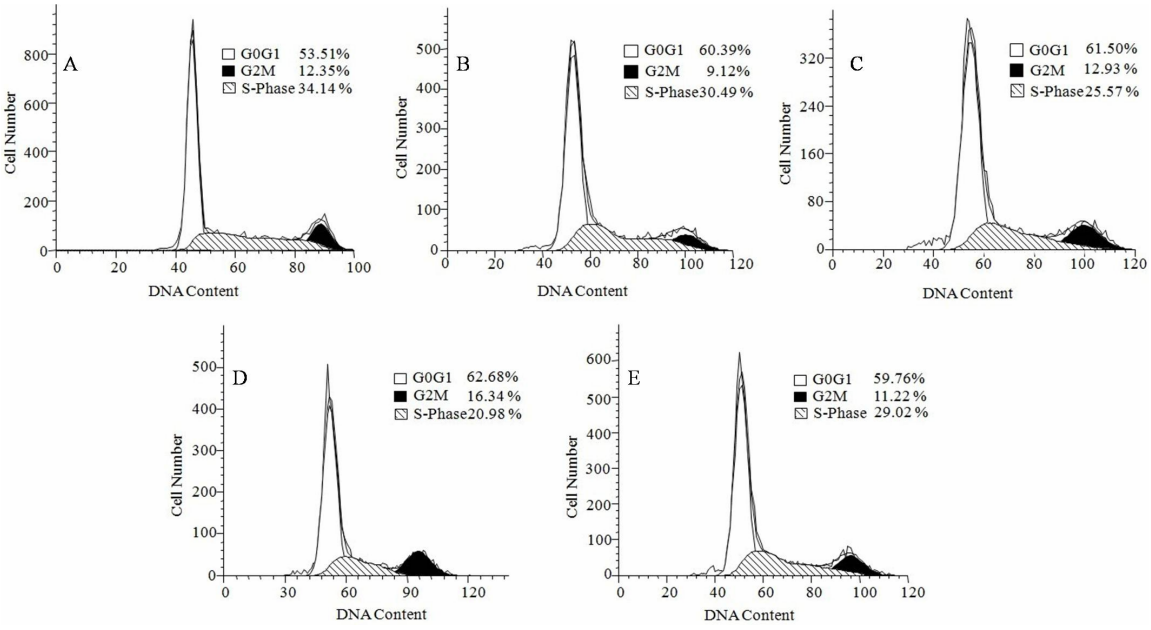

Fig. 3. Effect of shRNA plasmids on cell cycle in Hela cells. The data were analyzed with the ModFit LT FACS analysis software. Cells transfected with the control vector (A), shRNA-276 (B), shRNA-736 (C), shRNA-834 (D), shRNA-943 (E).

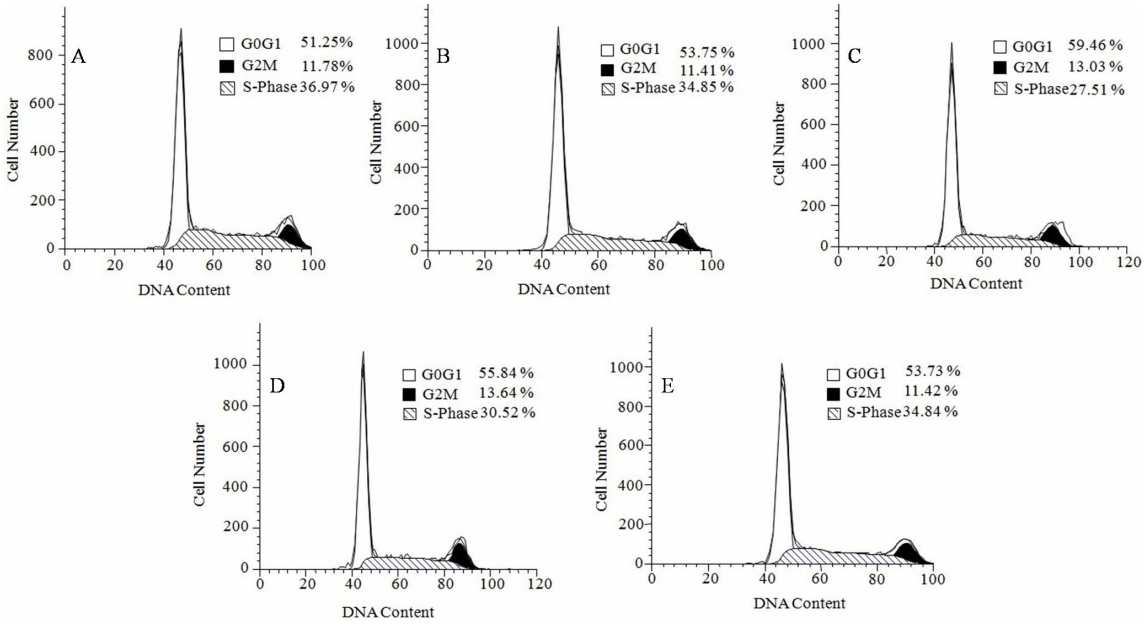

Fig. 4. Effect of shRNA plasmids on cell cycle in U-2 OS cells. The data were analyzed with the ModFit LT FACS analysis software. Cells transfected with the control vector (A), shRNA-276 (B), shRNA-736 (C), shRNA-834 (D), shRNA-943 (E).

the cells transfected with the different shRNA expression plasmids. According to the results of MTT and flow cytometry, we also found that the cell proliferation was suppressed by the shRNA plasmids. These findings demonstrated that *cdk2* plays a critical role both in Hela and U-2 OS cells. The shRNA-943 plasmid had the least efficiency in inhibiting *cdk2* expression in the four plasmids. It was probably because that it contains four Cs at the positions from 15 to 18, it only contains an A at the positions from 15 to 19 (35). The shRNA-276 plasmid was less effective than the shRNA-736 and shRNA-834 plasmids. This was probably because its target sequence located in the untranslated regions of *cdk2* sequence which can affect siRNA binding to RISC (36). Based on the results of RT-PCR and western blot, we found that the expression of endogenous gene *cdk2* mRNA was nearly completely inhibited both in Hela and U-2 OS cells 3 d after transfection. However, the suppression of the *cdk2* protein was less effective. It was probably because that degradation of the target gene expression by shRNA-mediated gene silencing occurs in the cytoplasm. Since there is expressed *cdk2* protein in the cytoplasm before gene silencing, therefore the *cdk2* protein expression existed at day 3 after transfection (37, 38).

The present study indicated that vector-mediated RNA interference could successfully inhibit the expression of *cdk2* in Hela and U-2 OS cells. The cell proliferation was suppressed and the cell cycle was arrested at G1 phase. These results manifested that *cdk2* plays an important role both in cell proliferation and cell cycle. Our study should be a powerful tool for investigating the mechanism of RNAi and the specific genes' function in mammalian cells. And it will provide a new approach for future clinical treatments in cervical cancer and osteosarcoma.

MATERIALS AND METHODS

Construction of RNAi plasmids

To generate an RNAi plasmid, subcloning an inverted repeat DNA template for the synthesis of siRNA into the relevant vector was involved. We selected the coding sequences for siRNA and analyzed by BLAST research to ensure that they did not have significant sequence homology with other genes (39). The sequence for the body of the siRNA for *cdk2* was taken from GenBank accession number NM001798, and the corresponding nucleotides of target sequences are 276-296, 736-756, 834-854 and 943-963. To construct the plasmid that contains DNA template for the synthesis of siRNA, four pairs of sense and antisense oligonucleotides were synthesized. They formed inverted repeat DNA templates after annealing the sense and antisense oligonucleotides. Then they were digested with double endonucleases (*Apa* I and *EcoR* I) and inserted into the BS/U6 vector (gift of Yang Shi, Harvard Medical School) digested with the same two endonucleases. The precise sequences of the inserted DNA templates under the control of the U6 promoter were confirmed by sequencing. These RNAi plasmids are named as shRNA-276, shRNA-736, shRNA- 834 and shRNA-943, respectively.

Cell culture and transfection

Hela and U-2 OS cells (Cell Resource Centre, Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA). The cells were maintained in a humidified 37°C incubator with 5% CO_2, fed every 3 days with complete medium and sub-cultured with 0.25% (w/v) Trypsin when 100% confluence was reached. One day before transfection, 2×10^5 cells per well were plated in 6-well plates. The transfection of different RNAi plasmids was performed using Lipofectamine 2,000 (Invitrogen, USA) according to the manufacturer's protocol. The parent plasmid BS/U6 was used as a negative control.

Western blotting

At 1, 2, and 3d after transfection, cells were washed three times with cold PBS and were lysed in ice-cold Lysis Buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 100 ug/ml PMSF). Thirty minutes later, the cells pellets were collected by scraping. Then they were transferred to new tubes and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was used for protein determination by the Bradford procedure (Bio-Rad, USA) and western blotting. The proteins were resolved on 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with the appropriate antibodies. The anti-CDK-2 antibody and anti-β-actin were obtained from Santa Cruz Biotechnology and used at a 1 : 500 dilution. The IgG-AP was used at a 1 : 5,000 dilution. The detection about anti-CDK-2 and anti-β-actin were performed with BCIP/NBT and DAB (Boster, China) respectively according to the manufacture's manuals.

RT-PCR

Total cellular RNA was isolated from the transfected cells by RNAiso reagent (Takara, Japan) according to the manufacturer's instruction. Before the DNA was amplified, the primers for *cdk2* gene amplification were chemically synthesized as follows: *cdk2*-F: 5' TTT CTG CCA TTC TCA TCG G 3'(19nt), *cdk2*-R: 3' CTT GGC TTG TAA TCA GGC ATA GA 3'(23nt). And the primers for β-actin were as follows: F: 5'ATC CTC ACCCTG AAG TAC CCC A-3', R: 5'CTC GGC CGT GGT GGT GAA GCT GTAGCC GCG CT-3'. The cDNA was synthesized by using total RNA (1 μg), oligo $(dt)_{18}$ primer (1 ul, 50 uM) and reverse transcriptase M-MLV (Takara, Japan). The reverse transcription was performed by first incubating at 70°C for 10 minutes and then at 42°C for 60 minutes followed by incubating at 70°C for 15 minutes. The three-step cycling for PCR was performed as follows: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C and extension for 45 seconds at 72°C. The 3-step cycle was repeated for 30 cycles and followed by a final extension at 72°C for 10 minutes. Each of the PCR products was separated on 1.5% agarose gel and visualized by ethidium bromide staining. We used the housekeeping gene of β-actin as an endogenous control.

MTT

One thousand cells were plated in 96 well plates per well, and were transfected according to the manufactures' instruction. At 1, 2, 3 and 4 d after transfection, 20 ul of MTT (5 mg/ml) was added to each well and the cells were incubated for 4 hours at 37°C with 5% CO_2. After incubation, the media was carefully removed from the plates and 200 ul of dimethyl sulfoxide was added to each well to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 492 nm using an automatic microplate reader (Labsystem, Finland).

Flow cytometry, FCM

The cells were harvested in 1.5 ml tubes 1 d post-transfection and fixed in 70% ice-cold ethanol and followed by RNAse A treatment, stained with 1 ml of propidium iodide for DNA content analysis by flow cytometry analysis on a FACS Calibur system (Becton Dickinson, USA). The data were collected and processed using the ModFit LT FACS analysis software.

Statistical analysis

All data are expressed as means ± SD. The data were analyzed statistically using the student's t test and $P < 0.05$ level of significance was chosen in all of our analyses. The control group was set as reference.

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