



siRNA-mediated silencing of FOXQ1 expression inhibits cell proliferation and induces apoptosis in human osteosarcoma cells

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Background: This study aimed to explore the expression of forkhead box Q1 (FOXQ1) and its effects on proliferation and apoptosis in human osteosarcoma cell line U2OS.

Methods: The expression of FOXQ1 was detected by Western blotting in osteosarcoma cell line U2OS. U2OS cells were divided into three groups: experimental group (FOXQ1-siRNA group), negative control group (NC-siRNA group) and blank group (untransfection group). FOXQ1-targeted siRNA and control siRNA were transfected into U2OS cells in FOXQ1-siRNA group and NC-siRNA group, respectively. The mRNA and protein levels of FOXQ1 were determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively. U2OS cell proliferation was detected by MTT assay and apoptosis was measured by flow cytometry.

Results: FOXQ1-targeted siRNA transfection significantly inhibited FOXQ1 gene expression at both mRNA and protein levels ($P < 0.05$). MTT assay result showed that the proliferation rate of U2OS cells was significantly lower in FOXQ1-siRNA group compared with NC-siRNA group ($P < 0.05$) and untransfection group ($P < 0.05$). Flow cytometry analysis showed that the apoptosis ratio was significantly higher in the experimental group than those of NC-siRNA group ($P < 0.05$) and untransfection group ($P < 0.05$).

Conclusions: *In vitro* FOXQ1-targeted siRNA could effectively inhibit the expression of FOXQ1 at both mRNA and protein levels in human osteosarcoma cell line U2OS. Downregulation of FOXQ1 expression inhibits cell proliferation and induces cell apoptosis of U2OS cells.

Keywords: Apoptosis; forkhead box Q1 (FOXQ1); osteosarcoma; proliferation; U2OS cell

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Introduction

Osteosarcoma (OS) is known as the most common malignant primary bone tumor in children and young adults (1,2). It causes death in patients as the main results of the high degree of malignancy and early occurrence of metastasis, with the 5-year survival rate of the non-metastatic patients reaching 65% (3). Therefore, it is in urgent need to identify novel diagnostic and prognostic biomarkers and potential therapeutic targets to improve the prognosis in patients with OS. Although numerous genetic

alterations have been reported to be associated with OS, molecular mechanisms underlying OS pathogenesis remain to be clarified. Forkhead box Q1 (FOXQ1) is a member of the forkhead transcription factor family, which is 2,338 base pairs in length and located in chromosome 6p25.3 (4). FOXQ1 is involved in chemoresistance, epithelial-mesenchymal transition, cell cycle progression, apoptosis, invasion, and metastasis of multiple cancers (5-8). Recently, studies have shown that FOXQ1 is overexpressed in lung cancer, breast cancer, and prostate cancer (9,10). In

this study, we investigated the effect of FOXQ1 on cell proliferation and apoptosis in OS cells by RNA interference.

Methods

Human OS cell lines

U2OS cells were preserved by the Department of Orthopaedic Surgery, Tangdu Hospital, the Fourth Military Medical University. U2OS cells were cultured in MEM medium (Corning, USA) contained 10% fetal bovine serum (Gibco, USA) at 37 °C in humidified 5% CO₂.

siRNA transfection

Specific siRNA directed against FOXQ1 gene was designed using the sense 5'-GGACACCUCCTACAGGUCTT-3' and antisense 5'-ACCGCCACGTGAAGGGTAATT-3'. The negative control siRNA (NC-siRNA) was designed using the sense 5'-ATCCCCGAACGUGACACGUAT-3'. Before transfection, U2OS cells were plated in six-well plates at a density of 1×10⁵ cells per well. When cell density is up to 60%, transfection assay was performed with FOXQ1-siRNA or NC-siRNA (the final concentration of siRNA was 100 nM) using Lipofectamine TM2000 Reagent (Invitrogen, USA) according to the manufacturer's instructions. After incubation for 8 h, cells were rinsed and replenished with MEM containing 10% FBS. After transfection for 48 h or the indicated time points, cells were collected for further experiments. The experiment was replicated three times.

Real-time qRT-PCR

Cells were collected 48 h after transfection and total RNA was isolation. Real-time qRT-PCR assay was performed using SYBR Green dye. The qPCR reaction amplification conditions were 94 °C for 8 min, 95 °C for 10 s, 60 °C for 1 min for 35 cycles, and 72 °C for 45 s. FOXQ1 mRNA expression was measured using the sense 5'-G-AAGAACTCCATCCGCCACA-3' and antisense 5'-GCCT-TAAACACCTGGTCCAATGTC-3' primers. The β-actin was used as a control using sense 5'-TGGTTACCCATTGGGAAGTGTGTC-3' and antisense 5'-TCACGGCCTGTTGTGTAGGTC-3' primers. Relative expression was calculated using the 2^{-ΔΔCt} method and normalized to the expression of β-actin.

Western blotting

U2OS cells were harvested after transfection for 48 h. Cellular protein isolation and protein concentration determination were performed for Western blotting. Proteins were electrophoresed on 12% SDS-PAGE and transferred into nitrocellulose membranes at 100 V for 90 min. Membranes were blocked with 5% nonfat milk in TBST at room temperature for 2 h and incubated respectively with rabbit anti-FOXQ1 antibody and rabbit anti-β-actin antibody (CST, USA) at 4 °C overnight with gentle rocking. After washing with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies [goat anti-rabbit IgG (Pierce, USA)] at room temperature for 2 h. After extensive washing with TBST, proteins were visualized by ECL detection. The gray values were analyzed using Quantity One software, and FOXQ1 expression was represented by the gray value ratio of FOXQ1 to β-actin.

Cell proliferation assay

The three groups of cells were harvested and seeded at a density of 0.8×10⁴ cells per well in 96-well plates and cultured at 37 °C overnight. Each group was made in quadruplicating. Transfection was performed with FOXQ1-siRNA and NC-siRNA separately as described above. After 24, 48, 72, and 96 h treatment, 20 μL of MTT reagent was added into each well and the cells were incubated at 37 °C for 4 h. Then 200 μL of dimethyl sulfoxide (DMSO) was added into each well, and the absorbance values were measured at 490 nm in an automatic microplate reader. The experiment was replicated three times.

Apoptosis detection

Three groups of cells were harvested 72 h after transfection. Double staining with annexin V and PI was conducted according to the manufacturer's protocol. The stained cells were detected using FACS. The experiment was replicated three times.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). The results were presented as the mean ± standard error. Quantitative data were performed by one-way ANOVA. Statistical multiple comparisons were performed with LSD test. P<0.05 was considered statistically significant.

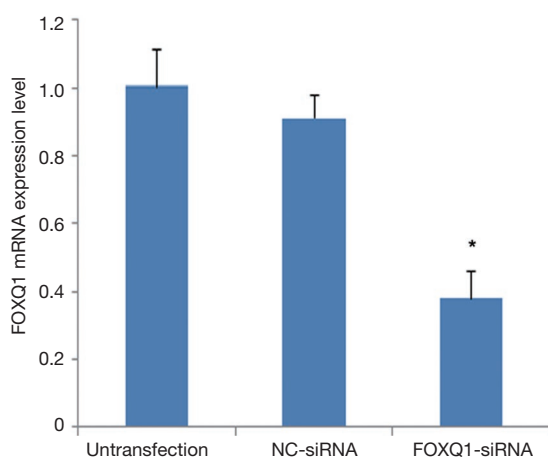


Figure 1 mRNA level of FOXQ1 in U2OS cells following siRNA transfection. mRNA expression of FOXQ1 was determined by qRT-PCR following siRNA-mediated knockdown. *, $P < 0.05$ vs. untransfection group or NC-siRNA group.

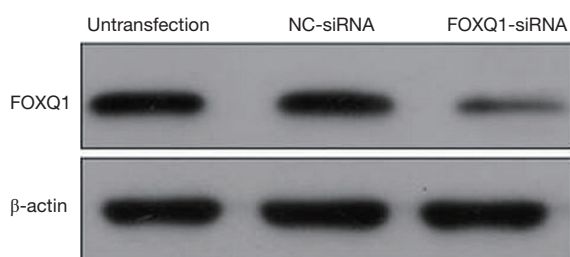


Figure 2 Protein level of FOXQ1 in U2OS cells following siRNA transfection. Protein expression of FOXQ1 was determined by Western blotting following siRNA-mediated knockdown.

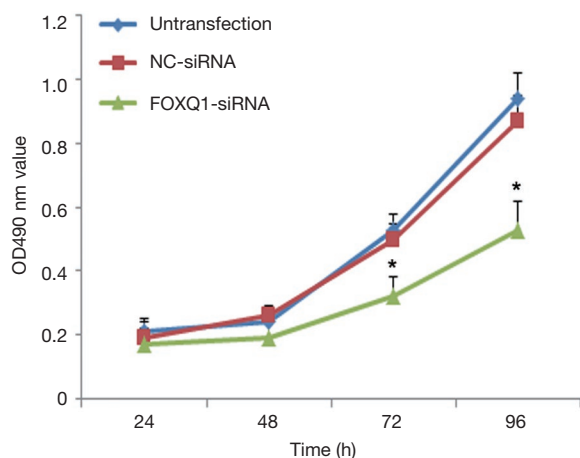


Figure 3 The effect of FOXQ1 knockdown on U2OS cell proliferation by MTT assay. *, $P < 0.05$ vs. untransfection group or NC-siRNA group.

Results

Special siRNA represses the expression of FOXQ1 mRNA expression

To explore the role of FOXQ1 in OS, inhibition of FOXQ1 by RNA interference is established in U2OS cells, as a high expression of FOXQ1 was detected in this cell line. We first performed the real-time qRT-PCR assay to determine the knockdown efficacy. The result showed that FOXQ1 mRNA expression was significantly lower in FOXQ1-siRNA group compared with those in the other two groups ($P < 0.05$, *Figure 1*). This result indicated that siRNA transfection effectively silencing FOXQ1 expression.

siRNA-mediated silencing suppress FOXQ1 protein expression

Furthermore, western blotting analysis showed that the protein level of FOXQ1 was remarkably depressed in FOXQ1-siRNA group compared with those in NC-siRNA group and untransfection group (*Figure 2*). This data confirmed that FOXQ1 expression was repressed effectively by siRNA-mediated silencing.

Downregulation of FOXQ1 expression inhibits cell proliferation

To assess the effect of FOXQ1 on the proliferation of U2OS cells, the cell viability of U2OS cells in untransfection group, NC-siRNA group, and FOXQ1-siRNA group were detected using MTT assay. The result showed that U2OS cells in FOXQ1-siRNA group proliferated more slowly than those in the other two groups (*Figure 3*). This result suggested that downregulation of FOXQ1 expression reduced U2OS cell proliferation *in vitro*.

Inhibition of FOXQ1 promotes apoptosis in U2OS cell line

Apoptosis is critical for cell proliferation. We next investigated whether downregulation of FOXQ1 has an effect on U2OS cell proliferation. The result of flow cytometry analysis showed that the apoptosis rate of U2OS cells in FOXQ1-siRNA group was significantly increased compared to those in NC-siRNA group and untransfection group (*Figure 4*), suggesting that inhibition of FOXQ1 expression enhanced the apoptosis of U2OS cells.

Discussion

FOXQ1 is a member of the FOX gene family, which is involved in embryonic development, cell cycle

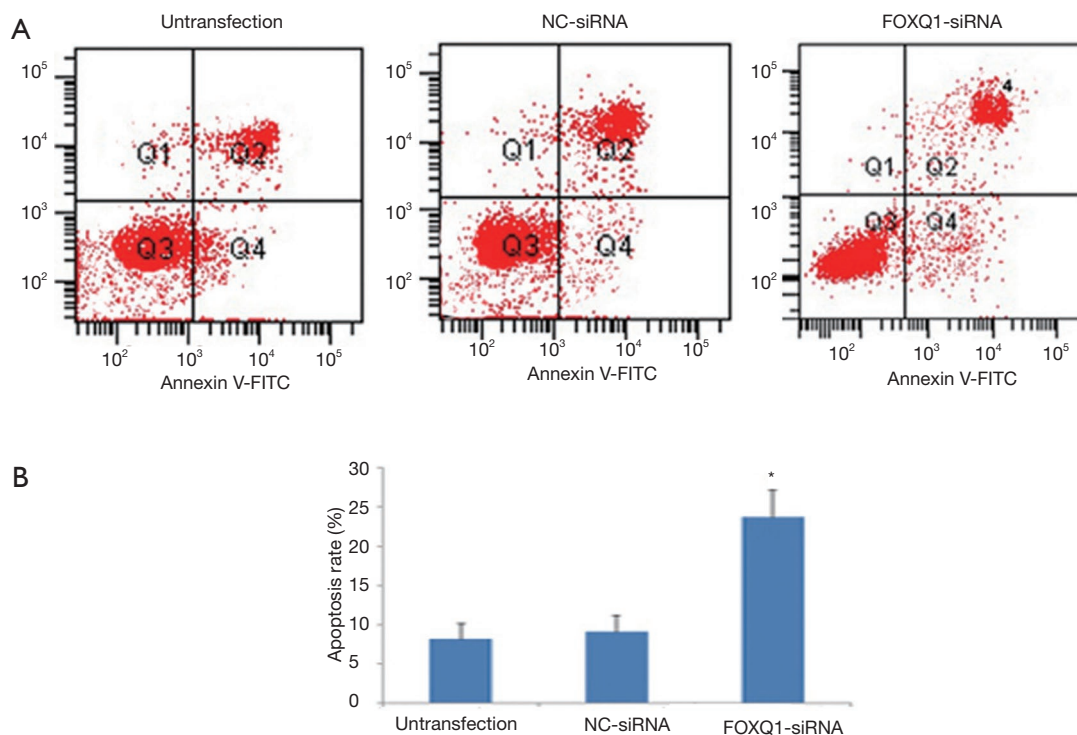


Figure 4 The effect of FOXQ1 knockdown on U2OS cell apoptosis by flow cytometry analysis. *, $P < 0.05$ vs. untransfection group or NC-siRNA group.

regulation, tissue-specific gene expression, cell signaling, and tumorigenesis (11-13). Recently, studies showed that FOXQ1 overexpressed in tumors is important in the progression and malignant proliferation of cancer cells (14). It has been reported that high FOXQ1 expression is correlated with large tumor diameter, high serum α -fetoprotein levels, and later stage grouping with tumor node metastasis classification (15). Feng *et al.* reported that FOXQ1 regulates EMT and increases chemosensitivity in non-small cell lung cancer cells (6), while Christensen *et al.* showed that the overexpression of FOXQ1 promotes cell proliferation, migration, and invasion of breast cancer cells (16). Pei *et al.* also demonstrated that FOXQ1 negatively modulates CDH1 to promote esophageal cancer proliferation and metastasis. These results suggest that FOXQ1 plays an important role in cancers.

Currently, despite the progress on OS treatment, the 5-year survival rate of Chinese patients remains to be 63.6%, and median survival rate to be 64.0% (17). Therefore, it is urgent to search novel molecular targets for the treatment of OS. A number of studies indicate that FOXQ1 is critical in tumorigenesis (18); however, the function and molecular mechanism of FOXQ1 involved in OS are still unclear. RNAi interference is a posttranscriptional sequence-

selective technique for the silencing of genes by suppressing gene expression in a sequence-specific method (19). Zhang *et al.* have reported that knockdown of FOXQ1 by siRNA significantly suppresses laryngeal cancer cell growth (Zhang, Li *et al.*, 2015). In this study, we found that FOXQ1 expression was detectable in U2OS cells and that siRNA-mediated silencing of FOXQ1 expression remarkably inhibited U2OS cell proliferation. Moreover, we found that knockdown of FOXQ1 induced apoptosis in U2OS cells, which was consistent with previous report that inhibition of FOXQ1 enhanced apoptosis of prostate cancer cells (Zhang, Wang *et al.*, 2016). It has been reported that FOXM1, another member of the FOX gene family, blocks cell cycle and promotes apoptosis in company with PIK-1, Survivin and CDC25B (Xu *et al.*, 2012). Whether FOXQ1 regulates OS cell apoptosis in a similar way is needed to be investigated in further studies.

In summary, our data provided evidence that siRNA-mediated targeting depression of FOXQ1 expression inhibits the proliferation and induces apoptosis in OS cells. Thus, FOXQ1 might be a novel molecular target for OS treatment.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2017.10.27>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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