

RUNX2 RNA interference inhibits the invasion of osteosarcoma

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Abstract. It has previously been demonstrated that the expression of the RUNX2 gene is increased in osteosarcoma tissues or cell lines; however, there is little research available on the effect of RUNX2 on osteosarcoma invasion. In the present study, small interfering (si)RNA to RUNX2 was designed and synthesized, and then transfected into SAOS-2 cells. The effects of RUNX2 RNA interference on the invasion of osteosarcoma cells were detected by the soft agar colony forming test and Transwell® chamber assay. The expression of the associated proteins, vascular endothelial growth factor (VEGF), matrix metalloprotein-2 (MMP-2) and MMP-9, was detected by western blot analysis. The results revealed that the number of cell colonies was reduced dose-dependently by the siRNA and that the number of cells permeating through the filter membrane was decreased following transfection with the siRNA. The inhibition of RUNX2 caused a notable decrease in VEGF, MMP-2 and MMP-9 expression (0.16 ± 0.04 , 0.16 ± 0.02 and 0.12 ± 0.02) compared with the empty vector (0.86 ± 0.22 , 0.74 ± 0.16 and 0.81 ± 0.16) and blank control (0.78 ± 0.12 , 0.82 ± 0.18 and 0.78 ± 0.14) groups, respectively ($P < 0.01$). It can therefore be concluded that RUNX2 siRNA inhibits the invasion of osteosarcoma cells by inhibiting the expression of VEGF, MMP-2 and MMP-9.

Introduction

Osteosarcoma is a common type of malignant tumor in adolescents and children and thus, its etiology and pathogenesis have previously been unknown. It accounts for ~2.4% of all malignancies in pediatric patients, and ~20% of all primary bone cancers (1). The estimated annual incidence rate of osteosarcoma in patients <20 years of age in the USA is 5.0 per million individuals, and the disease is slightly more

common in males (5.4 per million individuals) than females (4.0 per million individuals) (1). The prognosis of osteosarcoma is poor. Surgery is the main treatment, however, surgery alone may not prevent metastasis. However, surgery in combination with chemotherapy may prolong patient survival time (2). Certain studies have shown that RNA interference (RNAi) knockdown of cyclo-oxygenase 2 (3), ERK1/2 (4) or relaxin (5) inhibits the growth, invasion and migration of human osteosarcoma cells. The RUNX2 gene plays a crucial role in osteoblast differentiation and bone formation, and is closely correlated with the generation and development of osteosarcoma (6). Studies revealed that the expression of the RUNX2 gene was increased in osteosarcoma tissues or cell lines (7-10), but there is limited research available on the effect of RUNX2 on osteosarcoma invasion. In the present study, RUNX2 was downregulated in the osteosarcoma SAOS-2 cell line by RNAi, and its effects on cell invasion and the potential regulatory mechanism were studied.

Materials and methods

Experimental materials. The human osteosarcoma SAOS-2 cell line (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was stored in liquid nitrogen in the laboratory (Central Laboratory, Tongji Medical College, Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) was purchased from Gibco, Carlsbad, CA, USA. Rabbit anti-human RUNX2 (26 kDa; 1:1,000), matrix metalloprotein-2 (MMP-2; 1:1,000) and MMP-9 (1:1,000), and vascular endothelial growth factor (VEGF; 1:1,000) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Oligofectamine™ and RUNX2 siRNA double-stranded oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). Transwell® chamber models were obtained from Chemicon (Temecula, CA, USA). Western blot analysis kits were purchased from Wuhan Boster Biological Technology, Ltd., (Wuhan, China).

Construction of RUNX2 siRNA sequence and its transfection into the osteosarcoma SAOS-2 cell line. The two purchased RUNX2 siRNA oligonucleotides were identified with S1 (5'-UAACAGCAGAGGCAUUCGUAGCUC-3') and S2 (5'-GAGCUACGAAAGCCUCUGCUGUUA-3') in sequence determination, and were matched with the GenBank human RUNX2 cDNA sequence by Blast contrast. Additionally, the negative control sequence (Sn, UUCUCCGAACGUGUCACG

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UUUGUGC) was formulated and synthesized. Each sequence (100 nM) was transfected into the SAOS-2 cell line (1×10^5 cells/ml) by the Oligofectamine liposome. The cells were divided into five groups: The blank control (ConB), empty vector (ConN), S1 transfection (S1), S2 transfection (S2) and Sn transfection (Sn) groups. There was no difference between the treatments in each group, with the exception that ConB and ConN were transfected with phosphate-buffered saline and empty vector of the same concentration, respectively. Only the most effective siRNA was used in the following study.

Western blot detection of protein expression. Cells in the exponential growth phase were selected with cellular protein extracted by radioimmunoprecipitation assay buffer (Wuhan Boster Biological Technology, Ltd.) and the protein level was determined using the bicinchoninic acid protein extraction kit (AR0146; Wuhan Boster Biological Technology, Ltd.). Protein (50 μ g) was added with 2X loading buffer. Subsequent to a 5-min denaturation at 100°C, the proteins were separated by SDS-PAGE and then transferred onto a nitrocellulose filter. The filter was incubated with specific antibodies overnight at 4°C prior to being incubated with secondary antibodies, followed by enhanced chemiluminescence (Wuhan Boster Biological Technology, Ltd.) staining and autoradiography. Images were put into grayscale analysis using BandScan version 5.0 software (Glyko, Inc., Novato, CA, USA).

Soft agar assay for cell anchorage-independent growth. Cells in the exponential growth phase were suspended (1×10^3 cells/ml). Soft agar (5%) and nutritive medium were mixed at 1:9, agitated, added to the culture dish and coagulated at room temperature. Cell suspension (1.5 ml) was mixed with an equivalent volume of 5% soft agar, agitated and placed into the dish. Cell colony formation was observed following 2 weeks of culture at 37°C in air containing 5% CO₂. The rate of colony formation (%) was calculated based on the following formula: Colony formation rate = (number of colonies / number of cells incubated) \times 100.

In vitro invasion assay. A Transwell chamber (pore size, 5 μ m) and 24-well culture plates were purchased from Corning Incorporated (Corning, NY, USA). DMEM without and with 10% FBS was added to the top and bottom layers of the Transwell chamber, respectively. The cell suspension was adjusted to 1×10^5 cells/ml, 50 μ l of which was added to the upper chamber of the Transwell. At 24 h post-incubation, the cells adhering to the interior of the chamber were collected and fixed using 10% formalin, followed by Giemsa dyeing. The cells that had migrated through the membrane were counted. The membrane penetration rate = (number of cells penetrated/number of cells added to the upper chamber) \times 100.

Statistical analysis. Data are expressed as the mean \pm standard deviation, and t-tests were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Silencing effect of siRNA on RUNX2 protein. RUNX2 siRNA (100 nM) was transfected to the SAOS-2 cell line and western

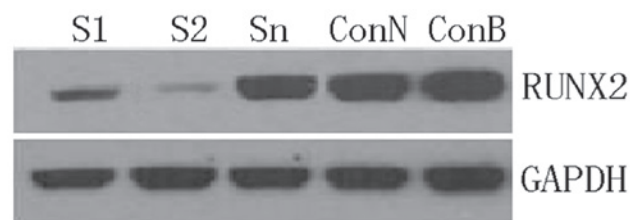


Figure 1. small interfering (si)RNA suppresses RUNX2 protein expression in the SAOS-2 cell line. RUNX2 exhibited high expression levels in ConN, ConB and Sn. Following siRNA transfection, RUNX2 was significantly downregulated in S1 and S2, but particularly S2.

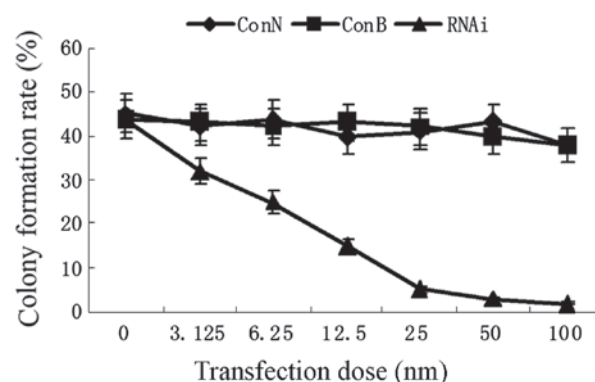


Figure 2. Cell colony formation rate is decreased gradually with increasing S2 small interfering RNA concentration (0, 3.125, 6.25, 12.5, 25, 50 and 100 nM) in the SAOS-2 cell line.

blot analysis was used to detect RUNX2 protein expression. RUNX2 protein expression in Sn, ConN and ConB demonstrated a grayscale ratio (RUNX2/GAPDH) of 1.26 ± 0.21 , 1.32 ± 0.23 and 1.34 ± 0.26 , respectively, with no significant differences in the three groups ($P > 0.05$). RUNX2 protein in S1 and S2 exhibited a lower expression level (grayscale ratios of 0.25 ± 0.04 and 0.12 ± 0.02 , respectively) compared with that of the other three control groups ($P < 0.01$), particularly in S2, in which the RUNX2 level decreased by almost 91% compared with the control groups (Fig. 1).

Effect of RUNX2 siRNA on the anchorage-independent growth of SAOS-2. Based on the finding that S2 exhibited the highest efficacy in interfering with RUNX2, S2 was used as the RUNX2-specific interference sequence and set as the RNAi group. Soft agar assay revealed that SAOS-2 formed colonies spontaneously in an *in vitro* culture system. The cell colony formation rate was decreased gradually with the increasing S2 siRNA concentration (0, 3.125, 6.25, 12.5, 25, 50 and 100 nM), while the colony formation rate in the ConN and ConB groups demonstrated only small changes (Fig. 2).

Effect of RUNX2 interference on SAOS-2 invasion. A Transwell chamber was employed to detect the invasion ability of SAOS-2 at 48 h after S2 siRNA transfection at various concentrations. The results revealed that RUNX2 interference decreased the percentage of membrane-permeating cells significantly compared with the control group (Fig. 3). Moreover, the cell membrane-penetrating rate was decreased gradually with the increasing siRNA concentration (Fig. 4).

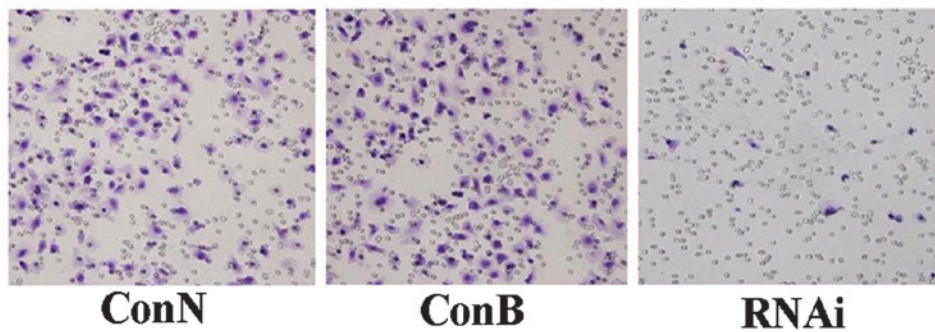


Figure 3. Decrease in membrane-penetrating cells following RUNX2 RNA interference in the SAOS-2 cell line.

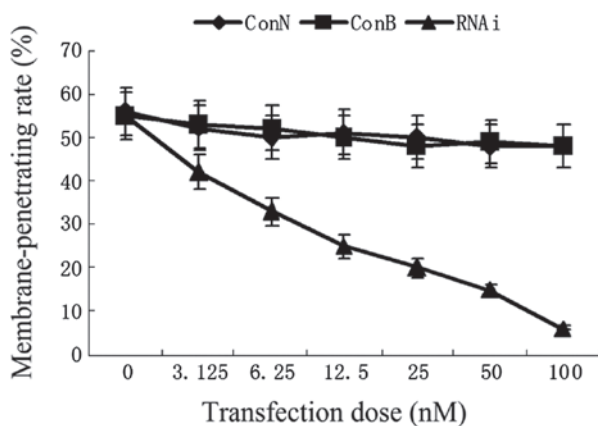


Figure 4. Cell membrane-penetrating rate is decreased gradually with the increasing small interfering RNA concentration in the SAOS-2 cell line.

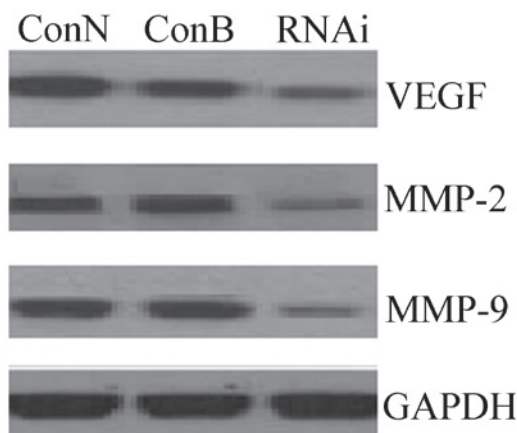


Figure 5. RUNX2 RNA interference downregulates the expression of vascular endothelial growth factor (VEGF) and matrix metalloprotein-2 (MMP-2) and MMP-9 proteins.

Effect of RUNX2 interference on VEGF, MMP-2 and MMP-9 proteins. In an attempt to elucidate the mechanism of RUNX2 in controlling osteosarcoma cell invasion, changes in VEGF, MMP-2 and MMP-9 expression levels were analyzed following RUNX2 interference. The results revealed that RUNX2 interference decreased VEGF expression significantly (0.16 ± 0.04) compared with levels in the ConN (0.86 ± 0.22) and ConB (0.78 ± 0.12) groups ($P < 0.01$). Levels of basal metalloproteinase family members MMP-2

and MMP-9 decreased significantly in the interference group (0.16 ± 0.02 and 0.12 ± 0.02 , respectively) compared with the ConN (0.74 ± 0.16 and 0.82 ± 0.18) and ConB (0.81 ± 0.16 and 0.78 ± 0.14) groups ($P < 0.01$) (Fig. 5).

Discussion

RUNX2 is a member of the RUNX family. As an osteoblast-specific transcriptional factor, it plays a dominant role in the growth and differentiation of osteoblasts and bone formation, and suppresses preosteoblast proliferation. RUNX2 expression is balanced in normal osteocytes, while it is highly expressed in osteosarcoma cells and forces their overgrowth, playing a similar role to oncogenes (11). It was previously reported that RUNX2 was upregulated in osteosarcoma tissues and that RUNX2 expression was associated with the clinical stage, metastasis and prognosis of the tumor (12). Other studies reported the correlation of RUNX2 expression with the clinical stage and progress of prostatic carcinoma (13) and thyroid cancer (14,15). The role and mechanism of RUNX2 in controlling osteosarcoma formation has previously been unknown, but it was reported that regulating RUNX2 expression could vary the local internal environment of osteocytes, affect osteosarcoma maturity, control apoptosis and matrix metalloproteinase activity and thereby affect osteosarcoma formation (16). It was demonstrated that downregulating RUNX2 could impair the activity of alkaline phosphatase and the deposition of calcium phosphate, and suppress osteosarcoma formation (17).

In the present study, the knockdown of RUNX2 using the RNAi technique was employed to observe the effect of the down-regulation of RUNX2 on the invasion ability of osteosarcoma. As a result, RUNX2-specific siRNA suppressed the expression of RUNX2 and achieved RUNX2 gene silencing after being transfected to SAOS-2 cells mediated by liposome (Fig. 1). Initially, a soft agar colony formation assay was performed to detect cell anchorage-independent growth. Anchorage dependence refers to cells that have to anchor with a given matrix to suppress apoptosis and survive. Tumor cells, on the other hand, would remain capable of growing in the absence of anchorage dependence (18). The soft agar colony assay is able to determine the anchorage-independent growth of tumor cells, as well as the malignancy, and the stronger invasion capacity of tumor cells is associated with a greater number of cell colonies (19). The present study revealed that RUNX2-specific siRNA suppresses the colony formation of SAOS-2 in soft agar in a concentration-dependent manner (Fig. 2). The migration and

invasion capacity of the tumors is correlated with the micro-environment for growth and the extracellular matrix (ECM). A Transwell chamber that imitates such an ECM is a reliable method of assaying the cell invasion ability (20). In the present study, following RUNX2 interference, the number of cells that passed through the Transwell were decreased significantly, and this decrease appeared to be siRNA concentration-dependent (Fig. 3). Preliminarily, this suggests that the interference of RUNX2 suppresses the invasion ability of SAOS-2.

The invasion and migration ability of tumors is strongly associated with their capacity to induce the production of proteinase, which is able to degrade the ECM and basement membrane (21). VEGF, which controls tumor vascularization, plays a key role in tumor formation, invasion and metastasis (22). However, the present study reveals that interfering with RUNX2 suppresses the invasion of SAOS-2 cells, while it decreases VEGF activity. MMPs are another type of molecule associated with tumor invasion. Among them, MMP-9 and MMP-2 regulate the activity of vascular endothelial cells and induce neovascularization. It has been demonstrated that RUNX2 controls the activity of MMP-13 in osteosarcoma cells (23,24), but the role of RUNX2 in MMP-2 and MMP-9 has not yet been reported. The findings of the present study indicate that RUNX2 suppression may inhibit the activity of MMP-2 and MMP-9, thus suppressing cell invasion.

In conclusion, RUNX2 RNAi attenuates the invasion ability of the osteosarcoma SAOS-2 cell line, and this may be attributed to the suppression of VEGF, MMP-2 and MMP-9. For this reason, RUNX2 is of crucial significance in regulating invasion and metastasis in osteosarcoma and is expected to become the targeted molecular therapy for osteosarcoma.

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