

Transcription factor NF-YA promotes a malignant phenotype by upregulating fatty acid synthase expression

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Abstract. Recent studies have revealed that increased expression of the alpha subunit of nuclear transcription factor Y (NF-YA) is associated with the malignant phenotype of various tumors. However, whether elevated expression of NF-YA promotes a malignant phenotype in osteosarcoma (OS), and the molecular mechanisms underlying this predicted effect is currently unknown. In the present study, small hairpin RNA (shRNA)-mediated knockdown of endogenous NF-YA significantly inhibited the migration and invasion capabilities of OS cells *in vitro*, whereas ectopic expression of NF-YA increased the migration and invasion capabilities of these cells. In addition, the induction of upregulated NF-YA expression on the malignant phenotype of OS cells was attenuated by silencing fatty acid synthase (FASN) expression. Furthermore, the expression level of FASN was increased by upregulating NF-YA, while decreased FASN expression was observed following NF-YA silencing in OS cells. The results of the present study suggest that NF-YA may promote a malignant phenotype in OS cells, in part, by activating the FASN signaling pathway, which may represent a promising target for the management of OS.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents, and demonstrates a high tendency to metastasize (1). The estimated worldwide incidence rate is 4,000,000 cases/year (2). Although the 5-year survival rates of patients with primary OS has been significantly improved from 30-50% to 60-90% by combining surgery with multiagent chemotherapy (3-5), the efficacy of chemotherapy is

reduced by the development of drug resistance (6). It is estimated that <30% of patients with recurrent disease achieve 5-year survival (7-9). Therefore, the identification of novel molecular targets and signaling pathways is necessary to improve the effectiveness of chemotherapy for the management of OS.

Nuclear transcription factor Y (NF-Y) is a ubiquitous protein, composed of three subunits; NF-YA, NF-YB and NF-YC. The genes that encode these proteins are highly conserved from yeast to mammals, with NF-YA acting as the regulatory subunit of the trimer (10). It has been demonstrated that NF-Y regulates gene expression by binding to promoter regions, and NF-Y is essential for cell proliferation (11-13). Multiple studies have recently demonstrated that increased expression of NF-Y in various cancers is associated with poor prognosis (14-16). However, whether elevated expression of NF-Y promotes a malignant phenotype in OS cells, and the molecular mechanisms underlying these putative effects remains unknown.

Fatty acid synthase (FASN) is an important enzyme involved in energy metabolism. Our previous studies suggested that FASN is overexpressed in OS cells and promoted its malignant phenotype (17,18). However, the mechanism underlying FASN overexpression remains unclear.

The present study aimed to determine whether NF-YA is involved in mediating the malignant phenotype of OS via regulation of FASN expression, by investigating the malignant phenotype of OS cells and FASN expression following knock-down or overexpression of NF-YA.

Materials and methods

Cell lines. The human OS cell lines, U2-OS and HOS, and the human osteoblast cell line, HOB, were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The U2-OS and HOS cells were cultured in Ham's/F-12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and HOB cells were cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc.), both supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37°C in 5% CO₂.

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Key words: alpha subunit of nuclear transcription factor Y, fatty acid synthase, osteosarcoma, malignant phenotype

Table I. Primer information.

Primer (product size)	Sequence (5'-3')
FASN (262 bp)	Forward: GTCGGAGAACTTGCAGGAGT Reverse: TCCTCGGAGTGAATCTGGGT
β -actin (295 bp)	Forward: TCACCCACACTGTGCCATCATCGA Reverse: CAGCGGAACCGCTCATTGCCAATGG
NF-YA (238 bp)	Forward: TTGTTGGTCAGGGTTTACAGC Reverse: ACGCTCCACGATGTCACTAA

FASN, fatty acid synthase; NF-YA, alpha subunit of nuclear transcription factor Y.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from OS and HOB cells was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA concentration was determined by measuring absorbance at a wavelength of 260 nm and purity was determined by calculating the 260/280 nm ratio with a BioPhotometer (Eppendorf, Hamburg, Germany). The mRNA expression levels of NF-YA and FASN were evaluated by RT-qPCR, using β -actin as the internal reference gene. The Two-Step RT kit (Promega Corporation, Madison, WI, USA) was used according to the manufacturer's protocol to synthesize cDNA, which was used as the template for amplification. TaqMan® Real-Time PCR Master Mixes (Thermo Fisher Scientific, Inc.) were used for amplification under the following cycling conditions: An initial denaturation step at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 20 sec and extension at 72°C for 20 sec. Data was normalized using the $2^{-\Delta\Delta C_q}$ method (19). All procedures were performed according to the manufacturer's protocols, with primer sequences as listed in Table I. A total of six independent experiments were performed over multiple days.

Lentivirus vector construction and cell transfection. HEK293 cells were used to produce lentiviral vectors, with the viral titer of 2×10^8 . To construct vectors for upregulating and downregulating NF-YA and FASN expression, the wild-type sequences and reverse complementary sequences (5'-ACTGAC TGACCAAACAGCAATAGTTCGACAGAGCACAGGACA CAAGGCCTGTTAC-3') were inserted into lentivirus vectors (Biomiga Inc., San Diego, CA, USA). U2-OS and HOS cells were transfected using polybrene (Genomeditech, Shanghai, China) with lentivirus vectors (multiplicity of infection=20) to upregulate NF-YA (Lv-up-NF-YA), downregulate NF-YA (Lv-down-NF-YA) and downregulate FASN (Lv-down-FASN). A non-targeting lentivirus vector (Lv-neg) was used as a negative control. The transfection efficiency was evaluated by fluorescence microscopy, as the lentiviral vectors expressed green fluorescent protein.

Western blot analysis. Total protein from cells was extracted using radioimmunoprecipitation assay lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) containing 60 μ g/ml phenylmethylsulfonyl fluoride, according to the manufacturer's protocol. Protein concentration was determined using the Bradford assay.

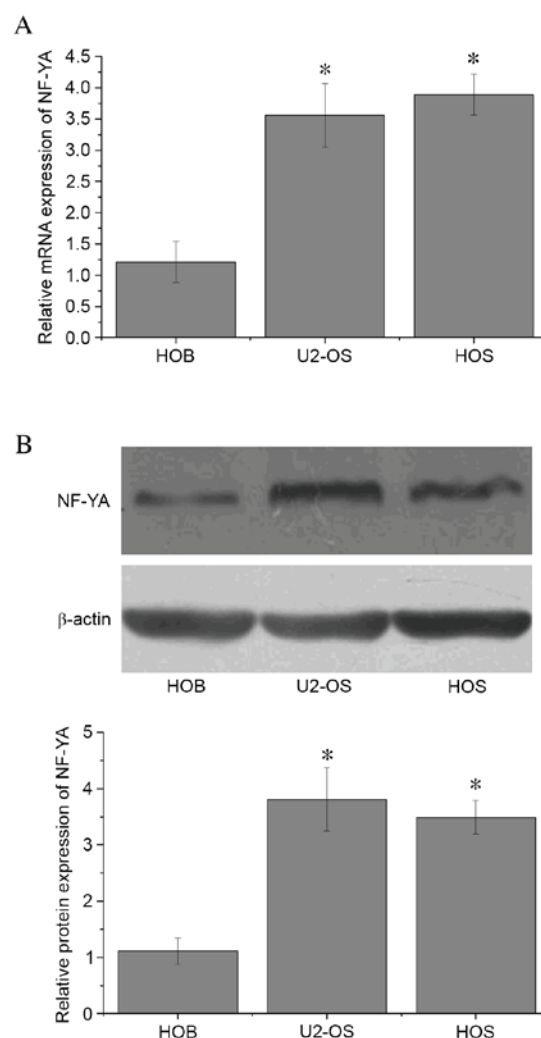


Figure 1. NF-YA mRNA and protein expression levels in human osteoblast HOB cells, and human osteosarcoma U2-OS and HOS cells. (A) mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction relative to β -actin. (B) Protein expression levels were detected by western blot and quantified relative to β -actin. Data are presented as the mean \pm standard deviation of six independent experiments. * $P < 0.05$ vs. HOB. NF-YA, alpha subunit of nuclear transcription factor Y.

Proteins (10 μ g) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with the following primary antibodies overnight

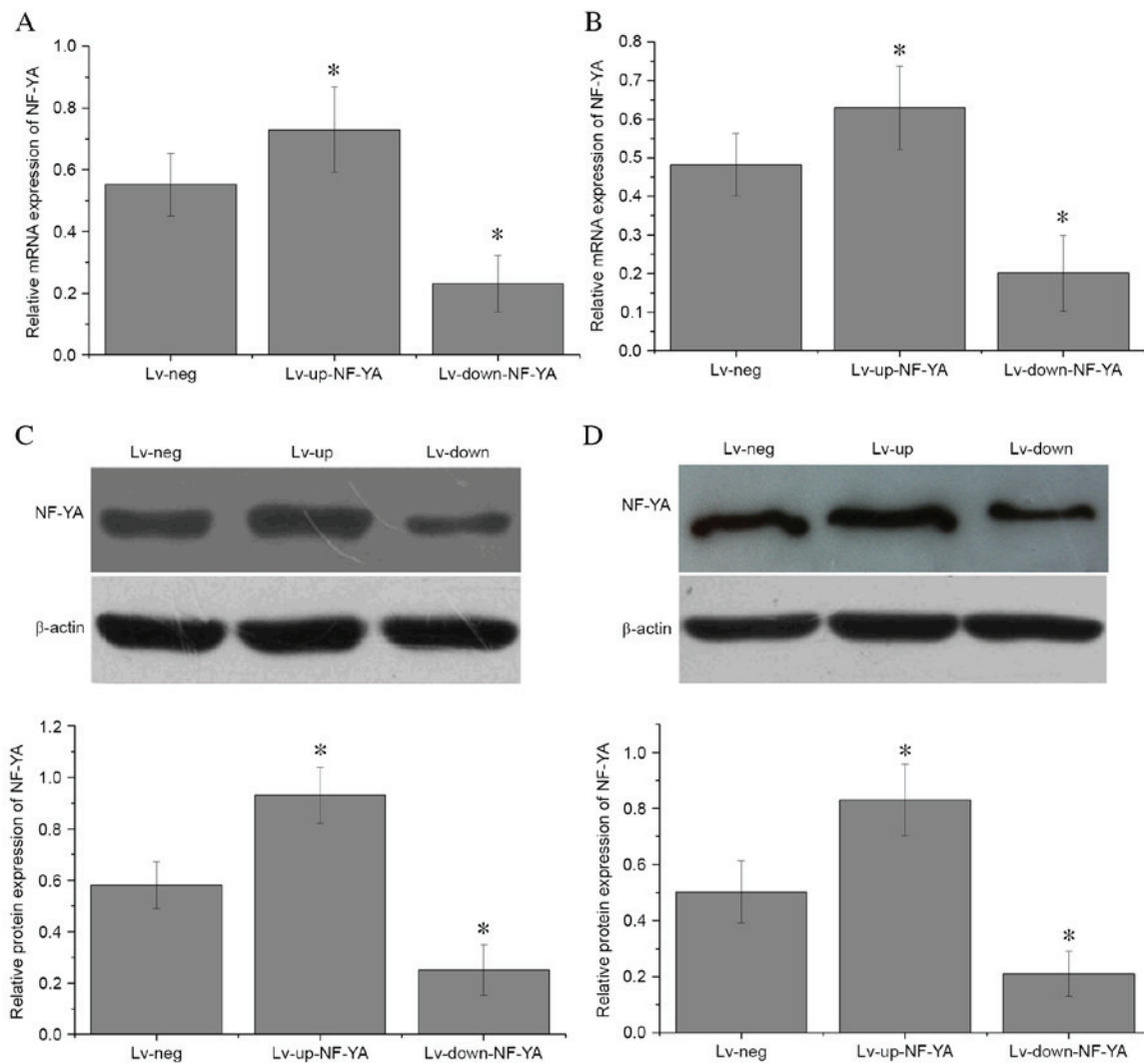


Figure 2. NF-YA mRNA and protein expression levels in OS cell lines following transfection with lentiviral vectors to upregulate and downregulate NF-YA. NF-YA mRNA expression levels in (A) U2-OS and (B) HOS cells were detected by reverse transcription-quantitative polymerase chain reaction relative to β -actin. NF-YA protein expression levels in (C) U2-OS and (D) HOS cells were detected by western blot and quantified relative to β -actin. Data are presented as the mean \pm standard deviation of six independent experiments. * $P < 0.05$ vs. Lv-neg. NF-YA, alpha subunit of nuclear transcription factor Y; Lv-neg, negative control lentivirus vector; Lv-up-NF-YA, lentivirus vector to upregulate NF-YA; Lv-down-NF-YA, lentivirus vector to downregulate NF-YA.

at 4°C: mouse anti-NF-YA (1:500; catalog no. sc-17753; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-FASN (1:500; catalog no. sc-55580; Santa Cruz Biotechnology, Inc.) and mouse anti- β -actin antibody (1:2,000; catalog no. sc-47778; Santa Cruz Biotechnology, Inc.). Following incubation with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000; catalog no. 610-103-043; Rockland Immunochemicals, Inc., Pottstown, PA, USA) for 1.5 h at room temperature, immunoreactive bands were visualized using an Enhanced Chemiluminescence reagent (Thermo Fisher Scientific, Inc.). The intensity of western blot bands was measured using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA). Six independent experiments were performed over multiple days.

Migration assays. Cell migration was assessed using a 'wound-healing assay', which measures the ability of cells to migrate into a two-dimensional space *in vitro*. In brief, cells were cultured to 80% confluence in 6-well tissue culture

dishes to a density of $\sim 5 \times 10^6$ cells/well. A line of cells was removed by scraping a line of cells from the center of the plate using a rubber policeman (Thermo Fisher Scientific, Inc.). Cultures were rinsed with PBS and fresh medium alone without 10% FBS was then added before the cells were incubated at 37°C for 24 h. Images were captured at 1 and 24 h using an electron microscope and camera (Canon, Inc., Tokyo, Japan), and the migrated distance was measured using ImageJ software version 1.48 (National Institutes of Health). For each sample, the cell migration rate was calculated by counting the number of migrated cells in three fields of view per well. A total of six independent experiments were performed on separate days.

Transwell invasion assays. Invasion of OS cells was measured using the BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. The medium in the lower chamber contained 5% fetal calf serum (Gibco; Thermo

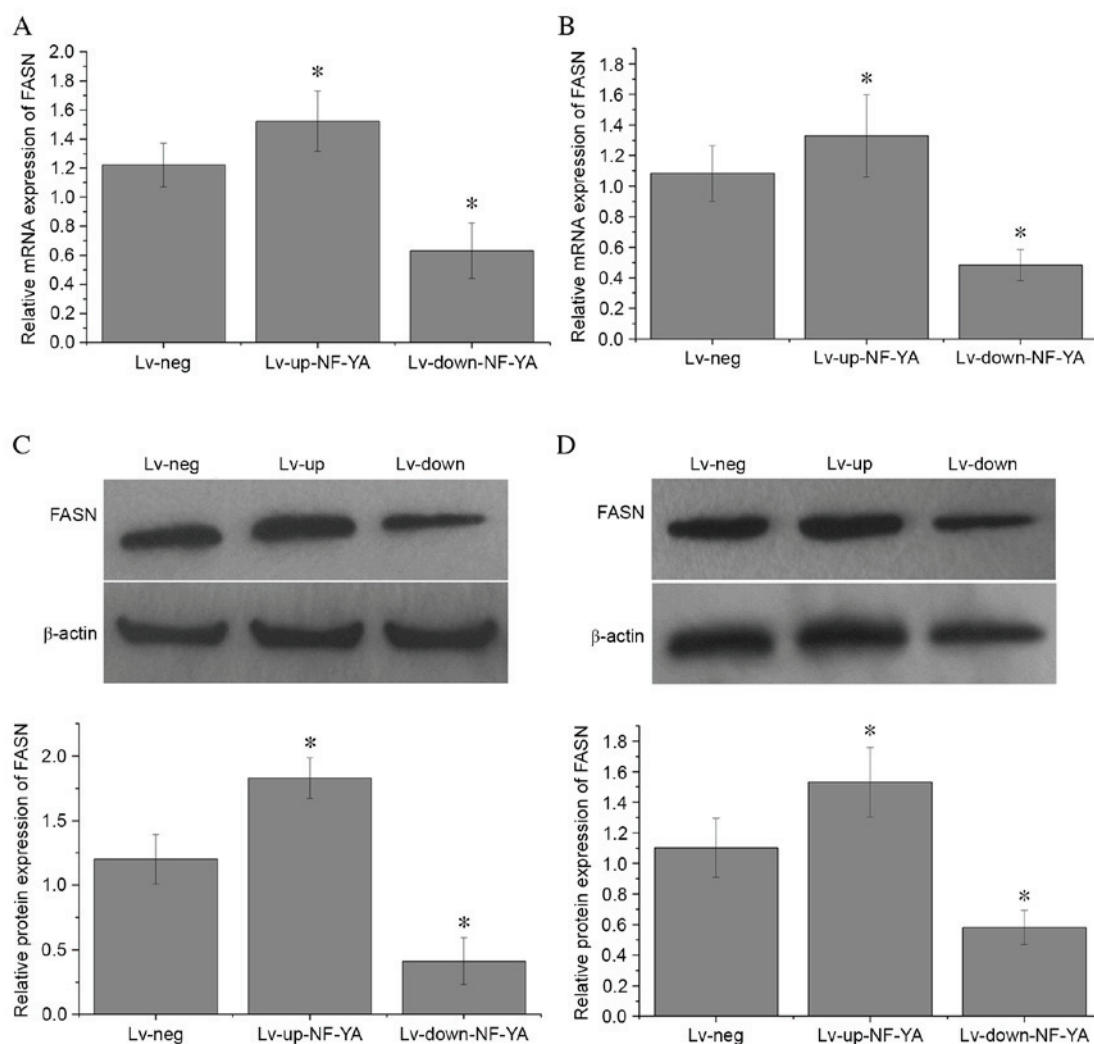


Figure 3. NF-YA regulates FASN expression in OS cell lines following transfection with lentivirus vectors to upregulate and downregulate NF-YA. FASN mRNA expression levels in (A) U2-OS and (B) HOS cells were detected by reverse transcription-quantitative polymerase chain reaction relative to β -actin. FASN protein expression levels in (C) U2-OS and (D) HOS cells were detected by western blot and quantified relative to β -actin. Data are presented as the mean \pm standard deviation of six independent experiments. * $P < 0.05$ vs. Lv-neg. NF-YA, alpha subunit of nuclear transcription factor Y; FASN, fatty acid synthase; Lv-neg, negative control lentivirus vector; Lv-up-NF-YA, lentivirus vector to upregulate NF-YA; Lv-down-NF-YA, lentivirus vector to downregulate NF-YA.

Fisher Scientific, Inc.) as a source of chemoattractants. Cells (1×10^3 /well) were suspended in serum-free medium and added to the upper chambers at the same time. Following 24 h of incubation, cells that passed through the Matrigel-coated membrane were fixed in methanol for 10 min and stained with Diff-Quik (Sysmex Corporation, Kobe, Japan). Images (original magnification, $\times 400$) were captured using an electron microscope and camera (Canon, Inc.). Images of three fields of view per membrane were captured and analyzed using ImageJ software version 1.48 (National Institutes of Health), with six independent experiments performed on separate days.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Student's t-tests were used for two-sample comparisons and one-way analysis of variance was used to compare >3 samples. $P < 0.05$ was considered to indicate a statistically significant difference. All analyses were performed using SPSS software (version, 13.0; SPSS, Inc., Chicago, IL, USA).

Results

Elevated NF-YA expression in OS cells. The mRNA and protein expression levels of NF-YA were assessed in U2-OS, HOS and HOB cells by RT-qPCR and western blot analyses, respectively. Expression levels of NF-YA mRNA (Fig. 1A) and protein (Fig. 1B) were significantly lower in the HOB osteoblast cell line when compared with the U2-OS and HOS OS cell lines, ($P = 0.01$; Fig. 1A). These results suggest that increased NF-YA expression may be a feature of OS cells.

Construction of lentivirus vectors and transfection into OS cells. U2-OS and HOS cells were transfected for 6 h with lentivirus vectors to upregulate (Lv-up-NF-YA) or downregulate (Lv-down-NF-YA) NF-YA expression. A non-targeting control (Lv-neg) was used as a negative control. NF-YA mRNA (Fig. 2A and B) and protein (Fig. 2C and D) expression levels were then assessed by RT-qPCR and western blot analyses, respectively. NF-YA mRNA expression was

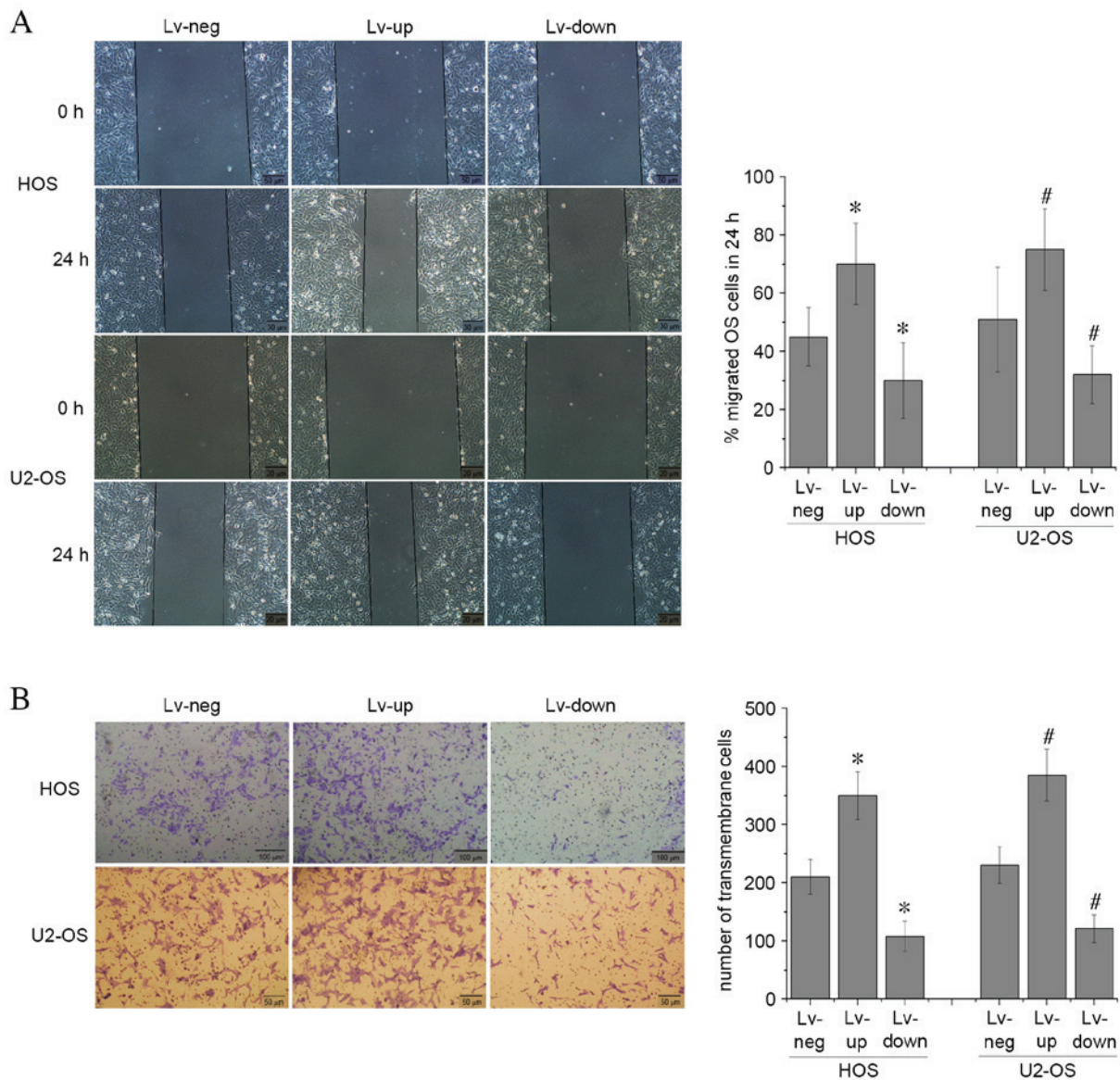


Figure 4. Effect of altered NF-YA expression on the migratory and invasive abilities of OS cells. Following transfection of U2-OS and HOS cells with lentivirus vectors to upregulate and downregulate NF-YA, (A) cell migration was assessed by wound healing assay, and (B) cell invasion was assessed by Transwell assay. Magnification, x400. Data are presented as the mean \pm standard deviation of six independent experiments. * $P < 0.05$ vs. Lv-neg in HOS cells; # $P < 0.05$ vs. Lv-neg in U2-OS cells. NF-YA, alpha subunit of nuclear transcription factor Y; Lv-neg, negative control lentivirus vector; Lv-up, lentivirus vector to upregulate NF-YA; Lv-down, lentivirus vector to downregulate NF-YA.

significantly decreased when compared with Lv-neg in U2-OS ($P = 0.01$; Fig. 2A) and HOS cells ($P = 0.01$; Fig. 2B) transfected with Lv-down-NF-YA. By contrast, NF-YA expression was significantly enhanced in U2-OS ($P = 0.03$; Fig. 2A) and HOS cells ($P = 0.01$; Fig. 2B) transfected with Lv-up-NF-YA compared with those transfected with Lv-neg. In addition, the protein expression levels of NF-YA in U2-OS ($P = 0.01$; Fig. 2C) and HOS cells ($P = 0.01$; Fig. 2D) transfected with Lv-up-NF-YA were significantly increased compared with Lv-neg-transfected cells. Furthermore, NF-YA protein expression was significantly lower in U2-OS ($P < 0.001$; Fig. 2C) and HOS cells ($P = 0.01$; Fig. 2D) transfected with Lv-down-NF-YA compared with those transfected with Lv-neg. These results indicate that the plasmids targeting NF-YA were constructed and transfected into OS cells successfully.

NF-YA regulates FASN expression in OS cells. In order to investigate whether NF-YA levels affect FASN expression in OS cells, the mRNA and protein expression levels of FASN were detected in U2-OS and HOS cells transfected with Lv-up-NF-YA, Lv-down-NF-YA and Lv-neg by RT-qPCR and western blot analyses, respectively. FASN mRNA expression was significantly decreased when compared with Lv-neg in U2-OS ($P = 0.02$; Fig. 3A) and HOS cells ($P = 0.01$; Fig. 3B) transfected with Lv-down-NF-YA. By contrast, FASN mRNA expression was increased in U2-OS ($P = 0.01$; Fig. 3A) and HOS cells ($P < 0.001$; Fig. 3B) transfected with Lv-up-NF-YA when compared with those transfected with Lv-neg. In addition, FASN protein expression levels in U2-OS ($P = 0.01$; Fig. 3C) and HOS cells ($P = 0.02$; Fig. 3D) transfected with Lv-up-NF-YA was significantly higher than those transfected with Lv-neg. FASN protein levels were significantly decreased in U2-OS

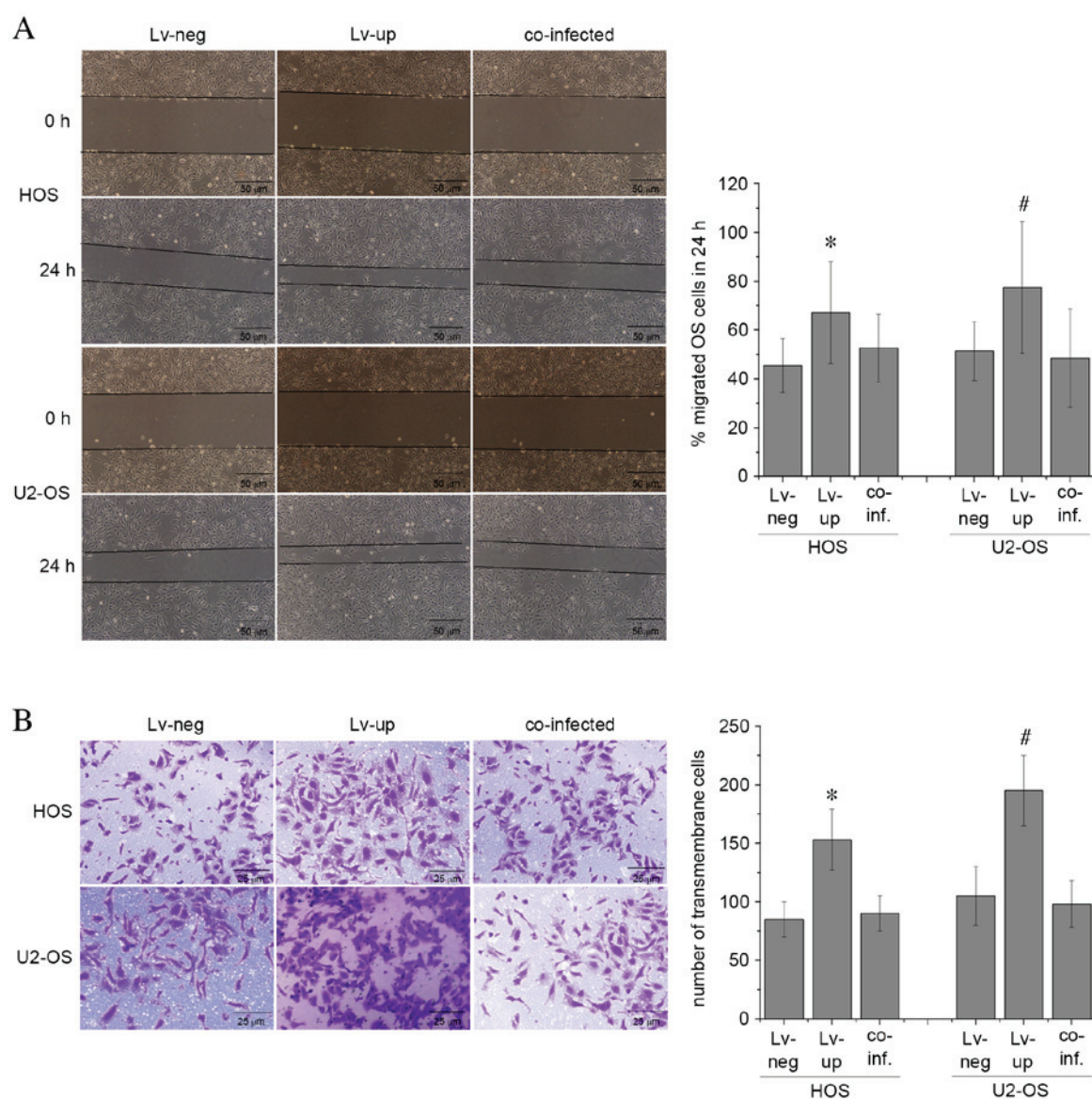


Figure 5. Effect on migratory and invasive abilities of OS cells following transfection of U2-OS and HOS cells with lentivirus vector to upregulate NF-YA, and co-infection with lentivirus vectors to upregulate NF-YA and downregulate FASN. (A) Cell migration was assessed by wound-healing assay, and (B) cell invasion was assessed using a Transwell assay. Magnification, $\times 400$. Data are presented as the mean \pm standard deviation of 6 independent experiments. * $P < 0.05$ vs. Lv-neg in HOS cells; # $P < 0.05$ vs. Lv-neg in U2-OS cells. NF-YA, alpha subunit of nuclear transcription factor Y; FASN, fatty acid synthase; Lv-neg, negative control lentivirus vector; Lv-up, lentivirus vectors to upregulate NF-YA; co-infected/co-inf., co-infection with lentivirus vectors to upregulate NF-YA and downregulate FASN.

($P = 0.01$; Fig. 3C) and HOS cells ($P = 0.01$; Fig. 3D) transfected with Lv-down-NF-YA compared with cells treated with Lv-neg. These findings indicate that alterations in NF-YA expression may affect FASN expression in OS cells.

NF-YA alters the malignant phenotype of OS cells *in vitro*.

In order to investigate the effect of altered NF-YA expression levels on the malignant phenotype of OS cells, cell migration and invasion abilities were assessed by wound healing and Transwell invasion assays, respectively, in U2-OS and HOS cells transfected with Lv-up-NF-YA, Lv-down-NF-YA and Lv-neg. The results revealed that OS cell migration (Fig. 4A) and invasion (Fig. 4B) capabilities were significantly increased in cells with elevated NF-YA levels when compared with cells transfected with Lv-neg. In addition, these markers of a malignant

phenotype were significantly inhibited in U2-OS and HOS cells following transfection with Lv-down-NF-YA compared with those transfected with Lv-neg ($P = 0.01$; Fig. 4). These findings suggest that elevated NF-YA levels may promote a malignant phenotype in OS cells *in vitro*.

NF-YA alters the malignant phenotype of OS by regulating FASN expression.

To investigate whether NF-YA facilitates the malignant phenotype of OS cells by activating FASN, the migration and invasion capabilities of U2-OS and HOS cells infected with Lv-neg, Lv-up-NF-YA, and co-infected with Lv-down-FASN and Lv-up-NF-YA were assessed. The proportion of migrated HOS and U2-OS cells was significantly higher following transfection with Lv-up-NF-YA when compared with cells transfected with Lv-neg ($P = 0.02$ and $P = 0.01$, respectively; Fig. 5A), with a

migration rate of $67.2 \pm 21\%$ in HOS and $77.5 \pm 27\%$ in U2-OS cells when transfected with Lv-up-NF-YA, compared with $45.5 \pm 11\%$ of HOS and $51.4 \pm 12\%$ of U2-OS cells in the Lv-neg group. Notably, this effect was abolished in the co-transfected cells, with migration rates of $52.6 \pm 14\%$ in HOS and $48.5 \pm 20\%$ in U2-OS cells following co-transfection with Lv-down-FASN and Lv-up-NF-YA. No statistically significant difference in cell migration was observed between co-transfected U2-OS and HOS cells when compared with Lv-neg-transfected cells ($P=0.07$ and $P=0.06$, respectively; Fig. 5A). The number of HOS and U2-OS cells that had traversed the membrane was significantly higher when transfected with Lv-up-NF-YA compared with Lv-neg ($P=0.01$ and $P=0.01$, respectively; Fig. 5B), with 153 ± 26 transmembrane HOS cells/high power field, $\times 400$ (Hp) and 195 ± 30 transmembrane U2-OS cells/Hp transfected with Lv-up-NF-YA, compared with 85 ± 15 transmembrane HOS cells/Hp and 105 ± 25 transmembrane U2-OS cells/Hp in the Lv-neg group. However, in the co-infected group, there were 90 ± 15 transmembrane HOS cells/Hp and 98 ± 20 transmembrane U2-OS cells/Hp, which was not significantly different when compared with HOS and U2-OS cells transfected with Lv-neg ($P=0.01$ and $P=0.01$, respectively; Fig. 5B). These results suggest that the effect of OS tumor cell migration and invasion mediated by NF-YA was reversed by silencing FASN. This indicates that NF-YA may promote the malignant phenotype of OS cells, in part, by regulating FASN expression.

Discussion

NF-Y is a ubiquitous protein, composed of 3 subunits, NF-YA, NF-YB and NF-YC, whose genes are highly conserved from yeast to mammals (20). While previous studies have aimed to understand the biological role of NF-Y, recent findings have revealed that NF-Y is involved in malignant tumor development (21-23). Clinically, elevated NF-Y genes indicate a poor prognosis in various cancers, such as breast and lung cancers (24). In the present study, expression of NF-YA was higher in OS cells compared with osteoblastic HOB cells. In addition, the malignant phenotype was inhibited in OS cells *in vitro* when NF-YA expression was silenced, while ectopic NF-YA expression promoted a malignant phenotype. These findings suggest that NF-YA may be involved in mediating the malignant phenotype of OS. However, the cellular and molecular mechanisms of this effect remain unclear.

FASN is an important enzyme for endogenous lipogenesis in mammals and is responsible for catalyzing the synthesis of long-chain fatty acids (25). Previous studies have demonstrated elevated FASN expression in a variety of human tumors, but low expression levels in normal tissues (26-28). Due to this increased expression in malignant tumor tissues, FASN may represent a novel diagnostic marker and therapeutic target. In addition, previous studies have revealed that inhibition of FASN promotes apoptosis, and reduces cell growth and metastasis *in vitro* and *in vivo* (29,30). FASN has been previously demonstrated to be overexpressed in OS tissues, while inhibition of FASN suppresses the malignant phenotype by inhibiting the phosphoinositide 3-kinase/Akt serine/threonine kinase 1/nuclear factor kappa κ -light-chain-enhancer of activated B cells signaling pathway (17,18). However, the molecular signaling pathways resulting from upregulated FASN expression in OS

remain to be fully elucidated. NF-Y is an important nuclear transcription factor that regulates the expression of numerous genes (31-33). Previous studies have demonstrated that NF-Y expression is a key component in the regulation of FASN expression by promoter activity in primary hepatocytes (34). In the present study, ectopic NF-YA expression was demonstrated to upregulate FASN expression in OS cells, while knockdown of NF-YA expression resulted in inhibition of FASN expression. In addition, the effect of NF-YA on OS cell migration and invasion was reversed by silencing FASN. These results suggest that NF-YA promotes cell proliferation, invasion and migration in OS, in part, via regulation of FASN expression.

The findings of the present study indicate that increased expression of NF-YA may promote a malignant phenotype in OS cells via modulation of FASN expression. However, further *in vivo* experiments are necessary to establish whether inhibition of NF-YA may represent a novel strategy in the management of OS.

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