

Inhibition of fatty acid synthase suppresses osteosarcoma cell invasion and migration via downregulation of the PI3K/Akt signaling pathway *in vitro*

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Abstract. In the present study, the effect of fatty acid synthase (FASN) inhibition on cell invasion and migration *in vitro* was investigated. A recombinant plasmid containing a microRNA targeting the FASN gene was used to inhibit FASN expression in U2-OS cells. Cell migration and invasion were investigated using wound healing and Transwell invasion assays. We found that cell invasion and migration were suppressed by inhibiting FASN. In addition, the effect of inhibition of FASN on phosphorylation of Akt was investigated by detecting the expression levels of pAkt using western blot analysis. Furthermore, protein expression levels of nuclear factor- κ B (NF- κ B; p65) and matrix metalloproteinase (MMP)-2 and -9 were also measured by western blot analysis. Results demonstrated that expression levels of pAkt, NF- κ B (p65) and MMP-2 and -9 proteins were reduced significantly by inhibiting FASN. Therefore, we confirmed that inhibition of FASN by RNA interference suppresses osteosarcoma cell metastasis via downregulation of the phosphoinositide 3-kinase/Akt/NF- κ B signaling pathway *in vitro*.

Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumors in children and adolescents. In the early 1970s, introduction of doxorubicin and methotrexate with leucovorin rescue demonstrated promise for the improvement of OS patient survival. The five-year survival rate for

patients treated with intensive multidrug chemotherapy and aggressive local control has been reported at 55-80% (1-3). However, despite the encouraging trend for longer survival, many patients still have a poor prognosis. Previous studies have estimated the five-year survival rate of patients with metastatic diseases to be <20% (4-6). Development of lung metastases is the main cause of mortality in patients with OS. Therefore, identification of the molecular mechanisms of metastasis in OS is likely to have a significant impact on management and prognosis of the disease.

Fatty acid metabolic pathways have been previously reported to be associated with carcinogenesis (7). Fatty acid synthase (FASN) is an important enzyme involved in endogenous lipogenesis in mammals and is responsible for catalyzing the synthesis of long-chain fatty acids. FASN has been identified as crucial for sustaining a number of biological features of cancer cells (8). The enzyme is expressed at high levels in a variety of human tumors (8-13), but remains at low levels in normal tissues. Various studies have reported that inhibition of FASN expression may suppress cancer cell proliferation *in vitro* and *in vivo* (14-18). In addition, FASN has also been hypothesized to contribute to cancer cell metastasis (19,20). However, it is currently unclear whether this molecule is involved in OS metastasis and the molecular mechanisms associated with FASN and metastasis remain unknown.

Matrix metalloproteinases (MMPs) are involved in degradation of the basement membrane and epimatrix, among which MMP-2 and -9 markedly correlate with tumor invasion and metastasis. MMP-2 and -9 are overexpressed in OS and promote OS cell migration and invasion by degrading components of the basement membrane and epimatrix. A large number of studies indicate that activation of the nuclear factor- κ B (NF- κ B) gene, an upstream regulator of MMPs, is closely associated with tumor invasion and migration (21,22). In addition, phosphorylation and activation of Akt has been recognized as an important regulatory factor in NF- κ B signaling. Specifically, activation of Akt has been identified to be essential for degradation of an inhibitor of NF- κ B, inhibitor of κ B (IkB) and NF- κ B activation mediated by IkB kinases

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(IKKs) (23). Previous studies have demonstrated that fatty acids synthesized by FASN are incorporated into membrane phospholipids, known modulators of Akt activation (24,25). Wang *et al* reported that inhibiting FASN decreases phosphorylation of Akt in ovarian cancer cells (26,27). Therefore, we hypothesize that inhibition of FASN may suppress osteosarcoma cell invasion and migration by downregulation of the phosphoinositide 3-kinase (PI3K)/Akt/NF- κ B signaling pathway.

In the present study, the effect of FASN inhibition on OS cell invasion and migration was investigated *in vitro*. In addition, the effect of inhibition of FASN on the PI3K/Akt/NF- κ B signaling pathway was investigated. Results indicate that inhibition of FASN may inhibit OS cell invasion and migration via downregulation of the PI3K/Akt/NF- κ B pathway *in vitro*.

Materials and methods

Construction of the recombinant plasmid containing miRNA targeting the FASN gene. The human cDNA sequence encoding FASN protein (NM_004104.4) was obtained from GenBank and miRNA and control single-strain DNA oligos were designed and synthesized using the following primer sequences: forward 5'-TGCTGAACTCCTGCAAGTTCTCCGACGTTTTGGCCACTGACTGACGTCGGAGATTGCAGGAGTT-3' and reverse 5'-CCTGAACTCCTGCAATCTCCGACGTCAGTCAGTGGCCAAAACGTCGGAGAACTTGCAGGAGTTTC-3'. Products were cloned into the express vector pcDNA6.2-GW/EmGFP-miR using the BLOCK-iT™ Pol II miR RNAi Expression Vector kit with EmGFP (K4936-00; Invitrogen Life Technologies, Carlsbad, CA, USA). The DNA sequence of the plasmid was confirmed using the PureLink HiPure Plasmid DNA kit (K2100-03; Invitrogen Life Technologies).

Cell culture and transfection. Human OS cell line, U2-OS, (Shanghai Cell Bank, Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO₂. U2-OS cells were seeded in 6-well plates at 30% confluence on the day prior to transfection. Transfection with recombinant plasmid targeting the FASN gene or negative plasmid was performed using Lipofectamine 2000 reagent. Transfection complexes were prepared according to the manufacturer's instructions (Invitrogen Life Technologies).

Western blot analysis. Total protein from the cells was extracted using RIPA lysis buffer containing 60 μ g/ml PMSF. Cell lysates were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blot analysis as described previously (28). Protein expression levels in cells transfected by recombinant plasmid were compared with those transfected by negative plasmid.

Real-time polymerase chain reaction (RT-PCR). RT-PCR was used to detect FASN mRNA levels. Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies). Total RNA concentration was determined by spectrophotometry at 260 nm and the purity was determined

by calculating the 260/280 ratio with a BioPhotometer (Eppendorf, Hamburg, Germany). RT-PCR and the Two-Step kit (Promega Corporation, Madison, WI, USA) were used to obtain cDNA according to the manufacturer's instructions, which was then used as the template for amplification. The following primers were used to amplify target sequences: FASN forward 5'-CCCACCTACGTACTGGCCTA-3' and reverse 5'-CTTGGCCTTGGGTGTGTACT-3', 294 bp; β -actin forward 5'-CGGGAAATCGTGCGTGAC-3' and reverse 5'-TGGAAGGTGGACAGCGAGG-3', 443 bp (Sangon, Shanghai, China). Following amplification, DNA electrophoresis was performed on standard 1% agarose gels and DNA was labeled and visualised using ethidium bromide. Images were captured using the Canon Digital IXUS 900Ti. FASN mRNA expression levels in cells transfected with recombinant plasmid was compared with cells containing the negative plasmid.

Wound healing assay. Cell migration was assessed by determining the ability of the cells to move into a cellular space in a two-dimensional *in vitro* 'wound healing assay'. In brief, cells were grown to confluence in 6-well tissue culture plastic dishes to a density of $\sim 5 \times 10^6$ cells/well. Cells were denuded by dragging a rubber policeman (Fisher Scientific, Hampton, NH, USA) through the center of the plate. Cultures were rinsed with PBS and replaced with fresh DMEM alone or containing 10% FBS, following which the cells were incubated at 37°C for 24 h. Images were captured at 0 and 24 h and the migrated distance was measured using ImageJ (NIH, Bethesda, MD, USA). Cell migration rate was calculated using 3 fields/area and presented as the average of 6 independent experiments performed over multiple days. The migration rate of cells transfected by recombinant plasmid targeting the FASN gene, was compared with cells transfected by negative plasmid.

Transwell invasion assay. Invasion of U2-OS cells was measured using the BD BioCoat™ BD Matrigel™ Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Medium in the lower chamber contained 5% fetal calf serum as a source of chemoattractants. Cells were suspended in DMEM and added to upper chambers at the same time. Cells that passed through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex, Kobe, Japan) and images were captured under a microscope (ECLIPSE-TS-100, Nikon, Japan; magnification, x400) at 0 and 24 h. Cell counts were performed using ImageJ. Values for invasion were obtained by counting 3 fields/membrane and presented as the average of 6 independent experiments performed over multiple days. The number of invaded cells transfected with recombinant plasmid targeting the FASN gene, was compared with cells transfected with negative plasmid.

Statistical analysis. All experiments were repeated 6 times. Data are expressed as the mean \pm SD of ≥ 3 experiments. Independent-samples T-test was performed for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference. All analyses were performed using SPSS version 13.0 (Statistical Software for Social Sciences, Chicago, IL, USA).

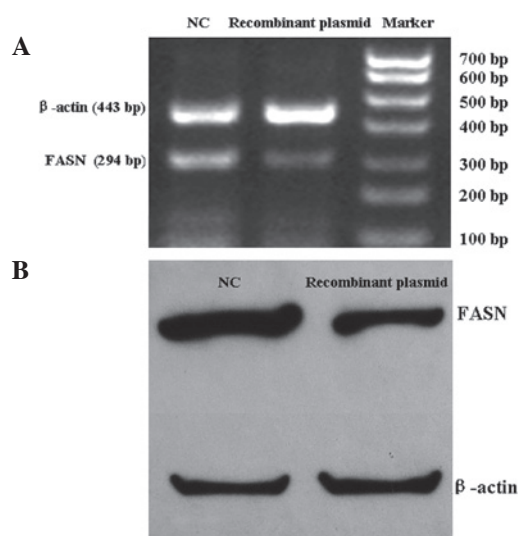


Figure 1. Inhibitory effect of recombinant plasmid on FASN expression. (A) RT-PCR analysis of FASN mRNA expression in U2-OS cells. FASN mRNA expression was lower in cells transfected with recombinant plasmid compared with negative control. (B) Western blot analysis of FASN protein expression in U2-OS cells. FASN protein expression was reduced in cells transfected with recombinant plasmid compared with negative control. FASN, fatty acid synthase; RT-PCR, real-time polymerase chain reaction; NC, negative control.

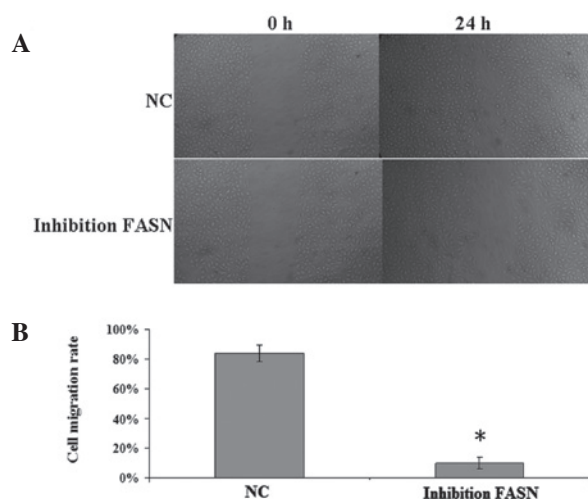


Figure 2. Effect of FASN inhibition by recombinant plasmid on U2-OS cell migration *in vitro*. (A) Wound healing assay. Migration distance was reduced in cells transfected with recombinant plasmid compared with negative control. (B) Quantification of wound healing assay. Data are presented as the mean \pm SD (n=6). *P<0.05, vs. negative control group. FASN, fatty acid synthase; NC, negative control.

Results

Effect of recombinant plasmid targeting FASN gene on FASN expression in U2-OS cells. Cultured U2-OS cells were transfected with the recombinant plasmid for 24 h. FASN mRNA and protein expression levels in U2-OS cells were detected by RT-PCR and western blot analysis (Fig. 1). FASN mRNA and protein expression levels in cells transfected with the recombinant plasmid were significantly lower than in those transfected with negative plasmid. These results indicate that recombinant

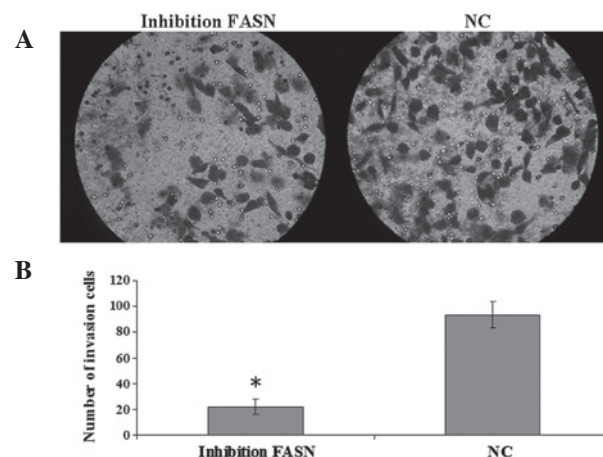


Figure 3. Effect of FASN inhibition by recombinant plasmid on U2-OS cell invasion *in vitro*. (A) Representative images of Transwell invasion assays using U2-OS cells at 24 h are presented for each group. (B) Quantification of Transwell invasion assay. The number of invaded cells was significantly lower in cells transfected with recombinant plasmids compared with the negative control. Data are presented as the mean \pm SD (n=6). *P<0.05, vs. negative control group. FASN, fatty acid synthase; NC, negative control.

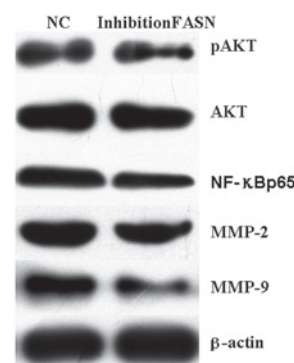


Figure 4. SDS-PAGE analysis of FASN inhibition and Akt phosphorylation and MMP-2, MMP-9 and NF- κ B (p65) protein expression in U2-OS cells. Cells were transfected with recombinant or negative plasmid for 24 h and protein expression levels of pAkt, MMP-2 and -9 and NF- κ B (p65) were determined. Results indicate that FASN inhibition downregulates phosphorylation of Akt and translocation of NF- κ B (p65) and inhibits expression of MMP-2 and -9 protein. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FASN, fatty acid synthase; MMP, matrix metalloproteinase; NF- κ B, nuclear factor- κ B; NC, negative control.

plasmid miRNA targeting the FASN gene may inhibit FASN expression in U2-OS cells.

Effect of FASN inhibition on U2-OS cell invasion and migration *in vitro*. The recombinant plasmid was transfected into U2-OS cells. Wound healing and Transwell invasion assays were performed to measure the migration and invasion of U2-OS cells and revealed that the number of transmembrane cells (22.2 ± 5.6 cells/membrane) and migration rate ($10 \pm 4\%$) in cells transfected by the recombinant plasmid were identified to be significantly reduced compared with cells transfected with negative plasmid (invasion, 93.7 ± 10.3 cells/membrane; migration; $84 \pm 5.4\%$; P<0.05; Figs. 2 and 3). The results indicate that FASN inhibition may suppress U2-OS cell invasion and migration *in vitro*.

Effect of FASN inhibition on the PI3K/Akt/NF- κ B signaling pathway. To investigate the effect of inhibition of FASN on phosphorylation of Akt, expression levels of pAkt protein in U2-OS cells were measured using western blot analysis. Results indicate that pAkt protein expression levels in cells transfected with recombinant plasmid were significantly lower than cells with negative plasmid (Fig. 4). This observation indicated that inhibition of FASN may decrease phosphorylation of Akt. In addition, protein expression levels of NF- κ B (p65), MMP-2 and -9 were detected. NF- κ B (p65), MMP-2 and -9 protein were decreased significantly in cells transfected with the recombinant plasmid compared with cells transfected with the negative plasmid. These observations demonstrate that inhibition of the FASN gene inhibits MMP-2 and -9 protein expression and nuclear transfer of NF- κ B in U2-OS cells (Fig. 4) and indicate that silencing FASN may downregulate the PI3K/Akt/NF- κ B pathway.

Discussion

OS is the most common childhood and adolescent primary malignant tumor. Metastasis to the lungs is one of the main causes of mortality in patients with OS. Therefore, study of the molecular mechanisms of metastasis of OS is important to improve survival of patients with metastatic disease.

Human FASN is a 270-kDa cytosolic dimeric enzyme, responsible for fatty acid synthesis. Endogenous fatty acid synthesis from the small carbon precursors acetyl-CoA and malonyl-CoA is dependent on the activity of FASN. In the majority of the cells, FASN is downregulated by dietary fatty acids, with the exception of lipogenic tissues, including the liver, lactating breast, fetal lung and adipose tissue. Previous studies have identified that neoplastic lipogenesis is essential for cancer cell survival (29). In addition, downregulation of FASN has been revealed to decrease invasion and migration in a variety of human tumors (19,20). Previously, we reported that cerulenin, an inhibitor of FASN inhibits OS cell proliferation *in vivo* and *in vitro* (30). However, it is currently unknown whether silencing FASN suppresses OS cell invasion and migration and the molecular mechanisms associated with this process have yet to be defined. In the present study, a recombinant plasmid, containing miRNA designed to target the FASN gene, was constructed for inhibition of FASN in OS cells. The inhibitory effect was investigated by RT-PCR and western blot analysis and the results demonstrated that FASN expression levels were significantly inhibited by the recombinant plasmid (Fig. 1). In addition, wound healing and Transwell invasion assays were performed to detect the migration and invasion of U2-OS cells. The migration rate of cells transfected by the recombinant plasmid was identified to be significantly lower than the negative plasmid cells (Fig. 2). Invasion was also inhibited (Fig. 3). Results indicate that silencing the FASN gene may inhibit OS cell invasion and migration *in vitro*.

The molecular mechanisms associated with FASN silencing and inhibition of OS cell migration and invasion were also analyzed. The role of the PI3K/Akt/NF- κ B signaling pathway in OS invasion and migration was confirmed. Upregulation of FASN expression in cancer cells has been previously associated with the PI3K/Akt signaling pathway (31,32). In addition, FASN inhibition

leads to downregulation of activated Akt and its downstream targets (33-35). Akt is essential for NF- κ B activation by stimulation of the IKK complex, which phosphorylates and inactivates I κ B, an inhibitor of NF- κ B. NF- κ B is composed of DNA-binding subunits (p50 and p52) and subunits with transcriptional activity (p65 and RelB or c-Rel), which dimerize in various combinations. The primary form of NF- κ B is a heterodimer of the p50 and p65 subunits and is localized mainly to the cytoplasm in an inactive form bound to I κ B. Previously, NF- κ B was demonstrated to upregulate MMP-9 (36). In addition, inhibition of NF- κ B was identified to downregulate MMP-2 (37). During the development of metastases, cancer cells must degrade the components of the extracellular matrix. MMPs, particularly MMP-2 and -9, are markedly associated with this process due to their capacity to degrade the extracellular matrix, promoting tumor invasion.

In the present study, pAkt protein expression levels were detected by western blot analysis to investigate whether silencing FASN led to downregulation of the PI3K/Akt/NF- κ B signaling pathway. Expression of pAkt protein was decreased in FASN-inhibited compared with negative control cells (Fig. 4), indicating that FASN inhibition downregulates phosphorylation of Akt. In addition, western blot analysis was performed to investigate expression levels of NF- κ B (p65) and MMP-2 and -9 protein. Again, protein expression levels were reduced in FASN-inhibited compared with negative control cells (Fig. 4), indicating that FASN inhibition reduces nuclear translocation of NF- κ B and attenuates activation of MMP-2 and -9 protein.

The present study demonstrates that inhibition of FASN may suppress OS cell invasion and migration via downregulation of the PI3K/Akt/NF- κ B pathway *in vitro*. Results indicate that targeting FASN and the PI3K/Akt/NF- κ B pathway may be a potential treatment strategy for treating OS metastases.

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