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Inhibition of fatty acid synthase suppresses U-2 OS cell invasion and migration via downregulating the activity of HER2/PI3K/AKT signaling pathway in vitro

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ABSTRACT

FASN plays an important role in the malignant phenotype of various tumors. Our previous studies show that inhibition FASN could induce apoptosis and inhibit proliferation in human osteosarcoma (OS) cell in vivo and vitro. The aim in this study was to investigate the effect of inhibition FASN on the activity of HER2/PI3K/AKT axis and invasion and migration of OS cell. The expression of FASN, HER2 and p-HER2(Y1248) proteins was detected by immunohistochemistry in OS tissues from 24 patients with pulmonary metastatic disease, and the relationship between FASN and p-HER2 as well as HER2 was investigated. The results showed that there was a positive correlation between FASN and HER2 as well as p-HER2 protein expression. The U-2 OS cells were transfected with either the FASN specific RNAi plasmid or the negative control RNAi plasmid. FASN mRNA was measured by RT-PCR. Western blot assays was performed to examine the protein expression of FASN, HER2, p-HER2(Y1248), PI3K, Akt and p-Akt (Ser473). Migration and invasion of cells were investigated by wound healing and transwell invasion assays. The results showed that the activity of HER2/PI3K/AKT signaling pathway was suppressed by inhibiting FASN. Meanwhile, the U-2OS cells migration and invasion were also impaired by inhibiting the activity of FASN/HER2/PI3K/AKT. Our results indicated that inhibition of FASN suppresses OS cell invasion and migration via down-regulation of the "HER2/PI3K/AKT" axis in vitro. FASN blocker may be a new therapeutic strategy in OS management.

1. Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumors in childhood and adolescents. With the advent of effective chemotherapy, the five-year survival rate for OS patients has been reported at 55–80% [1–4]. However, despite the encouraging trend to longer survival, many patients still face a dismal outcome. Numerous studies report that the five-year survival rate of patients with metastatic diseases was less than 20% [5–7]. Therefore, making clear the molecular mechanism of metastasis of OS to improve the curative effect has become one urgent problem to be solved.

Recently, studies show that targeting metabolic pathways such as glycolysis and lipid metabolism may represent a promising therapeutic strategy in cancer therapy [8]. Fatty acid synthase (FASN), a lipogenic multi-enzyme complex, is an enzyme crucial for endogenous lipogenesis in mammals, responsible for catalyzes the synthesis of long-chain fatty acids. In most normal cells, the level of FASN expression is low and difficult to find because of the presence of abundant amounts of dietary lipids [9]. However, FASN is expressed at high levels in a variety of human tumors [10,11], and has been strongly linked to tumor cell proliferation and apoptosis [12,13]. The metabolic products of the FASN complex are rapidly consumed by actively dividing cells and recent data demonstrates that FASN expression is important for tumor growth and survival, suggesting that FASN is a metabolic oncogene [14]. Our previously studies demonstrated that FASN may contribute to osteosarcoma cells metastasis [15,16]. However, its potential molecular mechanism is still unclear.

The HER2 proto-oncogene (also known as HER2 neu or c-erbB-2) is located at 17q21 and encodes a 185-kDa transmembrane

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tyrosine kinase glycoprotein with exten sive homology to the epidermal growth factor receptor [17]. HER2 has been associated with increased tumorigenicity and metastatic potential in various cancers, including soft-tissue sarcoma [18], osteosarcoma [19], breast [20]. HER2 overexpression increases the translation of FASN by altering the activity of the mTOR and PI3K/AKT signaling pathway in breast cancers cell [21], and inhibiting HER2/PI3K/AKT signaling pathway leads to blockade of FASN in Colorectal cancer cells [22]. However, various studies show that inhibiting FASN caused a marked decrease in the active forms of HER2 protein [23]. Those studies reveal a molecular connection between HER2 and FASN in cancer cells. Therefore, there is a complex molecular network linking HER2/PI3K/AKT signaling pathway with FASN, which might be functionally relevant to the biological behavior of tumor cells. However, the expression of HER2 in osteosarcoma is controversial [24], and the regulation molecular mechanism of HER2 and FASN interaction is still not clear in osteosarcoma.

In this study, we investigated the effect of inhibition FASN on the activity of HER2/PI3K/AKT signaling pathway, invasion and migration in U-2 OS cell.

2. Materials and methods

2.1. Antibodies

Rabbit monoclonal FASN, PI3K, phospho-Akt(Ser473), goat monoclonal Akt, mouse monoclonal β-Actin and polyclonal HER2 antibodies were purchased from Cell Signaling Technology Inc. Polyclonal phospho-HER2(Y1248) antibodies was from Abcam Inc.

2.2. Patient speciemens

A total of 24 samples of OS tissues were obtained from patients with pulmonary metastatic disease who underwent surgery in our hospital (The First Hospital Affiliated to Nanchang University, China) from 2005 to 2012. The pulmonary metastasis survey was performed with plain films and chest CT scans at first diagnosis. All the patients have no history of prior therapies with anticancer drugs or radiotherapy. The samples were fixed with 10% formalin and embedded in paraffin, and then, were cut into 4 μm sections. In all cases, informed consent was taken from related departments and persons, and the study had the approval from the Institute Ethics Committee.

2.3. Immunohistochemical analysis

Immunoperoxidase procedure(S-P procedure) and hematoxylin and eosin (H&E) staining were performed on paraffin-embedded sections. Antigen retrieval was performed with heating the sections in 10 mmol/L citrate buffer (pH 6.0) for 20 min. Anti-FASN, HER2 and p-HER2(Y1248) antibodies were used as the primary antibody at a final dilution as corresponding product specifications. Then the sections were chemiluminescence stained and counterstained using hematoxylin. Stained sections were evaluated and scored by two pathologic doctors in a blind manner without prior knowledge of the clinical pathological features of patients. According to the staining intensity by examining at least 500 cells in five representative areas, the expression level of FASN was judged and the intensity scores were recorded as follows: none, 0; weak, 1; moderate, 2; and intense, 3. According to the percentage of tumor cells with positive expression of FASN, the percentage scores were recorded: 0% (score 0); less than 10% (score 1), 10–49% (score 2), 50–79% (score 3), and 80–100% (score 4). The final score was averaged with the scores from the two pathologic doctors; these scores were calculated by multiplying the intensity score to the percentage score. For example, when a specimen contained 50% of the tumor cells with moderate intensity, the final score is 4 ($2 \times 2 = 4$). The section with a final score less than 4 were considered as (−), score 4 were considered as (+), score 6 score as (++), and much 6 were considered as (+++). For HER2 and p-HER2, only specimens with more than 25% highly (+++) or moderately (++) positive cells were classified as positive.

2.4. FASN-specific RNAi plasmid construction

The human cDNA sequence encoding FASN protein (NM_004104.4) was obtained from GenBank. Four pairs of FASN-specific RNAi oligos were designed by Block-iTTM RNAi Designer and synthesized (Table 1). Products were cloned into the express vector pcDNA6.2-GW/EmGFP-miR using the BLOCK-i™ 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). After being annealed, connected and transformed, four different FASN-specific RNAi plasmids (named as miR-FASN-1 to miR-FASN-4) were synthesized according to the manufacturer's instructions (Invitrogen).

2.5. Cell culture and transfection

The human OS cell line U-2 OS was purchased from American Type Culture Collection (Manassas, VA), and routinely cultured in RPMI-1640 (HyClone) supplemented with 10% fetal bovine serum (Sigma) in a humidified 37 °C incubator containing 5% CO_2. U-2 OS cells were seeded in 6-well plates at 40% confluence on the day prior to transfection. U-2 OS cells were transfected with miR-FASN or miR-Neg using Lipofectamine 2000 according to the Invitrogen technical bulletin.

Table 1
Four pairs of FASN-specific RNAi and one pair of negative control sequence of oligonucleotide.

Oligo name	Sequence of oligonucleotide from 5′ to 3′
FASN-1F	TGCTGAACTCCTGCAAGTTCTCCGACGTTTTGGCCACTGACTGACGTCGGAGATTGCAGGAGTT
FASN-1R	CCTGAACTCCTGCAATCTCCGACGTCAGTCAGTGGCCAAAACGTCGGAGAACTTGCAGGAGTTC
FASN-2F	TGCTGTGAAGTCGAAGAAGAAGGAGAGTTTTGGCCACTGACTGACTCTCCTTCCTTCGACTTCA
FASN-2R	CCTGTGAAGTCGAAGGAAGGAGAGTCAGTCAGTGGCCAAAACTCTCCTTCTTCTTCGACTTCAC
FASN-3F	TGCTGTCAGGAAGATAGCCATGCCGAGTTTTGGCCACTGACTGACTCGGCATGTATCTTCCTGA
FASN-3R	CCTGTCAGGAAGATACATGCCGAGTCAGTCAGTGGCCAAAACTCGGCATGGCTATCTTCCTGAC
FASN-4F	TGCTGAACTGCTGCACGAAGAAGCATGTTTTGGCCACTGACTGACATGCTTCTGTGCAGCAGTT
FASN-4R	CCTGAACTGCTGCACAGAAGCATGTCAGTCAGTGGCCAAAACATGCTTCTTCGTGCAGCAGTTC
Negative control	
Negative-F	tgctgAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
Negative-R	cctgAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTc

2.6. RT-PCR

Semi-quantitive 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). The Two-Step kit (Promega Corporation, Madison, WI, USA) was 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. All experiments were repeated by six times over multiple days.

2.7. Western blot analysis

Total protein from the cells was extracted using RIPA lysis buffer containing 60 μg/ml PMSF. Protein concentration was determined by Bradford assay. Equal amounts of protein were electrophoresed by 8% SDS–PAGE and transferred onto a pure Nitrocellulose blotting membrane (0.22 μm). Membranes were blocked with 5% skim milk for 1 h at room temperature, then blocked with primary antibodies (rabbit anti-FASN IgG, 1:200; rabbit anti-PI3K IgG, 1:1000; rabbit anti-pAKT IgG, 1:800; rabbit anti-pHER2 IgG, 1:400; rabbit anti-HER2 IgG, 1:1000; goat anti-AKT IgG, 1:1000) over night at 4 °C. Membranes were washed before incubated with appropriate peroxidase-conjugated secondary antibodies (anti-rabbit, anti-goat and anti-mouse, 1:2000).The immune complexes were detected with pro-light HRP Kit (TIAN GEN, China). β-Actin (1:2000, Cell Signaling Technology Inc.) protein expression was used as a normalization control for protein loading. All experiments were repeated by six times over multiple days.

2.8. 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 5×10^6 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 under a microscope (ECLIPSE-TS-100, Nikon, Japan; magnification, 100×) at 0 and 24 h and the migrated distance was measured using Image J (NIH, Bethesda, MD, USA). The migration rate of cells transfected with the miR-FASN was compared with cells transfected with miR-Neg. All experiments were repeated by six times over multiple days.

2.9. Transwell invasion assay

Invasion of U-2 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, 400×) at 24 h later. Cell counts were performed using Image J. The migration rate of cells transfected with miR-FASN was compared with those transfected with miR-Neg. All experiments were repeated by six times over multiple days.

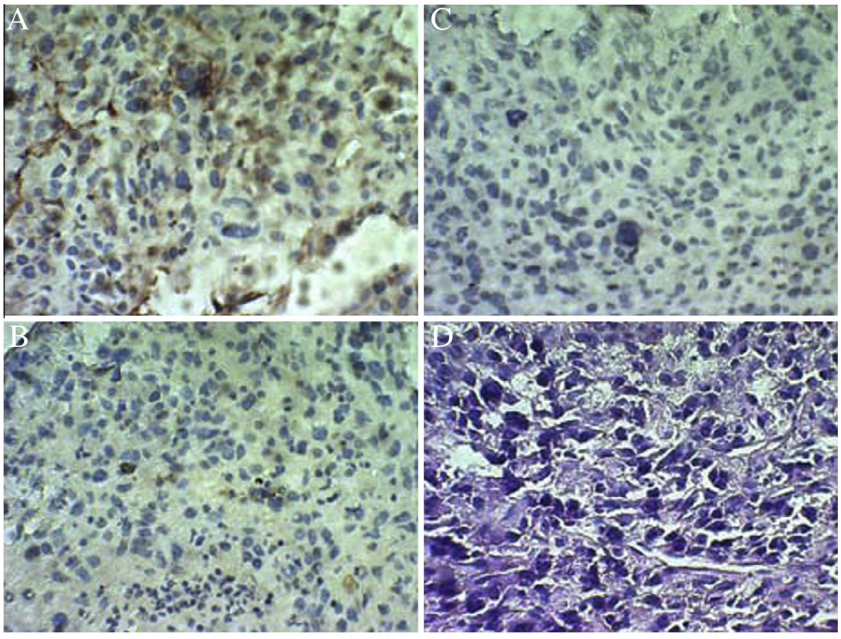

Fig. 1. Immunohistochemical staining to examine FASN, HER2 and P-HER2(Y1248) expression (A, B, C, respectively) and HE staining (D) in osteosarcoma (OS) tissues with pulmonary metastasis (400×). (A) FASN protein was showed brownish-yellow particle deposition and expressed in the cytoplasm. (B) HER2 was brownish-yellow particle deposition and expressed in cytoplasm and nucleus. (C) p-HER2 was brownish-yellow particle deposition and expressed in cytoplasm and nucleus. (D) Cells in osteosarcoma (OS) tissues with pulmonary metastasis were polygonal and short spindle, with large pleomorphic nuclei and abundant cytoplasm.

2.10. Statistical analysis

The correlation of FASN with HER2 and p-HER2 protein in OS tissues was evaluated using the Wilcoxon rank Sum Test. All Measurement data were presented as $\bar{X} \pm SD$. The 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 (Chicago, IL, USA).

3. Results

3.1. Positive correlation between FASN and p-HER2(Y1248), HER2 protein expression in tissues of OS with pulmonary metastasis

In order to investigate the relationship of FASN with p-HER2 and p-HER2 in OS with pulmonary metastatic disease, the FASN, HER2 and p-HER2 protein in 24 samples from patients with pulmonary metastatic disease was detected by immunohistochemistry. FASN protein was expressed in the cytoplasm (Fig. 1A), which was consistent with previous reports [19]. HER2 and p-HER2 were both in cytoplasm and nucleus (Fig. 1B and C). The positive expression rate of FASN and HER2 was 83.3% (20/24) and 41.7% (10/24), respectively. The relationship was significant between HER2 and FASN protein expression level (Spearman's rho, *rs* = 0.502). Interesting, the positive expression rate of p-HER2 protein was 37.5% (9/24). There was a significant positive correlation between FASN and p-HER2 protein expression (Spearman's rho, *rs* = 0.444).

3.2. FASN-specific RNAi plasmids inhibiting the expression of FASN in U-2 OS cell

The U-2 OS cells were transiently transfected with four different FASN-specific RNAi plasmids or miR-Neg for 6 h and then cultured for 48 h. The FASN mRNA and protein expression in U-2 OS cells was detected by RT-PCR and Western blotting. The results showed FASN mRNA and protein expression in U-2 OS cells were inhibited by RNAi, and when compared with miR-FASN-2 and miR-FASN-3 groups, FASN protein and mRNA expression in U-2 OS cells transfected with miR-FASN-1 or miR-FASN-4 was obviously lower ($p \leqslant 0.05$) (Fig. 2A–D). Therefore, miR-FASN-1 and miR-FASN-4 were selected for positive groups in invasion and migration experiments.

3.3. Silencing FASN down-regulates HER2/PI3K/AKT signaling pathway in U-2 OS cell

In order to investigate the effect of inhibiting FASN on the activity of p-HER2/PI3K/AKT signaling pathway in U-2OS cell, the U-2 OS cells were transfected with miR-FASN or miR-Neg for 6 h, and then cultured for 48 h. The phosphorylation of HER2 was measured using Western blot analysis in U-2 OS cells by detecting the p-HER2 (Y1248) protein. Results revealed that p-HER2 or HER2 protein expression in cells transfected with recombinant plasmid was significantly lower than cells treated with miR-Neg. Interestingly, after inhibiting p-HER2 or HER2, the protein expression of PI3K, Akt and p-Akt was dramatically declined (Fig. 3).

3.4. Inhibition of the FASN/HER2/PI3K/AKT signaling pathway blocks U-2 OS cells invasion and migration

The invasion and migration of U-2 OS cells were evaluated using the wound healing and transwell invasion assays. In the transwell assays, the invaded cells in FASN-silenced groups were significantly lower than the groups transfected with miR-Neg

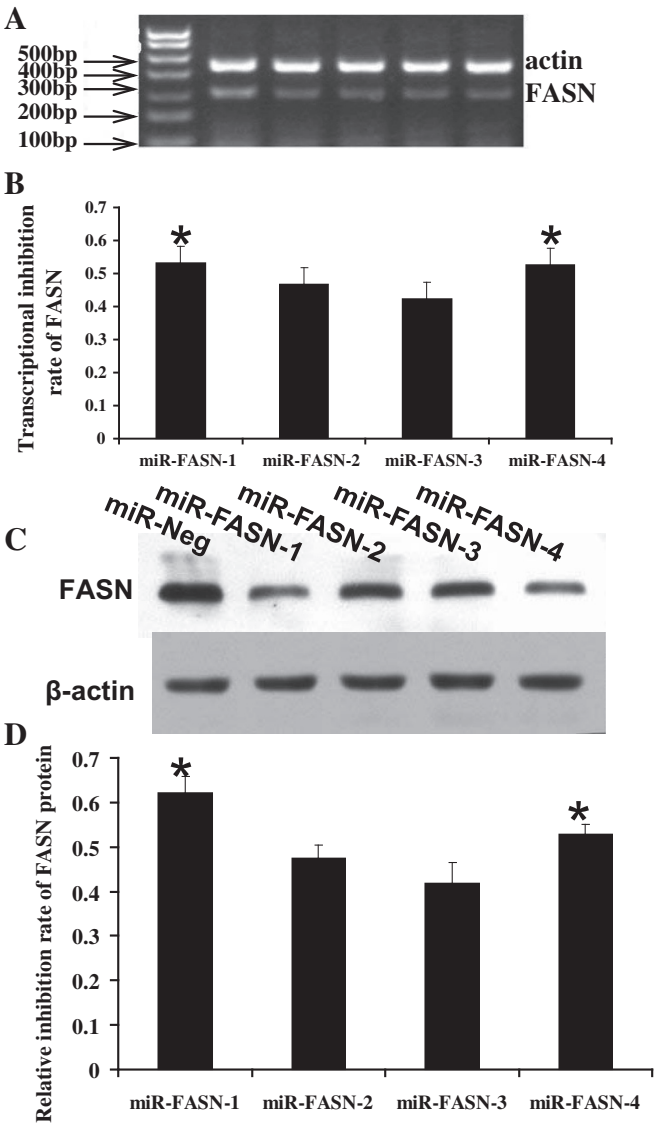

Fig. 2. The effect of miR-FASN plasmids on the expression of FASN in U-2 OS cells. (A) The expression of FASN mRNA was measured by RT-PCR. (B) The inhibition effectiveness of miR-FASN plasmids on FASN mRNA expression was assessed using inhibition rate. The inhibition rate of FASN mRNA expression was measured by the ratio of gray value of FASN/β-Actin. The inhibition rate in cells transfected with miR-FASN-1 and miR-FASN-4 was higher than in those transfected with miR-FASN-2, 3. Columns, mean ($n = 6$); bars, SD; $^{*}p < 0.05$ VS miR-FASN-2, 3 groups. (C) The expression of FASN protein was measured by Western blot. (D) The inhibition effectiveness of miR-FASN plasmids on FASN protein expression was assessed using inhibition rate. The inhibition rate of FASN protein was measured by the ratio of gray value of FASN/β-Actin. Columns, mean ($n = 6$); bars, SD; $^{*}p < 0.05$ VS miR-FASN-2, 3 groups.

($P < 0.05$) (Fig. 4A and B). As shown in Fig. 4C and D, the migration rate in FASN-silenced groups was significantly lower than the groups transfected with miR-Neg ($p < 0.05$).

4. Discussion

Fatty acid metabolic pathways play an important role in linking to carcinogenesis [14]. FASN is an enzyme crucial for endogenous lipogenesis in mammals, responsible for catalyzing the synthesis of long-chain fatty acids. In cancer cells, FASN is commonly increased providing cancer cells with extra source of cellular fatty

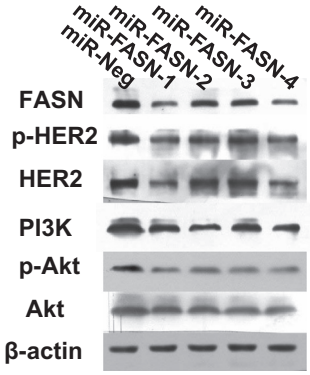

Fig. 3. Down-regulation of FASN suppressed the expression of p-HER2, HER2, PI3K, P-Akt and Akt proteins in U-2 OS cells. U-2 OS cells were seeded in 6-well plates at 40% confluence on the day prior to transfection. The cells transfected with miR-FASN or miR-Neg plasmid for 6 h, and then cultured for 48 h. The cells were lysed and protein was quantified, then separated with 8% SDS–PAGE and assayed by Western-blot. This is a representative image of the six experiments shown for each group. It showed that with the silencing of FASN, the HER2, P-HER2, PI3K, Akt and p-Akt proteins were blockaded in varying degrees, which indicated that FASN may down-regulate the HER2/PI3K/AKT signaling pathway.

acid. FASN may directly lead the cellular to play a key role in tumor initiation and propagation for malignant fatty acid accumulation, and influence many metabolic processes of cancer cells. Inhibition of tumor FASN activity by inhibitors reduces tumor proliferation, induces apoptosis and decreases metastasis in vitro and vivo [15,25,26]. Our previous studies show that FASN may be involved in OS metastasis [21], and inhibiting FASN suppresses U-2 OS cell growth and induces apoptosis in vitro [20]. So, the effect of inhibiting FASN on invasion and migration was investigated in this study only. We found that inhibition of FASN expression blocked the invasion and metastasis of U-2 OS cells in vitro. It indicated that mediating FASN could effectively change malignant phenotype of U-2 OS cells, and FASN could be recognized as a promising target of OS.

Modulation of HER2 is also tightly regulated in normal cellular function. Various studies show that HER2 overexpression is important in oncogenic transformation, tumorigenesis and metastasis [27]. However, the expression of HER2 in OS is controversial [24]. In this study, the HER2 and p-HER2 protein in samples from OS patients with pulmonary metastatic disease was detected by IHC. With the emergence of new adjuvant chemotherapy, the number of OS tissue samples meeting the requirement of this research, which had not received chemotherapy, is very limited, and in this study we used 24 OS tissues samples collected in recent years. The results showed that the positive expression of HER2 and p-HER2 protein was found and located in cytoplasm and nucleus. The positive expression rate of HER2 and p-HER2 was 41.7% and 37.5%, respectively. These results suggested that HER2 may be a promising target in the treatment of OS metastasis.

It has been shown that the oncogenic effects of HER2 mainly depend on the preservation of the "lipogenic phenotype". HER2 activates downstream PI3K/AKT, MAPK and mTOR signaling pathways with subsequent transcriptional activation of FASN expression [28,29]. However, it is interesting that inhibition of FASN restrains the activity of HER2. In present study, to explore a possible connection between FASN and HER2 or p-HER2 with in tissues of osteosarcoma with pulmonary metastasis, immunohistochemical staining analyses was conducted on OS samples from patients with pulmonary metastatic disease, and the results revealed a significant positive correlation between FASN and HER2 or p-HER2. Those results indicated that there may be a feedback regulation between FASN and HER2.

Therefore, we speculated that knockdown of FASN could decrease HER2 and p-HER2 in OS cell. To define our hypothesis, the

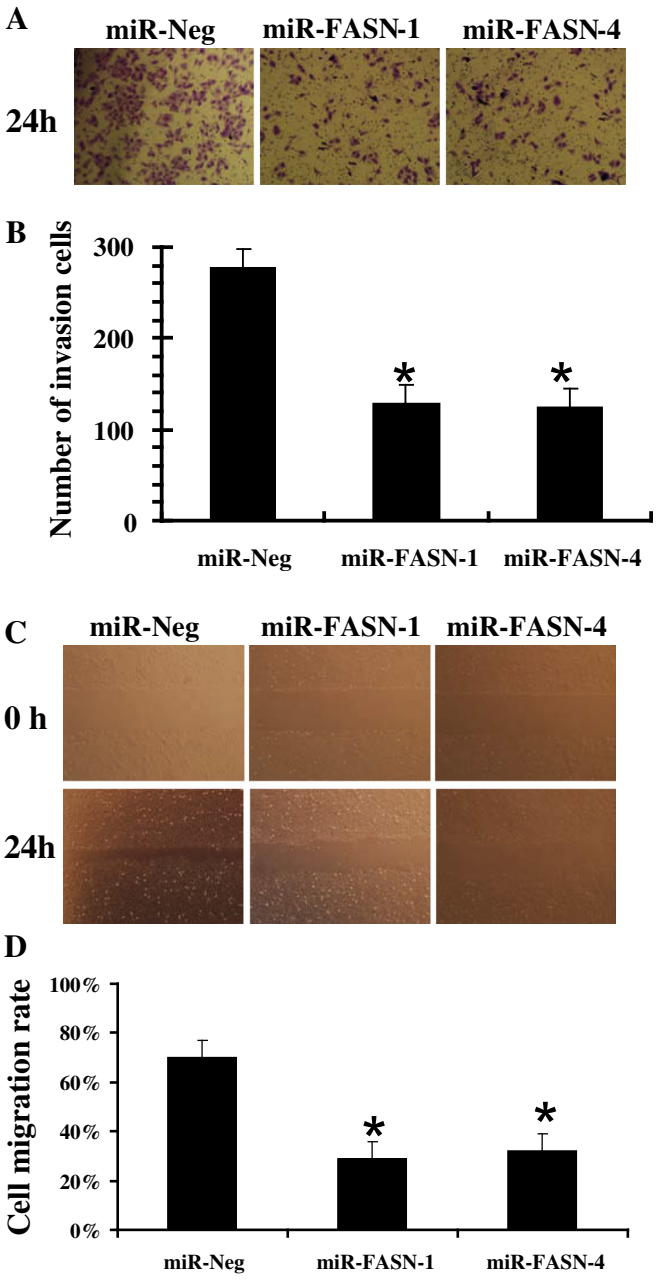

Fig. 4. (A) A representative image of the transwell invasion assays is shown for each group. (B) Quantification of cell invasion. Columns, mean ($n = 6$); bars, SD; $^{*}p < 0.05$ versus miR-Neg group. It indicated that cell invasion is significantly inhibited by blockade of FASN. (C) A representative image of the wound healing assays is shown for each group. (D) Quantification of cell migration. Columns, mean (n = 6); bars, SD; $^{*}p < 0.05$ VS miR-Neg group. It indicates that inhibiting the expression of FASN in the U-2 OS cells suppressed cell migration.

FASN expression in U-2 OS cell was inhibited by RNAi. The results revealed that the HER2 and p-HER2 protein was significantly lower in U-2 OS cells treated with miR-FASN than in those transfected by miR-Neg. Interesting, after inhibiting p-HER2 and HER2, the protein expression of PI3K, Akt and p-Akt was dramatically declined. It suggested that silencing FASN results in down-regulation of HER2/PI3K/AKT signaling pathway in OS.

In summary, our findings suggested that inhibition of FASN suppresses U-2 OS cell invasion and migration via down-regulation HER2/PI3K/AKT signaling pathway in vitro. However, previous studies showed that tumor microenvironment might influence tumor progression, invasion and cell migration. So, further

experiments in vivo are necessary to be performed to make it clear whether the FASN and HER2 blockers could represent a new molecular strategy in the management of OS with metastases.

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