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Original Article

FTO and Smad6 involved in honokiol-induced osteosarcoma cell apoptosis

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Abstract: Osteosarcomas are the most common malignant bone tumors in children and adolescents. Early pulmonary metastasis often occurs, with poor prognosis. The current study aimed to investigate honokiol-induced cell apoptosis in human osteosarcoma MG63 cells. Cell viability was detected via CCK8 assays. Cell apoptosis was assessed using annexin V-PI double-labeling staining. Expression of genes and proteins was analyzed using real-time PCR and Western blotting, respectively. Cells were transfected with siRNAs, as a gene-silencing method. Results showed that honokiol induced cell death, in a concentration-dependent manner, and suppressed osteosarcoma cell proliferation. Moreover, mRNA and protein expression of fat mass and obesity (FTO) levels were significantly lower in osteosarcoma cells with honokiol administration, compared to the control group. In contrast, protein expression of Smad6 was significantly higher in osteosarcoma cells with honokiol administration group, compared to the honokiol + Smad6 SiRNA group. Smad6 siRNA transfection may suppress protein expression levels of Smad6. Moreover, using differentially-expressed genes microarrays, a significant correlation was found between FTO-transfer and downregulated Smad6. The current study demonstrated that honokiol may induce osteosarcoma cell apoptosis through downregulation of FTO and upregulation of Smad6. There may be cross-talk between FTO and Smad6 in cell apoptosis progression.

Keywords: FTO, Smad6, honokiol, osteosarcoma

Introduction

Osteosarcomas are the most common malignant bone tumors in children and adolescents. Early pulmonary metastasis is common, with a poor prognosis [1]. A second peak of incidence has been identified in elderly adults, associated with defective bone remodeling [2]. Five-year disease-free survival rates have increased up to 70% with current protocols, including a combination of limb salvage and neoadjuvant chemotherapy. Although chemotherapeutic agents, such as cisplatin, have obvious killing effects on osteosarcoma cells, toxic side effects and resistance effects, after long-term application, are huge obstacles for clinical doctors [3, 4]. Therefore, it is important to explore novel and effective adjuvant therapy drugs.

Honokiol, a small molecular weight natural product that is isolated and purified from *Magnolia officinalis*, has been shown to possess potent anti-oxidative [5], anti-inflammatory [6], anti-neoplastic, and anti-angiogenic properties [7, 8]. Functional studies have revealed that honokiol may induce cell apoptosis in human chondrosarcoma cells *in vitro* and reduce tumor volume *in vivo* [8]. Moreover, honokiol has been shown to significantly inhibit cyclosporine A-induced and Ras-mediated survival of renal cancer cells through downregulation of vascular endothelial growth factor (VEGF) and cytoprotective enzyme HO-1 [9]. Interestingly, honokiol analogs have shown much higher growth inhibitory activity in A549 human lung cancer cells and significant increases in cell population in the G0-G1 phase [10].

Fat mass and obesity (FTO) associated genes have been found, according to several genome wide association studies (GWAS), to be associated with obesity and type II diabetes mellitus

[11]. FTO has been shown to be expressed in the pancreas, skeletal muscle, white adipose tissue, and mammary glands [11-14]. Although roles remain essentially unclear, it seems that FTO is associated with increased risk of cancer, including breast cancer [12, 15], endometrial cancer [16], pancreatic cancer [17], prostate cancer [18], colorectal cancer [19], and lung cancer [20]. Results have been inconsistent. However, the effects of FTO on osteosarcomas have been associated with Smad6. This is an important consideration when prescribing FTO for malignancies. In a study concerning chicken sternal embryonic chondrocytes, a morpholino antisense oligonucleotide complementary to Smad6 reduced expression of Smad6 proteins and enhanced the stimulatory effects of BMP-2 on both colX and alkaline phosphatase activity. In contrast, overexpression of Smad6 blocked BMP-2 mediated induction of the type X collagen promoter [21]. Smad6 participates in an important negative feedback loop. In this loop, BMP-2 mediated effects on chondrocyte differentiation are reduced by induction of Smad6 [21].

Although the effects of honokiol-induced tumor apoptosis have been studied in some cancers [1, 2, 8-10], the roles of honokiol in the process of cell apoptosis in osteosarcomas remain largely unknown. Therefore, the aim of the present work was to investigate the involvement of apoptosis mechanisms of honokiol in human osteosarcoma cell lines.

Materials and methods

Cell culturing

Human osteosarcoma MG63 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM (Dulbecco's Modified Eagle's medium; Invitrogen), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/mL of penicillin, and 100 mg/mL of streptomycin (Invitrogen) at 37°C in a humidified 5% CO_2 and 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with various concentrations of honokiol (50 μg/mL, 100 μg/mL).

Cell viability detection via CCK8

Human osteosarcoma MG63 cells in FBS-free medium were treated with honokiol (0-100 μg/mL) for 24 or 48 hours. Next, 10 μL of CCK8 (Dojindo, Kumamoto, Japan) was added to the cells. Cell viability was measured at 490 nm using an ELISA reader (BioTek, Winooski, VT, USA), according to manufacturer instructions.

Quantification of apoptosis by flow cytometry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues exposed on the cell surface of apoptotic cells. The cells were treated with vehicle or honokiol for indicated time intervals. After treatment, the cells were washed twice with PBS (pH = 7.4). They were re-suspended in a staining buffer containing 1 μg/ml PI and 0.025 μg/mL annexin V-FITC. Double-labeling was performed at room temperature for 10 minutes in the dark, followed by flow cytometric analysis. The cells were immediately analyzed using FACScan and the Cellquest program (Becton Dickinson).

Quantitative assessment of apoptotic cells was also assessed using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis. The BD ApoAlert™ DNA Fragmentation Assay Kit was employed. Briefly, the cells were incubated with honokiol at indicated times. They were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% of sodium citrate. After washing, the cells were incubated with the reaction mixture for 60 minutes at 37°C. The stained cells were then analyzed using a fluorescence microscope.

Real-time polymerase chain reaction (RT-PCR)

Total mRNA was extracted from osteosarcoma cells, according to TRIzol manufacturer protocol (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of cDNAs was performed with reverse transcription reactions with 4 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas), as described by the manufacturer. First strand cDNAs served as the template for regular polymerase chain reac-

Table 1. Primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
FTO	TGACCCAGCCTATGGTTTGC	CAACCCTGTTGCACATTCCC
Smad6	AGGGCTTCAGCGGATTTCTG	GCTAGGGCATGAACCTCCTC
GAPDH	CATGGTTCACACCCATGACG	CCACTAGGCGCTCACTGTTCTC

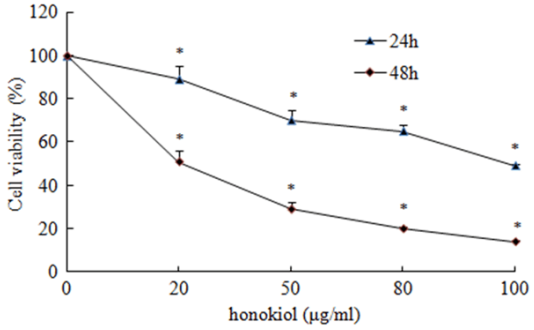

Figure 1. CCK-8 analysis of the effects of different concentrations of honokiol on the viability of human osteosarcoma MG63 cells. MG63 cells were incubated with various concentrations of honokiol for 24 h or 48 hours. Cell viability was examined via CCK8 assays. Values are expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$ compared with controls.

tions (PCR), performed using a DNA Engine (ABI 7300). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as an internal control, was used to normalize data, determining relative expression levels of the target genes. PCR primers used in this study are shown in **Table 1**. The products of RT-PCR were then submitted to gel electrophoresis for further confirmation.

Western blotting

Osteosarcoma cells were extracted in Laemmli buffer (Boston Bioproducts, Worcester, MA, USA). This was followed by 5-10 minutes of boiling and centrifugation, obtaining the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with antibodies. After three washings with TBST, the membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG, at a dilution of 1:15,000. After 2 hours of incubation at room temperature, the membranes were washed three times with TBST. Blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals underwent densitometric assessment (Odyssey Application Software version 3.0) and were normalized to the β-actin signals, correcting for unequal loading using mouse monoclonal anti-β-actin antibodies (Bioworld Technology, USA).

siRNA transfection

For the current study, siRNAs against human Smad6 and control siRNA were purchased from Santa Cruz Biotechnology. The cells were transfected with siRNAs (at a final concentration of 100 nM) using Lipofectamine 2000 (Invitrogen Life Technology), according to manufacturer instructions.

Statistical analysis

Data are reported as mean ± standard errors of mean (SEM) for each group. Statistical analyses were performed using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed with one-way ANOVA. This was followed by Tukey's multiple comparison testing, as a post-test, comparing group means if overall $P < 0.05$. P values < 0.05 indicate statistical significance.

Results

Honokiol-induced cell apoptosis in human osteosarcoma MG63 cells

Evaluating the potential roles of honokiol in apoptosis of human osteosarcoma MG63 cells, this study analyzed the effects of honokiol on cell survival in human osteosarcoma MG63 cells. Treatment of MG63 cells with honokiol induced cell death, in a concentration-dependent manner, according to CCK8 assays (**Figure 1**). In addition, immunofluorescence staining results showed that honokiol could promote osteosarcoma cell apoptosis (**Figure 2**). Moreover, Annexin V-PI double-labeling, a hallmark of early and late phase of apoptosis, was used for detection of the effects of honokiol on cell apoptosis. Consistent with immunofluorescence staining, results showed that the propor-

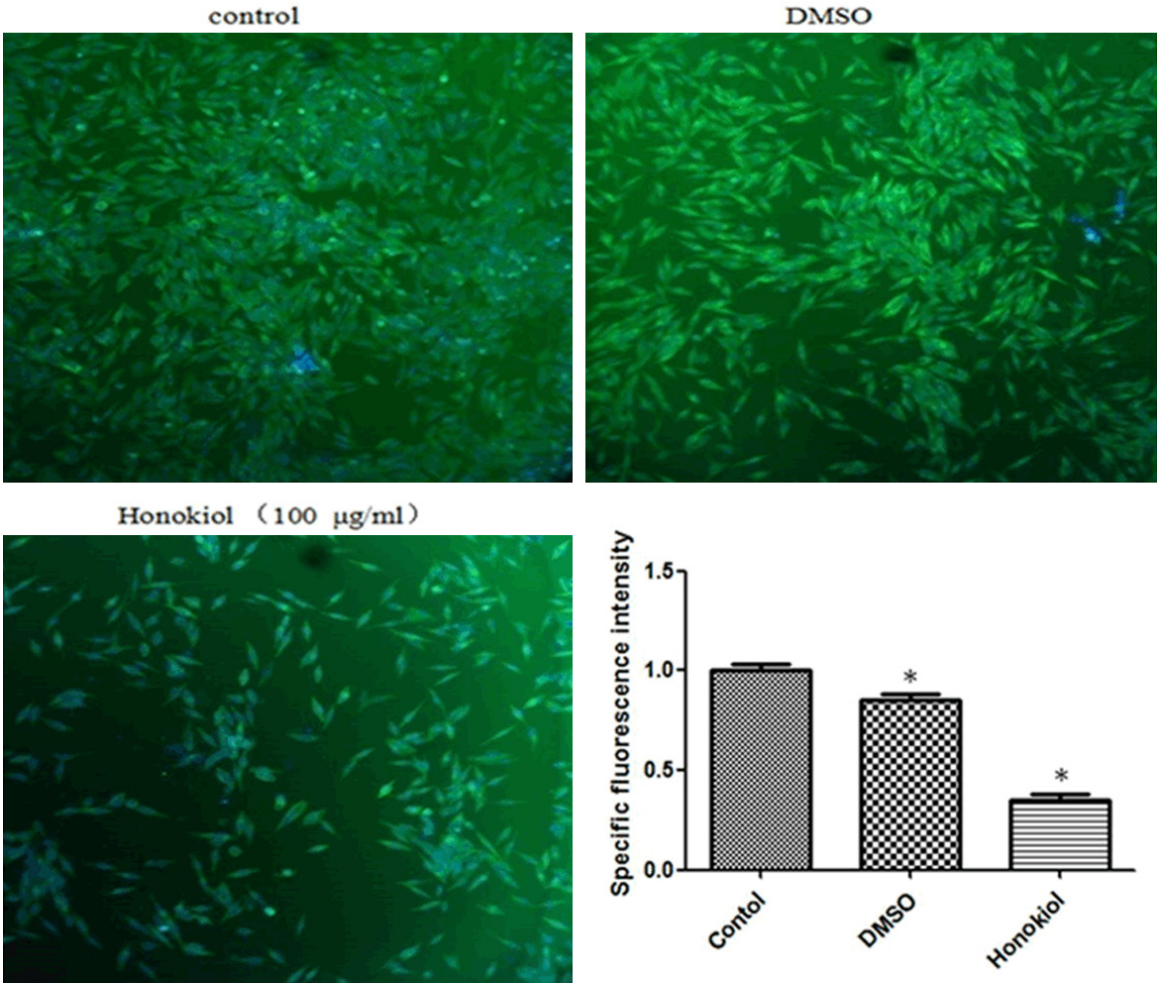

Figure 2. Immunofluorescence staining analysis of the death of MG63 cells. Representative microscopic images of nuclear DAPI staining (blue), TUNEL immunofluorescent staining (green) in MG63 cells of control, DMSO, or honokiol (100 μg/mL) groups. Scale bar = 100 μm.

tion of early and late phases of apoptosis cells had increased, compared to the control group (**Figure 3**).

Honokiol increases mRNA and protein expression of FTO in osteosarcoma cells

Aiming to explore the effects of FTO on osteosarcoma progression, the current study investigated whether FTO is involved in the promotion of apoptosis of MG63 cells induced by honokiol. Results showed that both mRNA and protein expression levels of FTO were significantly lower in osteosarcoma cells with honokiol administration, compared to the control group (**Figure 4**). Therefore, current data suggests that suppression of expression of FTO is involved in honokiol-mediated cell death.

Differentially-expressed mRNAs in human osteosarcoma MG63 cells

Microarray data of FTO-none human osteosarcoma MG63 cells was treated as a control in the selection of differentially-expressed genes related to FTO-transfer. After the removal of redundant and unannotated sequences, with FDR < 1%, 7 genes were found to be significantly upregulated. Ten genes were significantly downregulated ($P < 0.0001$) in the FTO-transfer group, compared to that in the FTO-none group. Moreover, mRNA expression of Smad6 reached the lowest levels in the FTO-transfer group (**Figure 5A**, **5B**). Results suggest that Smad6 is involved in honokiol-induced osteosarcoma cells apoptosis. To confirm, RT-PCR and Western blotting were performed, evaluat-

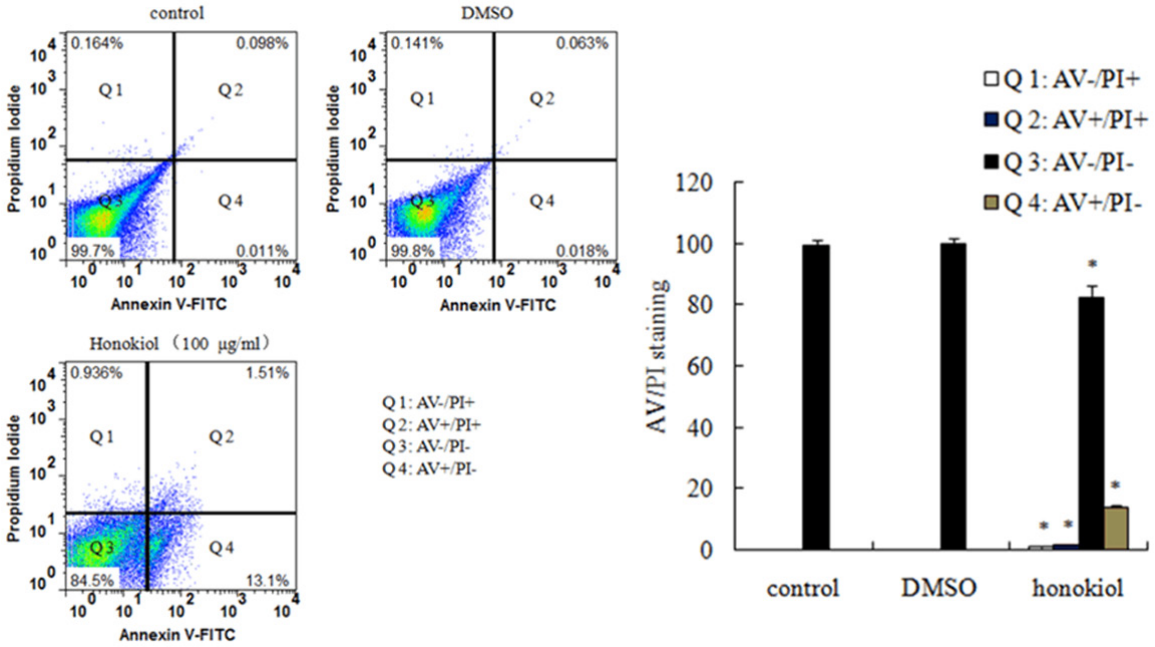

Figure 3. Honokiol induced apoptosis of MG63 cells. MG63 cells were treated with vehicle, DMSO, or honokiol (100 μg/mL) for 24 hours. The percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining. Values are expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$, versus control group.

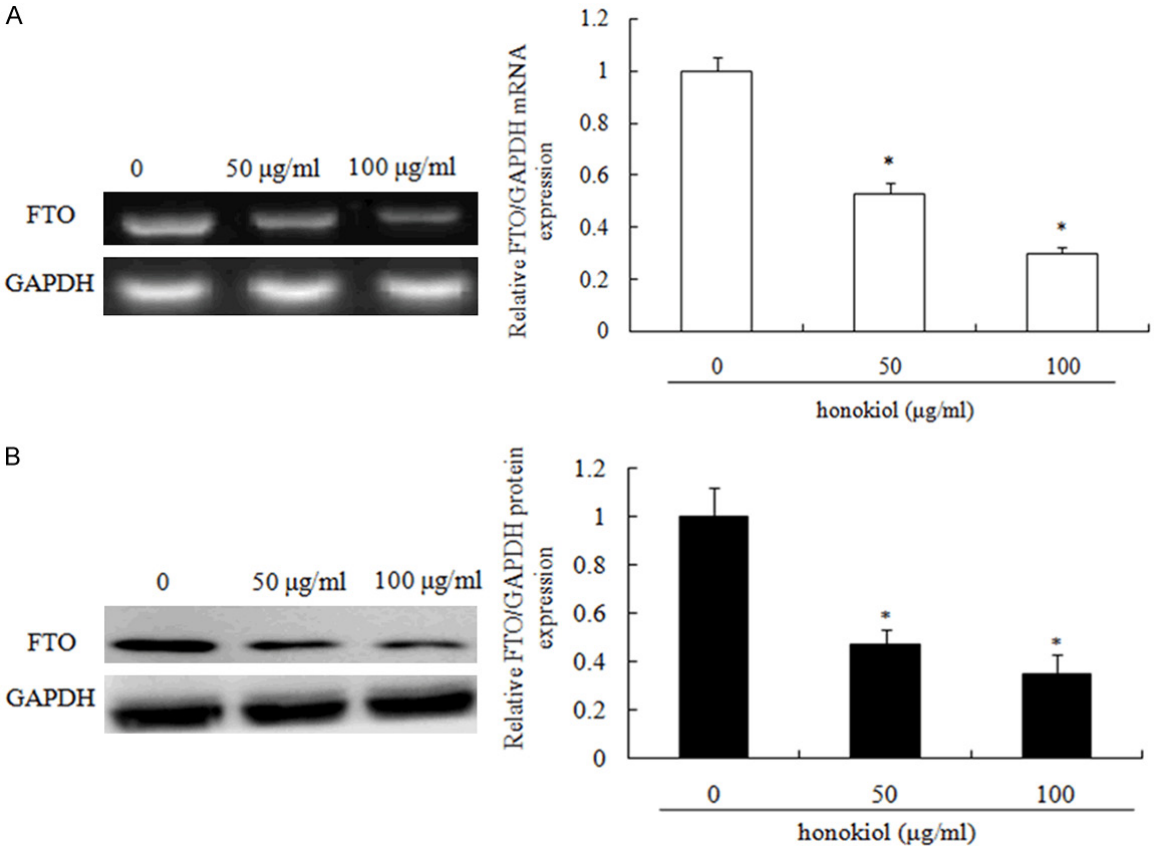

Figure 4. Detection of mRNA and protein expression levels of FTO and Smad6 in osteosarcoma cells in the presence of honokiol. MG63 cells were treated with honokiol at different concentrations (50 μg/mL or 100 μg/mL) for 24 hours. Next, (A) mRNA and (B) protein expression levels of FTO were measured using real-time PCR and Western blotting, respectively. Values are expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$, versus control group.

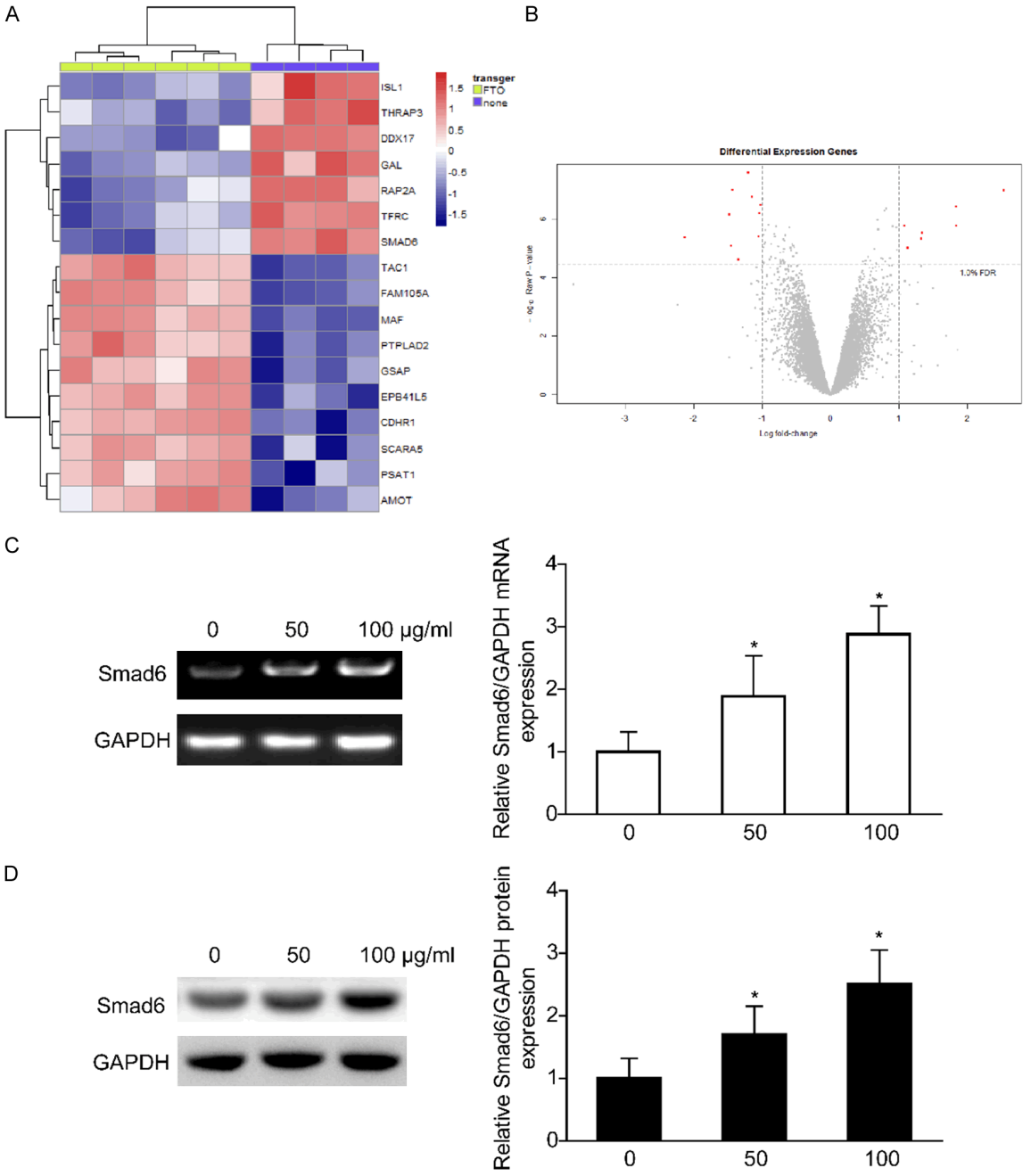

Figure 5. Differentially-expressed mRNAs in human osteosarcoma MG63 cells. Differentially-expressed mRNAs chosen with FDR < 1%. (A, B) Hierarchical clustering of differentially-expressed mRNAs in FTO-none group and FTO-transfer group. Red indicates high relative expression, while blue indicates low relative expression. The codes are log2-transformed values. Fold changes >1 and *P*-values < 0.05 are considered significant. MG63 cells were treated with honokiol at different concentrations (50 μg/mL or 100 μg/mL) for 24 hours. Afterward, (C) mRNA and (D) protein expression levels of Smad6 were measured using RT-PCR and Western blotting, respectively.

ing the effects of honokiol on expression of Smad6 in MG63 cells. Results were consistence with the hypothesis that honokiol increases mRNA and protein levels of Smad6 (**Figure 5C**, **5D**).

Honokiol-induced osteosarcoma cell apoptosis suppressed by Smad6 siRNA

Smad6 participates in an important negative feedback loop. In this loop, BMP-2 mediates

effects on chondrocyte differentiation [21]. Exploring the function of Smad6 on cell apoptosis in the presence of honokiol, siRNAs targeting Smad6 were used to downregulate Smad6 expression in MG63 cells. **Figure 6A** shows the knockdown efficiency of Smad6 siRNA-1/-2. **Figure 6B** shows that honokiol decreased cell viability levels in osteosarcoma MG63 cells. However, the roles of inhibition of the growth induced by honokiol was abolished when MG63 cells were transfected with Smad6 SiRNA on the basis of honokiol administration. Annexin V-PI double-labeling results also showed that the proportion of apoptotic cells had increased with honokiol administration, but Smad6 SiRNA impaired the apoptosis promotion of MG63 cells induced by honokiol administration (**Figure 6C**). Therefore, results suggest that honokiol induced osteosarcoma cell apoptosis through upregulating Smad6 expression.

Discussion

The current study investigated the pharmacological mechanisms of honokiol in human osteosarcoma MG63 cells. This study proposed that there may be crosstalk between FTO and Smad6 in cell apoptosis progress. The present study demonstrated that honokiol may induce osteosarcoma cell apoptosis, at least partially, through downregulation of FTO and upregulation of Smad6.

According to CCK8 assays and Annexin V-PI double-labeling staining, honokiol induces cell death through apoptotic mechanisms, with the proportion of apoptotic cells increasing. Honokiol had been shown to possess the effects of cytoprotective autophagy in prostate cancer cells. It has also been shown to induce apoptosis in human colorectal cancer cells [22, 23]. Few studies have investigated honokiol on osteocytes. A previous study focused on osteoblasts, finding that honokiol played a vital role in bone remodeling [24]. Honokiol may have positive effects on skeletal structure, acting as a dual anabolic/anti-catabolic agent for the amelioration of multiple bone diseases [24, 25]. The roles of honokiol in the process of cell apoptosis in chondrosarcomas provide evidence that honokiol reduced cells survival and tumor growth in human chondrosarcoma cells *in vitro* and *in vivo* [8]. Although the effects of honokiol-induced tumor apoptosis have been studied in some cancers [1, 2, 8-10], the roles of honokiol in the process of cell apoptosis in human osteosarcoma MG63 cells remain largely unknown. To the best of our knowledge, the current study is the first attempt to determine whether fat mass and obesity-associated genes are involved in honokiol suppression of osteosarcoma cell proliferation. Present data provides evidence that honokiol reduced cell survival and tumor growth in human osteosarcoma MG63 cells *in vitro*.

Present data shows that Smad6 was downregulated in FTO overexpression of osteosarcoma cells, according to microarray assays and qRT-PCR. Previous studies have confirmed that Smad signaling impacts the progression of tumor-induced bone disease. Moreover, Runx2 in prostate cancer cells plays a significant role in intratibial prostate cancer-related tumor growth and bone loss through mechanisms mediated by Runx2-Smad signaling pathways [26]. Interestingly, immunohistochemical analysis of phosphorylated Smad1 and Smad2 showed nuclear expression of both proteins in 70% of the osteosarcoma samples, at levels comparable to osteoblastomas. Cases with lower expression showed significantly worse disease-free survival. This may imply that Smad signaling pathways in osteosarcomas might change tumor aggravation levels [27]. Moreover, overexpression of Smad6 blocks BMP-2 mediated chondrocyte differentiation [21]. More importantly, this study further confirmed that mRNA and protein expression levels of Smad6 were significantly increased in osteosarcoma cells with honokiol administration. Therefore, Smad6 might be involved in honokiol-induced osteosarcoma cell apoptosis. Aiming to identify whether an siRNA targeting human Smad gene was negatively correlated with honokiol-induced osteosarcoma cell apoptosis, it was found that Smad6 SiRNA could regulate protein expression levels of endogenous Smad6 and attenuate honokiol-induced osteosarcoma cell apoptosis.

Taken together, results suggest that honokiol induced cell death and suppressed osteosarcoma cell proliferation. Underlying mechanisms were mediated, at least partially, through downregulation of FTO and upregulation of Smad6. Honokiol provides an intriguing explanation of cellular and molecular mechanisms responsible for human osteosarcoma cell apoptosis.

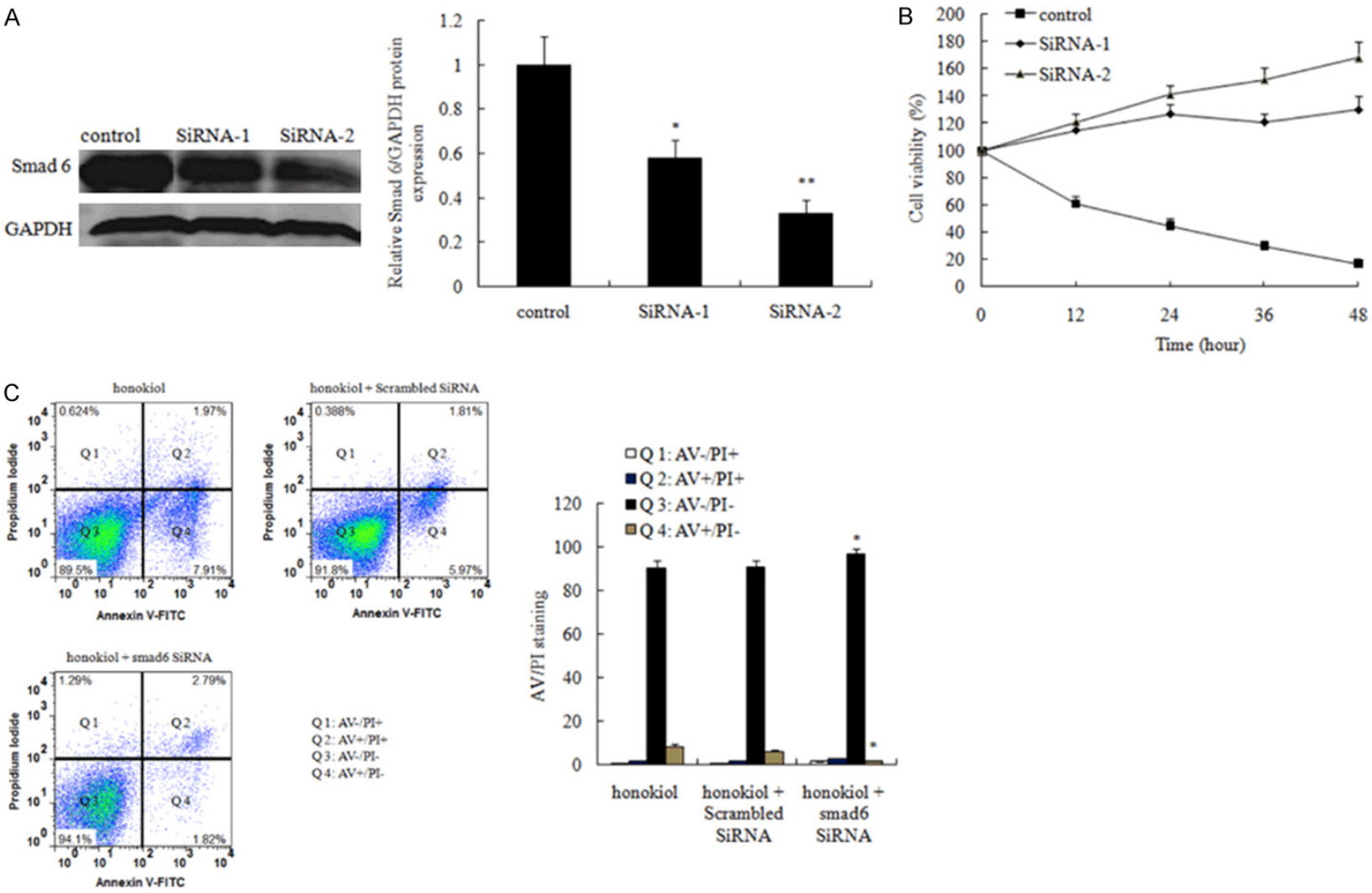

Figure 6. Validation of the possible signaling pathway involved in honokiol-induced osteosarcoma cell apoptosis. Human osteosarcoma MG63 cells were treated with Honokiol (100 μg/mL) and siRNA-1 (honokiol + Smad6 siRNA-1) or siRNA-2 (honokiol + Smad6 siRNA-2) for 24 hours. Next, (A) protein expression levels of Smad6 were measured by Western blotting; (B) Cell viability was examined by CCK8 assays; (C) The percentage of apoptotic cells was analyzed by flow cytometric analysis of annexin V/PI double staining. Values are expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$, versus the control group.

Thus, it may be an effective adjuvant therapy drug for clinical treatment.

Disclosure of conflict of interest

None.

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