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# Suppression of liver receptor homolog-1 by microRNA-451 represses the proliferation of osteosarcoma cells

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ABSTRACT

Liver receptor homolog-1 (LRH-1) plays an important role in the onset and progression of many cancer types. However, the role of LRH-1 in osteosarcoma has not been well investigated. In this study, the critical role of LRH-1 in osteosarcoma cells was described. Quantitative polymerase chain reaction and Western blot analysis results revealed that LRH-1 was highly overexpressed in osteosarcoma cells. LRH-1 was knocked down by small interfering RNA (siRNA), and this phenomenon significantly inhibited osteosarcoma cell proliferation. Bioinformatics analysis results showed that LRH-1 contained putative binding sites of microRNA-451 (miR-451); this result was further validated through a dual-luciferase activity reporter assay. miR-451 was overexpressed in osteosarcoma cells through transfection of miR-451 mimics; miR-451 overexpression then significantly inhibited LRH-1 expression and cell proliferation. The loss of LRH-1 by siRNA or miR-451 mimics significantly impaired Wnt/β-catenin activity, leading to G0/G1 cell cycle arrest. Results showed that LRH-1 is implicated in osteosarcoma. Therefore, miR-451-induced suppression of LRH-1 can be a novel therapy to treat osteosarcoma.



## 1. Introduction

Osteosarcoma is one of the most common malignant bone tumors affecting adolescents and children worldwide [1]. Osteosarcoma is also a highly metastatic tumor with a high degree of resistance to drugs and radiation [2]. However, effective treatments to improve the survival rate of patients with osteosarcoma remain insufficient [3]. Although an improved understanding of the mechanism of osteosarcoma may help develop effective therapeutic methods, specific molecular mechanisms underlying the onset and progression of osteosarcoma remain largely unknown. Indeed, novel molecular targets in the development of osteosarcoma are of great importance to treat this disease.

The role of liver receptor homolog-1 (LRH-1), also named as nuclear receptor subfamily 5 group A member 2, has been implicated in the development of different cancer types [4]. LRH-1 is a member of orphan nuclear receptor family involved in regulating many functions, including metabolism, differentiation, and development [5–7]. LRH-1 also regulates multiple biological processes, such as bile acid metabolism [8], reverse cholesterol transport [9], and glucose homeostasis [10]. Furthermore, LRH-1 plays an important role in various human cancers, including breast, liver, gastric, colon, and pancreatic cancers [6]. In breast cancer, LRH-1 is highly expressed; LRH-1 suppression blocks tumor cell proliferation, invasion, and migration [11–13]. LRH-1 overexpression in pancreatic and hepatic cancer cells promotes colony formation, cell proliferation, and tumor progression [14]. These findings suggest that LRH-1 can be involved in the onset and progression of cancers. However, the critical role of LRH-1 in osteosarcoma remains poorly understood.

With the critical role of LRH-1 in cancer progression, targeting LRH-1 may be applied to develop novel cancer therapy. In addition to chemical inhibitors of gene expression, microRNAs (miRNAs) have been considered as a novel tool to regulate gene expression [15]. miRNAs are small endogenously expressed non-coding RNAs that can bind the 3′-untranslated region (UTR) of mRNA of their target genes, thereby inhibiting protein translation [16]. miRNAs have also been implicated in the development of osteosarcoma [17,18]. For instance, miRNAs are differently expressed in human osteosarcoma cells; in particular, miR-9/99/148a/181a/195 are overexpressed, whereas miR-143/145/335/539 are downregulated [19]. miRNAs with different expressions in circulation have been proposed as a novel class of osteosarcoma biomarkers [20–23]. The

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expression of miRNAs, such as miR-9/34a/192, in tumor tissues is also associated with prognosis of patients with osteosarcoma [24,25]. miR-217 suppresses tumor growth and metastasis of osteosarcoma by targeting Wiskott-Aldrich syndrome protein family member 3 [26]. Zhang et al. [27] demonstrated that miR-301a regulates chemotherapy resistance of osteosarcoma by targeting AMP-activated protein kinase alpha 1. Therefore, miRNA-targeted therapy can be applied to treat osteosarcoma.

In this study, the potential role of LRH-1 in osteosarcoma was investigated. Our results indicated that LRH-1 was significantly overexpressed in osteosarcoma cells; LRH-1 was also knocked down, and this phenomenon inhibited tumor cell proliferation. Bioinformatics analysis results revealed that LRH-1 contained putative binding sites of miR-451; these findings were validated through a dual-luciferase activity reporter assay. miR-451 overexpression also significantly inhibited LRH-1 expression in osteosarcoma cells and induced a similar biological effect of the small interfering RNA (siRNA) of LRH-1 on cell proliferation. This study suggested that LRH-1 plays an important role in regulating osteosarcoma cell proliferation; thus, targeting LRH-1 by miR-451 can be applied to treat osteosarcoma.

## 2. Materials and methods

### 2.1. Cell culture

Human osteosarcoma U2OS and MG63 cell lines and human normal fetal osteoblastic cell line hFOB1.19 (hFOB) were obtained from American Type Culture Collection (Manassas, VA, USA). hFOB was used as a control. These cells were maintained in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C and 5% $CO_2$ in a humidified incubator.

### 2.2. Cell transfection

The siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) of LRH-1 was transfected into cells according to standard transfection protocol. In brief, the cells were plated on a six-well tissue culture plate at $2 \times 10^5$ cells per well and cultured until 60%–80% confluency was reached. In transfection, siRNA was diluted in a transfection medium containing transfection reagent to obtain a concentration of 0.01 μg/μl and incubated for 30 min at room temperature. The transfection mixture was then added to each well and incubated for 48 h miR-451 mimics and negative control (NC) miRNAs were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). miRNAs were introduced to cells by using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 50 nM according to the manufacturer's instructions.

### 2.3. Quantitative real-time PCR (qPCR)

Total RNAs in cells were extracted using an miRNeasy mini kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. cDNA was generated using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). Reverse transcription was conducted using an miScript reverse transcription kit (Qiagen) to analyze miRNA expression. qPCR was performed in an ABI7500 real-time PCR detection system (Applied Biosystems, Carlsbad, CA, USA) by using SYBR Green Master Mix (Life Technologies). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA were used as internal references of mRNA and miRNA expressions, respectively.

### 2.4. Western blot analysis

Cells were harvested and lysed in a lysis buffer. Protein concentrations were determined using a BCA kit (Beyotime, Haimen, China). An equal amount of protein was loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were separated through SDS-PAGE and then transferred to a nitrocellulose membrane (Miltenyi Biotec, Auburn, CA, USA). The membrane was blocked by 2.5% nonfat milk and incubated with anti-LRH-1 and anti-GAPDH antibodies (Bioss, Beijing, China) at 4 °C overnight. The membrane was labeled with secondary antibodies (1:2000; Bioss) and the protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Biosciences, Little Chalfont, UK). Band intensity was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

### 2.5. Cell proliferation assay

Cell proliferation was assessed through 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. In brief, cells were seeded into a 96-well plate and transfected with siRNA or miRNAs. After the cells were incubated for 48 h, the old medium was replaced with an equal volume of fresh medium. MTT (0.5 mg/ml in PBS) was then added at 20 μl per well and incubated for another 4 h. Afterward, the medium was discarded and 150 μl of dimethyl sulfoxide was added to each well to dissolve formazan crystal. Absorbance was read at 490 nm by using a microplate reader (ThermoElectron Corporation, Vantaa, Finland).

### 2.6. Cell cycle analysis

Cell cycle distribution was determined through fluorescence-activated cell sorting. The cells were serum starved for 24 h, transfected with siRNA or miRNAs, and incubated for 24 h. Afterward, the cells were harvested, washed with ice-cold PBS, fixed in 70% ethanol, and stained with PI/RNase fluorescent probe solution (Molecular Probes, Eugene, OR, USA) for 30 min in the dark. Cell cycle distribution was assessed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

### 2.7. Dual-luciferase reporter assay

The 3′-UTR of LRH-1 containing the putative binding site of miR-451 was amplified and subcloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The vector was co-transfected with miR-451 mimics into human embryo kidney 293 cells for 48 h. The cells were harvested and relative luciferase activity was detected using a dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions.

### 2.8. Wnt signaling activity assay

The cells were introduced using a TCF-responsive reporter, a TOPFlash firefly luciferase reporter vector (Addgene, Cambridge, MA, USA), and *Renilla* luciferase vectors phRL-TK (Promega). After siRNA or miRNA transfection was performed for 24 h, the cells were harvested and luciferase activities were quantified using a dual-luciferase reporter assay kit (Promega).

### 2.9. Data analysis

Data were presented as mean ± standard deviation (SD). Differences were determined through one-way ANOVA in SPSS

version 11.5 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. LRH-1 is highly expressed in human osteosarcoma cells

To investigate whether LRH-1 plays a key role in human osteosarcoma, we initially detected the expression profile of LRH-1 in human osteosarcoma cell lines. The results showed that the mRNA expression level of LRH-1 was significantly higher in human osteosarcoma U2OS and MG63 (Fig. 1A) cells than in normal control hFOB cells. Western blot analysis results also revealed that the protein expression level of LRH-1 was more abundant in U2OS and MG63 (Fig. 1B) cells than in control hFOB cells.

### 3.2. LRH-1 silencing impairs osteosarcoma cell proliferation

To determine the regulatory function of LRH-1 in osteosarcoma cells, we silenced LRH-1 expression by using siRNA that targeted LRH-1. The protein expression of LRH-1 in U2OS (Fig. 2A) and MG63 (Fig. 2B) cells was suppressed, and this finding was confirmed through Western blot analysis. We then evaluated the effect of LRH-1 expression silencing on cell proliferation by conducting an MTT assay. The results showed that LRH-1 was knocked down, and this knockdown significantly inhibited U2OS (Fig. 2C) and MG63 (Fig. 2D) cell proliferation. Furthermore, LRH-1 silencing markedly increased G0/G1 phase population in U2OS (Fig. 2E) and MG63 (Fig. 2F) cells compared with that in control cells.

### 3.3. LRH-1 is a direct target gene of miR-451

Gene expression regulated by miRNAs can be applied to treat diseases, including cancer [28]. Thus far, miRNAs that target and regulate LRH-1 have been rarely investigated. In this study, LRH-1 was predicted as a putative target gene of miR-451 through bioinformatics analysis (Fig. 3A). To verify their relationship, we performed a dual-luciferase reporter assay. The results showed that miR-451 mimics remarkably decreased the luciferase activity in the 3′-UTR of wild-type (WT) LRH-1 transfected cells (Fig. 3B). By contrast, miR-451 mimics did not evidently affect the 3′-UTR of mutant-type (MT) LRH-1 transfected cells. We further determined whether miR-451 regulates LRH-1 expression in human osteosarcoma cells. mRNA and protein expressions of LRH-1 were detected in miR-451 mimic-transfected cells. The mRNA expression level of LRH-1 was significantly decreased by miR-451 mimics (Fig. 3C and D). Western blot analysis results showed that the protein expression level of LRH-1 was also downregulated by miR-451 mimics (Fig. 3E and F).

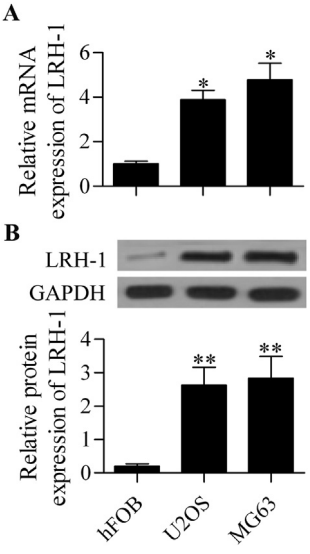


**Fig. 1.** Detection of LRH-1 expression in human osteosarcoma cells. (A) qPCR analysis of the mRNA expression of LRH-1 in human osteosarcoma U2OS and MG63 cells. (B) Western blot analysis of the protein expression of LRH-1 in U2OS and MG63 cells. Relative protein expression was analyzed with Image-Pro Plus 6.0 software and normalized with GAPDH. Human normal fetal osteoblastic hFOB cells were used as control. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. hFOB.

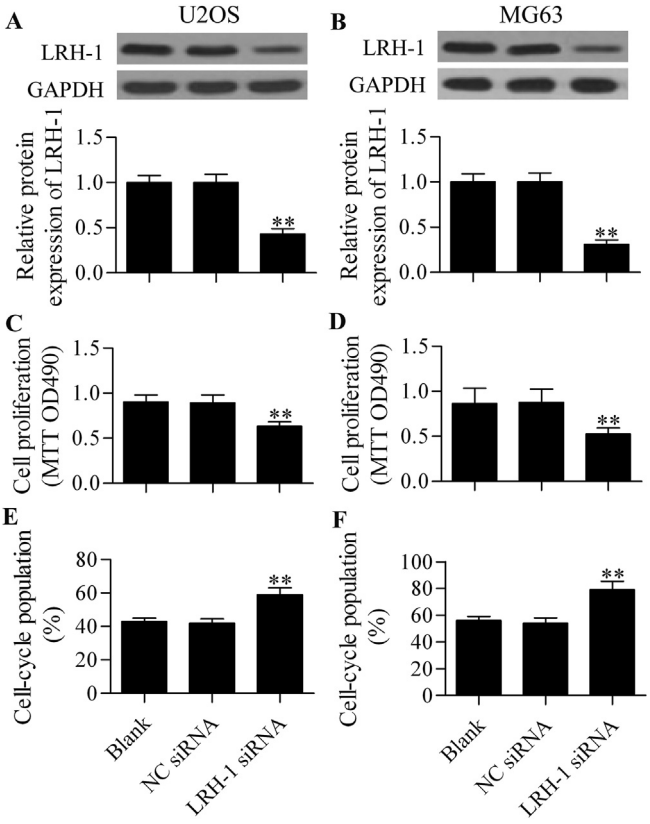


**Fig. 2.** Effect of LRH-1 silencing on osteosarcoma cell proliferation. Western blot analysis of the protein expression of LRH-1 in U2OS (A) and MG63 (B) transfected with the siRNA of LRH-1 for 48 h. NC siRNA, cells transfected with negative control (NC) siRNA; Blank, cells without any transfection. $^{**}p < 0.01$ vs. blank or NC siRNA. Effect of LRH-1 suppression on U2OS (C) and MG63 (D) cell proliferation detected through MTT assay. Cells were transfected with the siRNA of LRH-1 for 48 h $^{**}p < 0.01$ vs. blank or NC siRNA. Analysis of cell cycle population in G0/G1 phase after LRH-1 was knocked down in U2OS (E) and MG63 (F) cells. $^{**}p < 0.01$ vs. blank or NC siRNA.

### 3.4. miR-451 overexpression induces the inhibitory effect of the siRNA of LRH-1 on cell proliferation

Considering that miR-451 regulates LRH-1 expression, we can speculate that miR-451 can modulate osteosarcoma cell proliferation. The results showed that miR-451 overexpression in osteosarcoma cells significantly suppressed cell proliferation (Fig. 4A and B). Furthermore, G0/G1 phase population in osteosarcoma cells was remarkably increased when miR-451 was overexpressed (Fig. 4C and D).

### 3.5. Loss of LRH-1 in osteosarcoma cells impairs the activity of Wnt signaling

LRH-1 regulates cell proliferation by modulating Wnt/β-catenin [14]. To investigate whether the loss of LRH-1 impairs Wnt

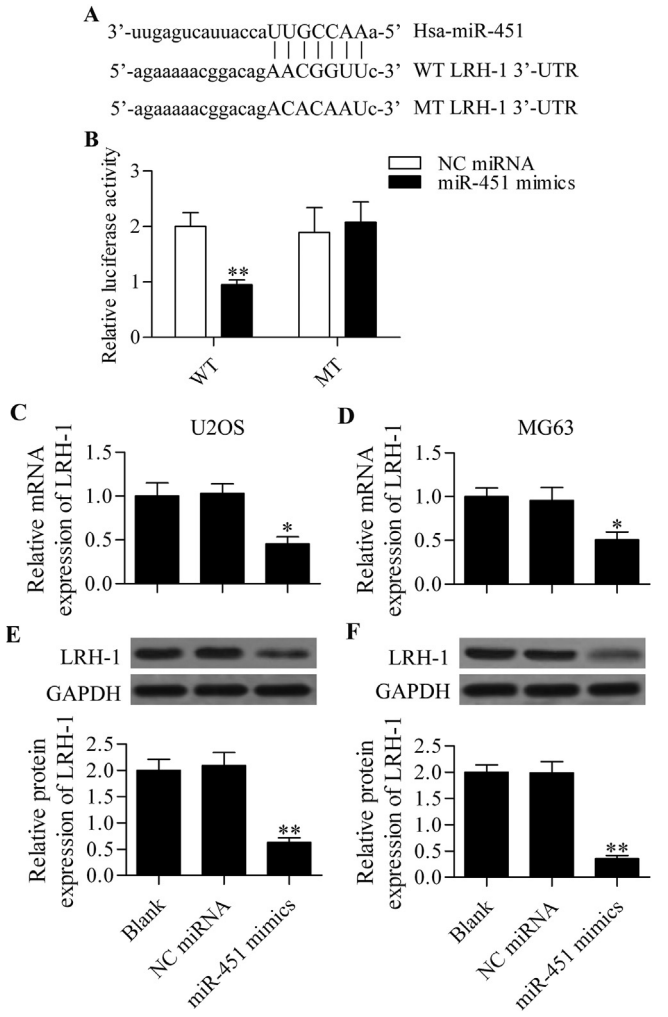


**Fig. 3.** LRH-1 is a direct target gene of miR-451. (A) Schematic of the 3′-UTR of LRH-1 containing wild-type (WT) or mutant-type (MT) miR-451 binding site. (B) Dual-luciferase reporter assay of cells co-transfected with miR-451 or NC miRNAs and 3′-UTR of WT or MT LRH-1. **p < 0.01 vs. NC siRNA. qPCR analysis of LRH-1 expression in U2OS (C) and MG63 (D) cells transfected with miR-451 mimics for 48 h. NC miRNA, cells transfected with negative control (NC) miRNA; Blank, cells without any transfection. *p < 0.05 vs. blank or NC miRNA. Protein expression of LRH-1 in U2OS (E) and MG63 (F) cells was detected through Western blot analysis after miR-451 mimics were transfected for 48 h **p < 0.01 vs. blank or NC miRNA.

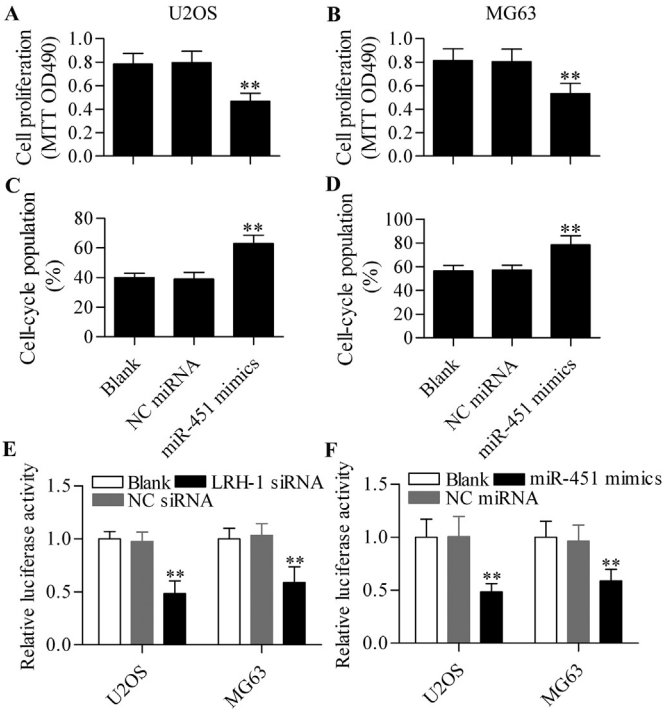


**Fig. 4.** miR-451 suppresses osteosarcoma cell proliferation and Wnt activity. Effect of miR-451 overexpression on U2OS (A) and MG63 (B) cell proliferation detected through MTT assay. Cells were transfected with miR-451 mimics for 48 h **p < 0.01 vs. blank or NC miRNA. Analysis of cell cycle population in the G0/G1 phase of U2OS (C) and MG63 (D) cells after these cells were transfected with miR-451 mimics. **p < 0.01 vs. blank or NC miRNA. Effect of LRH-1 siRNA (A) or miR-451 mimics (B) on Wnt signaling activity detected through TCF-dependent TOPFlash reporter activity assay. **p < 0.01 vs. blank or NC.

signaling in osteosarcoma cells, we detected Wnt signaling activity by performing a luciferase assay. The results showed that siRNA-silenced LRH-1 significantly decreased Wnt activity compared with that of the control sample (Fig. 4E). miR-451-induced inhibition of LRH-1 also impaired Wnt activity in osteosarcoma cells (Fig. 4F). Therefore, siRNA- or miR-451 overexpression-induced inhibition of LRH-1 impaired Wnt signaling activity.

## 4. Discussion

To the best of our knowledge, this study is the first to demonstrate the critical role of LRH-1 in osteosarcoma. We found that the mRNA and protein of LRH-1 were significantly overexpressed in human osteosarcoma U2OS and MG63 cells. LRH-1 was silenced by a specific siRNA; LRH-1 silencing then impaired osteosarcoma cell proliferation. We also observed that miR-451 targeted the 3′-UTR of LRH-1 and regulated LRH-1 expression in osteosarcoma cells. LRH-1 was also inhibited by miR-451 mimics; this inhibition significantly impaired cell proliferation and Wnt signaling activity in osteosarcoma cells. Therefore, targeting LRH-1 by miR-451 could be applied to repress osteosarcoma.

LRH-1 is an estrogen receptor target gene, which is highly induced by 17β-estradiol; this finding indicates that LRH-1 exhibits a potential oncogenic role in breast cancer [11]. LRH-1 also regulates estrogen receptor expression in breast cancer cells [29]. Likewise, LRH-1 regulates proliferation, migration, and invasion of breast cancer cells [13,30]. A high LRH-1 expression has also been detected in gastric cancer tissues; LRH-1 overexpression promotes the proliferation of gastric cancer cells [31]. Benod et al. [32] reported that LRH-1 is highly overexpressed in pancreatic cancer tissues; LRH-1 blocked by specific siRNA significantly inhibits the proliferation of pancreatic cancer cells. Consistent with these findings, our data suggested that LRH-1 is an oncogene implicated in osteosarcoma; this oncogene is abundant in osteosarcoma cells. Furthermore, LRH-1 silencing by specific siRNA effectively inhibits osteosarcoma cell proliferation. Our data implied that LRH-1 may be used as a therapeutic target to treat osteosarcoma.

Considering the critical role of LRH-1 in cancers, we believe that targeting LRH-1 may help develop effective therapies to treat LRH-1-induced cancers. Bayrer et al. [33] demonstrated that LRH-1 silencing significantly inhibits cell proliferation and gene expression programs in colon cancer cell lines. SR1848, a small molecule repressor of LRH-1, can effectively inhibit LRH-1 activity and impair the proliferation of liver and ovarian cancer cells [34]. In addition to chemical inhibitors, miRNAs have been considered as a novel tool to regulate gene expression [15]. In our study, the 3′-UTR of LRH-1 contained the putative binding sites of miR-451, and this finding was confirmed through dual-luciferase activity reporter assay. miR-

451 is a tumor suppressor in various cancers, including lung cancer [35], liver cancer [36], and bladder cancer [37]. High miR-451 expression in pre-treatment samples is correlated with positive response to chemotherapy of patients with osteosarcoma [38]. Decreased miR-451 expression has also been found in many osteosarcoma cell lines [39]. Xu et al. [40] reported that miR-451 is downregulated in osteosarcoma cell lines and tumor tissues; miR-451 overexpression also inhibits the proliferation of osteosarcoma cells. Likewise, Yuan et al. [41] demonstrated that miR-451 expression is significantly lower in osteosarcoma tissues, particularly in osteosarcoma tissues exhibiting positive distant metastasis, advanced clinical stage, and chemotherapy resistance. Osteosarcoma cell proliferation and migration are also significantly inhibited by miR-451 transfection [41]. However, target genes regulated by miR-451 in osteosarcoma cells have been rarely investigated. miR-451 targets chemokine ligand 16 to regulate growth and invasion of osteosarcoma cells [42]. Our results indicated that LRH-1 is a predicted target gene of miR-451. Therefore, high LRH-1 expression in osteosarcoma cells may be attributed to the decreased miR-451 expression. We further demonstrated that LRH-1 expression was significantly inhibited by miR-451 mimics. miR-451 overexpression also significantly suppressed the proliferation of osteosarcoma cells. The results implied that osteosarcoma can be repressed by inhibiting LRH-1 expression, as induced by miR-451.

LRH-1 is a coactivator of Wnt/β-catenin that synergistically activates cell cycle-related genes, including cyclin E1, cyclin D1, and c-Myc, to promote cell proliferation [14]. Wnt/β-catenin pathway has been proposed as a critical signaling pathway involved in the initiation and progression of osteosarcoma [43]. Our results showed that siRNA- or miR-451-induced inhibition of LRH-1 significantly decreases the activity of Wnt/β-catenin signaling pathway in osteosarcoma. Our results also explained why the loss of LRH-1 inhibits proliferation and causes G0/G1 cell cycle arrest of osteosarcoma cells.

In this study, LRH-1 was overexpressed in osteosarcoma cells. siRNA-induced suppression of LRH-1 significantly repressed cell proliferation mediated by G0/G1 cell cycle arrest. Furthermore, miR-451 directly targeted and regulated LRH-1 expression in osteosarcoma cells; miR-451 also caused a similar biological effect to the siRNA of LRH-1. The loss of LRH-1 by its siRNA or miR-451 mimics markedly impaired the activity of Wnt/β-catenin in osteosarcoma cells. Therefore, LRH-1 plays an important role in osteosarcoma; miR-451-induced inhibition of LRH-1 can be a novel therapy to treat this disease.

## Conflict of interests

None.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.013.

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