HIF-1-mediated expression of Foxo1 serves an important role in the proliferation and apoptosis of osteoblasts derived from children's iliac cancellous bone

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Abstract. Activation of the transcription factor hypoxia inducible factor-1α (HIF-1α) is considered critical for the stimulation of osteogenic markers including runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin, which are closely associated with forkhead boxclass O1 (Foxo1) levels in osteoblasts. The present study explored the associations between HIF-1α and Foxo1 in the regulation of cell viability, proliferation and apoptosis of osteoblasts. Osteoblasts obtained from children's iliac cancellous bone were used in the present study, which were confirmed by immunofluorescence staining for the osteoblast marker osteocalcin. The results revealed that the levels of reactive oxygen species and apoptosis were markedly increased in cells with knockdown of HIF-1α. By contrast, these were reduced in response to overexpressed HIF-1α. In addition, HIF-1α overexpression significantly stimulated cell viability, which was suppressed by silencing HIF-1α. HIF-1α overexpression also significantly increased the transcriptional and translational levels of Foxo1. Conversely, silencing HIF-1α markedly suppressed the expression levels of Foxo1. Furthermore, silencing HIF-1α reduced the expression of osteogenic markers, including Runx2, ALP and osteocalcin. Runx2 and ALP expression induced by HIF1α were markedly reversed by Foxo1 small interfering (si)RNA, whereas osteocalcin was not significantly affected by Foxo1 siRNA. Therefore, the cooperation of and interactions between HIF-1α and Foxo1 may be involved in the regulation of osteoblast markers, and serve a pivotal role in the proliferation and apoptosis of osteoblast. The HIF1α-induced expression of Runx2 and ALP may be completely dependent on the expression levels of Foxo1, and in turn, osteocalcin may be partially dependent on Foxo1 expression.

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Introduction

Osteoporosis and fractures are the most common orthopedic diseases in the elderly (1). have been plagued by the delayed healing and nonunion of fractures, bone defects and osteoporosis have become a growing concern for orthopedic clinicians, and the outcome of current clinical treatments are not very satisfactory. Therefore, in order to prevent and reduce the occurrence of osteoporosis and fracture, understanding the mechanisms underlying osteoporosis and fracture healing has become important in research associated with bone injury.

The maintenance of the normal functional state of the body is dependent on an appropriate supply of oxygen. In addition, maintaining homeostasis in regard to oxygen levels is a prerequisite for cell life activity (2), and tissue oxygen concentrations must be precisely controlled to fluctuate only within a very small range (2,3). Due to a reduction in blood supply following bone or soft tissue injury, the microenvironment surrounding lesions enters a hypoxia state (4). Thus, angiogenesis serves an important role in the process of fracture healing (5,6). Osteogenesis is closely associated with angiogenesis in the formation and repair of bone (5-7). Vessels carry oxygen and nutrients; however, they also serve a pivotal role in the formation, reshaping and alteration of bone through interactions between osteoblasts, osteocytes or osteoclasts and cytokines in the blood vessels (5-7). Hypoxia is considered to be an important stimulus in angiogenesis (8); this stimulus is now thought to be achieved by hypoxia inducible factor-1α (HIF-1α) (9). HIF-1α is a key regulator of vertebrate adaptation to hypoxia (9). The study of HIF-1α expression levels, its function and hypoxia status under physiological and pathological conditions in the skeletal system has become an area of growing interest (10,11). There are two main aspects associated with the regulatory function of HIF-1α in fracture healing. The first being that HIF-1α can induce the formation of blood vessels during the healing process of the fracture by stimulating the expression of vascular endothelial growth factor (VEGF) (10,11). Secondly, HIF-1α is directly involved with the regulation of cell functioning, including in osteoblasts,

osteoclasts and chondrocytes (10,11). However, the detailed mechanisms of HIF-1α in the proliferation, apoptosis and differentiation of osteoblasts have not been fully elucidated. Previous studies have revealed that blocking expression of runt-related transcription factor 2 (Runx2) and HIF-1α inhibited the formation of heterotopic ossification (11,12). Runx2 can stabilize HIF-1α structure via the inhibition of HIF-1α ubiquitination in order to promote angiogenesis in growth plate hypertrophic chondrocytes (12).

Forkhead box class O1 (Foxo1) is one of the earliest members identified in the Foxo family, and is also the most representative of the Foxo family. Previous studies had demonstrated that they serve an important role in a number of physiological and pathological processes, including proliferation, apoptosis, phagocytosis, metabolism, inflammation, differentiation and oxidative stress (13,14). Previous studies revealed that Foxo1 mediates dendritic cells and macrophages in order to regulate associated target genes in inflammatory responses (15); osteoclasts, dendritic cells and macrophages share a common precursor cell line. However, only Foxo1 is the transcription factor required for osteoblast proliferation and the maintenance of the body's redox balance (16). In addition, previous studies have demonstrated that interactions and cooperation between Foxo1and Runx2 serve a key role in the transcriptional regulation of osteoblast markers, including alkaline phosphatase (ALP), and osteocalcin (17,18). Runx2, ALP and osteocalcin are closely associated with the development of osteoblasts.

In addition, orthopedic diseases in children and adolescents, such as osteoporosis, and children with avascular necrosis and non-traumatic avascular necrosis of the femoral head, have received little attention when compared with orthopedic diseases observed in the elderly (19-21). A previous study revealed that the negative associations between HIF-1α and the rate of bone cell apoptosis was involved in the non-traumatic avascular necrosis of the femoral head (22). Furthermore, there has been no report regarding the associations between HIF-1α and Foxo1. Thus, in the present study, children's iliac cancellous bone was used to determine whether HIF-1α regulates the proliferation, differentiation and apoptosis of osteoblasts through the regulation of Foxo1 expression.

Materials and methods

Cell culture. Bone tissues were obtained between February 2015 and March 2017 from children with congenital dislocation of the hip when they underwent surgery for extra iliac bone at Department of Orthopedics, the Children's Hospital, Zhejiang University School of Medicine. The present study was approved by the institutional review board of The Children's Hospital (Zhejiang, China) and written informed consent was obtained from the parents of each participant. Only children who were not taking hormones or other drugs, and had no metabolic bone disease were enrolled, comprising 2 males and 2 females, aged 3-5 years old. The obtained bone tissues were maintained aseptically, and placed in DMEM/F-12 serum-free medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) for storage at 4˚C. Bone tissues were repeatedly rinsed with 0.9% sterile saline until the rinse solution was clear without any precipitates, and were washed twice with Dulbecco's PBS. The bone tissue was cut to a size with ~1 mm^3 volume and digested with 0.25% trypsin at 37˚C for 30 min.

Bone particles were then digested 4 times using 0.1% collagenase II at 37˚C for 30 min. The cells were collected by centrifugation at 500 x g for 10 min at 4˚C. They were inoculated into four 100 ml flasks, DMEM/F-12 serum-free medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added and the cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. According to levels of the growth, culture medium was replaced in the first 3-5 days, then it was subsequently replaced every 2-3 days. When the primary cells were grown into monolayers, the cells were digested with 0.25% trypsin for 3-5 min at 37˚C to continue subculture. In addition to natural purification, enzymatic digestion and repeated adherence methods were used to purify cells (23).

Identification of osteoblasts. The isolated cells were cultured in primary culture, and morphological observation and imaging were performed under an inverted phase contrast microscope when the cells were subcultured to 80% confluence.

Cell osteocalcin immunofluorescence staining was also performed to identify osteoblasts. Osteoblasts were inoculated on coverslips and the medium was discarded when the cells reached 80% confluency; cells were then fixed with 95% ethanol for 10 min. Cell climbing slices were washed with PBS three times for 5 min each, incubated at room temperature with 0.5% Triton X-100 for 10 min, and then washed 3 times with PBS for 5 min each. Subsequently, once the slices were incubated with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature, the anti-osteocalcin antibody (ab13418; 31:100; Abcam, Cambridge, UK) was added for incubation overnight at 4˚C; this was followed by 3 washes with PBS for 5 min. The slices were then incubated with a TRITC-labeled secondary antibody (YB1130; 1:50; Dako; Agilent Technologies GmbH, Waldbronn, Germany) for 45 min at room temperature. Subsequently, DAPI staining was performed to stain the nuclei for 15 min at room temperature, which was followed by 3 washes with PBS for 5 min each. Then five fields were randomly selected from each section and observed and imaged under a laser confocal microscope.

Cell transfection. HIF-1α small interfering (si)RNA (5'CCA ACCTCAGTGTGGGT-AT3') and negative siRNA control (5'CCATGTAG-GCGCAGTCTAT3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and recombinant plasmid containing HIF-1α (Addgene, Inc., Cambridge, MA, USA) were transfected into cells with Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Briefly, prior treatment with siRNA, cells were seeded in 6-well plates and grown to 50% confluence. Transfection of 50 nM siRNA in cells was carried out using Lipofectamine 2000® following the manufacturer's protocols. Cells were then incubated for 5 h at 37˚C and the medium was replaced with complete DMEM medium (Sigma-Aldrich; Merck KGaA). Cells were harvested at least 24 h following transfection for use in the following experiments.

Cell viability assay. Cell viability was determined using a Cell Counting kit (CCK)-8 assay. Cells collected at 24,

48, 72 and 96 h following transfection were inoculated in 96-well plates ($2x10^5$ cells/well) and 20 μl of CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well. Following incubation for 4 h at 37˚C, the absorption was read at 450 nm on an ELISA reader (ELx800™; BioTek Instruments, Minneapolis, MN, USA).

Reactive oxygen species (ROS) assay. Cells were harvested from all groups [control, negative control (NC), HIF1a, Mock and siHIF1a] and washed with PBS following transfection for 24 h. Cells were then incubated in 20 μM 2',7'-Dichorofluorescin diacetate (Sigma-Aldrich; Merck KGaA) at 37˚C for 1 h following the manufacturer's protocols. Following washing with PBS, ROS levels in cells were determined using FACS Aria II flow cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was performed using FlowJo version 7.6 (FlowJo LLC, Ashland, OR, USA).

Apoptosis determination by flow cytometry assay. Cells collected from all groups (control, NC, HIF1a, Mock and siHIF1a) were digested using 0.25% trypsin-EDTA for 3-5 min at 37˚C. Subsequently, cells were harvested at a density of $1x10^6$ by centrifugation at 500 x g for 4 min at 4˚C. Cells were washed with PBS and then placed in binding buffer (140 mM NaCl and 2·5 mM CaCl2 in 10 mM HEPES/NaOH; pH 7·4). A total of 5 μl propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled Annexin V (Biodesign International; Meridian Life Science, Inc., Memphis, TN, USA) were added to cells for incubation at room temperature for 10 min. Samples were analyzed by FACS Aria II flow cytometer (BD Biosciences) and data analysis was performed using FlowJo version 7.6 (FlowJo LLC). Those that were Annexin V-FITC positive and PI negative were considered early apoptotic cells, and late apoptotic cells were indicated by Annexin V-FITC positive and PI positive.

Western blot assay. Cells were collected from all groups and washed twice with ice-cold PBS. Cells were then lysed in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.25% deoxycholate, and protease and phosphatase inhibitors) at 37˚C for 30 min and centrifuged for 20 min at 6,000 x g at 4˚C. The supernatants were collected and 50 μg of cell lysate was used to separate proteins by 10% SDS-PAGE, which were then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature. Subsequently, the blots were incubated with primary antibodies against apoptosis-inducing factor (AIF; ab32516; 1:1,000; Abcam), B-cell lymphoma 2 (Bcl-2; ab32124; 1:1,000; Abcam), Bcl-2-associated X protein (Bax; ab32503; 1:2,000; Abcam), caspase-3 (ab13585; 1:1,000; Abcam), Runx2 (ab76956; 1:1,000; Abcam), ALP (ab224335; 1:1,000; Abcam), osteocalcin (ab13420; 1:1,000; Abcam), F-actin (ab205; 1:500; Abcam) and Foxo1 (ab207204; 1:1,000; Abcam), at the appropriate dilution at 4˚C overnight. The blots were then washed three times with TBS and incubated with horseradish peroxidase-conjugated secondary antibodies (P0260; 1:2,000 dilution; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for at room temperature for 1 h. The protein-antibody complexes were detected using an enhanced chemiluminescence system (GE Healthcare Life Sciences, Little Chalfont, UK). ImageJ software (version 1.42; National Institutes of Health, Bethesda, MD, USA) was used to determine densitometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Cells collected from all groups were washed twice with ice-cold PBS. RNA was isolated using the RNeasy mini-kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols. Reverse transcription was carried out at with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocols. The temperature protocol used for RT-PCR was: 30˚C for 10 min, 42˚C for 30 min, 99˚C for 5 min and 4˚C for 5 min. Subsequently, qPCR was performed using the iQ SYBR-Green Supermix (Bio-Rad Laboratories, Inc.) and iCycleriQ thermal cycler (Bio-Rad Laboratories, Inc.) following the manufacturer's protocols. The thermocycling conditions for qPCR were: 45˚C for 10 min, 95˚C for 10 min, 40 cycles of 95˚C 15 sec and 60˚C for 45 sec. Each sample was performed in duplicate. Data was calculated using the $2^{-\Delta\Delta Cq}$ method (24) and relative expression was normalized to housekeeping gene (GAPDH). The primer sequences used were as follows: HIF-1α forward, 5'-TCCAAGAAGCCCTAACGTGT-3' and reverse, 5'-TGATCGTCTGGCTGCTGTAA-3'; AIF forward, 5'-TCTACCCTCTATGCCAGGACT-3' and reverse, 5'-ACCCAGATGTTAGAGCGTGC-3'; Bax forward, 5'-TCATGGGCTGGACACTGGAC-3' and reverse, 5'-CACAGTCCAAGGCAGTGGGA-3'; Bcl-2 forward, 5'-TGGGCCACAAGTGAAGTCAA-3' and reverse, 5'-TGATGCGGAAGTCACCGAAA-3'; caspase-3 forward, 5'-TCTGGTTTTCGGTGGGTGTG-3' and reverse, 5'-GTCGGCCTCCACTGGTATTT-3'; Foxo1 forward, 5'-GCGCTTAGACTGTGACATGG-3' and reverse, 5'-ACTAACCCTCAGCCTGACAC-3'; Runx2 forward, 5'-CTGTGGTTACTGTCATGGCG-3' and reverse, 5'-AGGTAGCTACTTGGGGAGGA-3'; ALP forward, 5'-GTCAGTGGGAGTGGTAACCA-3' and reverse, 5'-ACATGTACTTTCGGCCTCCA-3'; Osteocalcin forward, 5'-AATCCGGACTGTGACGAGTT-3' and reverse, 5'-TTATTTGGGAGCAGCTGGGA-3'; and GAPDH forward, 5'-CGGGAAACTGTGGCGTGATG-3', and reverse 5'-ATGACCTTGCCCACAGCCTT-3'.

Statistical analysis. All of the experimental data were expressed as the mean ± standard deviation. A t-test was used for comparisons between two groups and a one-way analysis of variance was performed for multiple comparisons and Bonferroni post hoc test was used for pairwise comparison. All experiments were repeated at least 3 times. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of osteoblasts by morphology and fluorescence immunity. The primary cultured human osteoblasts were observed under an inverted phase contrast microscope and revealed spherical morphology prior to adherent growth. Following incubation, the cells adhered and distributed evenly on the wall of the bottle. The cells were irregularly shaped,

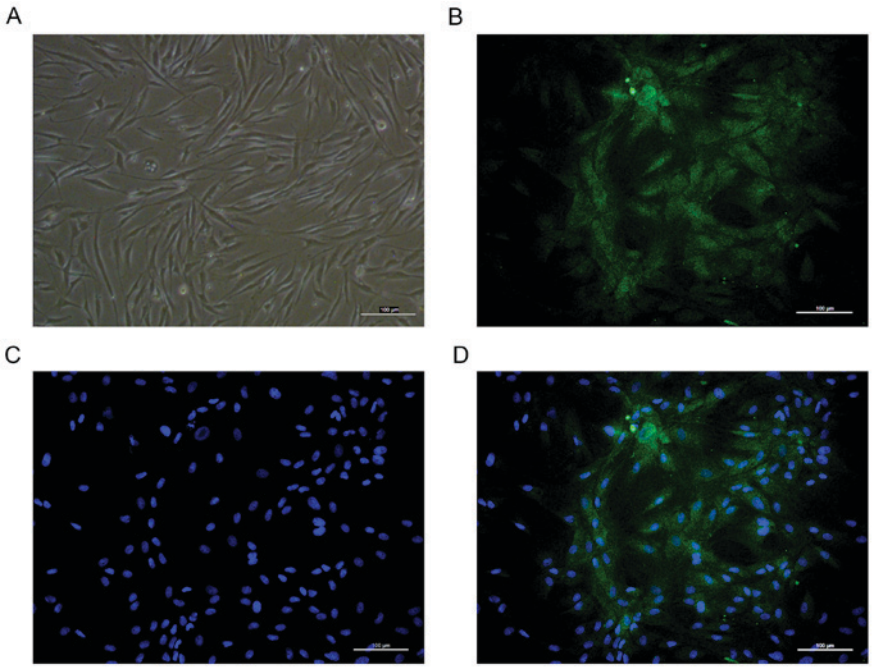

Figure 1. Osteoblasts were obtained from children's iliac cancellous bone following several purification processes. (A) The primary cultured children's osteoblasts were observed under an inverted phase contrast microscope. (B) Osteocalcin in osteoblast cytoplasm was stained in green and (C) osteocalcin in the nuclei was stained blue with DAPI; (D) these images were then merged (scale bars, 100 μm).

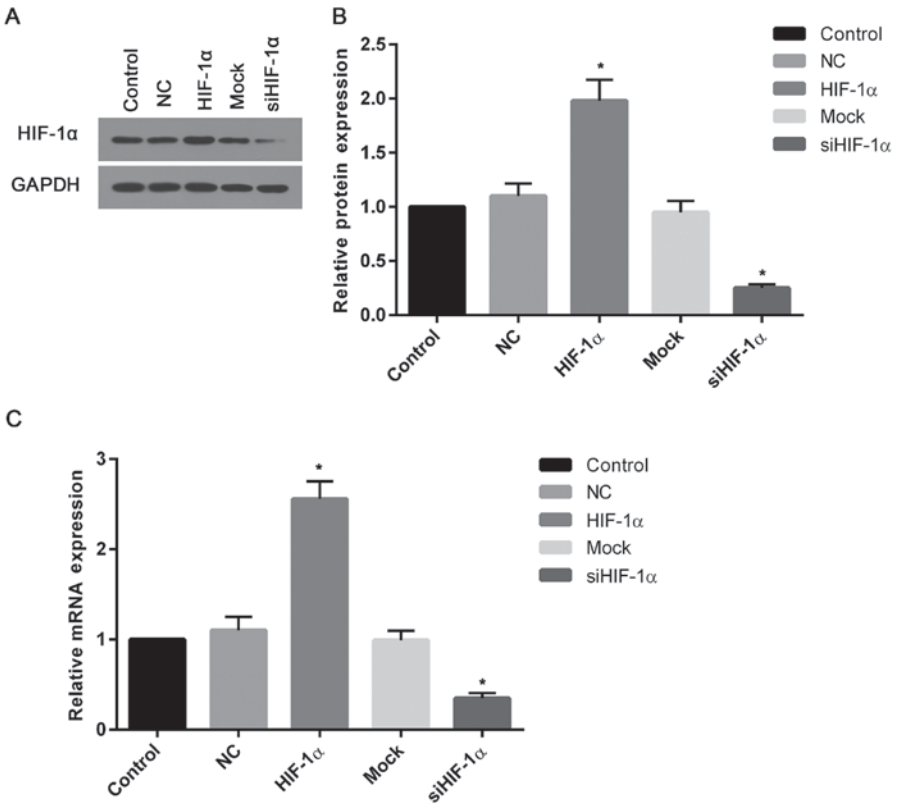

Figure 2. Western blotting and a RT-qPCR assay confirmed the transfection effectiveness. (A) Western blot analysis was performed on transfected cells and (B) revealed that HIF-1α protein levels were increased in cells treated with recombinant HIF-1α and decreased in cells transfected with HIF-1α siRNA. (C) RT-qPCR demonstrated that HIF-1α mRNA levels were significantly upregulated in cells treated with recombinant HIF-1α and downregulated in cells transfected with HIF-1α siRNA. *P<0.05 vs. control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HIF-1α, hypoxia inducible factor-1α; siRNA, small interfering RNA; NC, negative control.

with long fusiform, star or irregular polygons. The cytoplasm was homogeneous and the central nucleus was round, oval centered or biased (Fig. 1A). To further confirm the osteoblasts obtained, the present study performed immunofluorescence

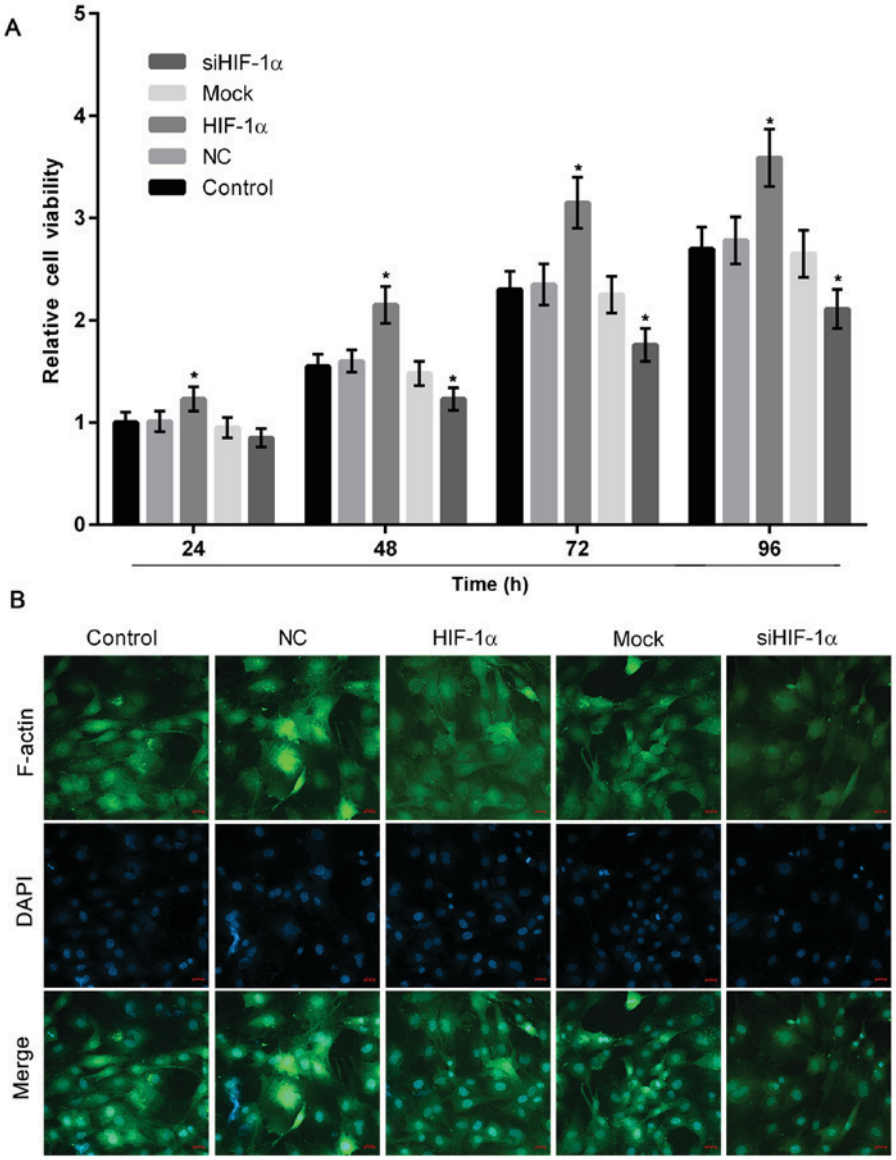

Figure 3. Altered expression of HIF-1α influenced the cell viability and proliferation. (A) A Cell Counting kit-8 assay revealed that the cell viability increased in cells that overexpressed HIF-1α in a time-dependent manner. By contrast, the cell viability was decreased in cells treated with HIF-1α siRNA when compared with control. (B) Immunofluorescence detection of F-actin demonstrated that the expression of F-actin and osteoblast proliferation were higher in the group overexpressing HIF1 when compared with those in group with HIF1 siRNA treatment (scale bars, 20 μm). *P<0.05 vs. control. HIF-1α, hypoxia inducible factor-1α; siRNA, small interfering RNA; NC, negative control.

staining of osteocalcin and osteoblasts were observed to exhibit intense cytoplasmic staining for osteocalcin (Fig. 1B-D).

Expression of HIF-1α at the protein and mRNA levels. To assess the transfection efficiency, the present study determined the transcriptional and translational levels of HIF-1α in cells. The western blotting and mRNA assays demonstrated that the levels of HIF-1α protein and mRNA in cells transfected with recombinant HIF-1α were ~2 and 2.5-fold greater than that of the control (Fig. 2A and B). However, the expression of HIF-1α protein and mRNA in cells treated with HIF-1α siRNA were markedly suppressed when compared with the control (Fig. 2C).

HIF-1α overexpression or knockdown induces or suppresses the proliferation of osteoblasts. To evaluate the effect of differentiated HIF-1α expression in osteoblasts, the present study performed a CCK-8 assay, which revealed that HIF-1α overexpression significantly stimulated osteoblast proliferation, while downregulation of HIF-1α significantly decreased the growth of osteoblasts when compared with the control (Fig. 3A). Furthermore, in the HIF-1α overexpression group, F-actin positive immunofluorescence staining was greater and osteoblast proliferation was higher, when compared with the group with HIF-1α downregulation (Fig. 3B).

ROS levels increase in cells with HIF-1α downregulation. To measure the levels of oxidative stress in cells up- or down-regulated HIF-1α, the ROS levels were determined. When compared with the control and NC groups, the relative ROS level in cells treated with recombinant HIF-1α was markedly reduced. By contrast, the relative ROS level in cells treated with HIF-1α siRNA was elevated when compared with the control and mock groups (Fig. 4).

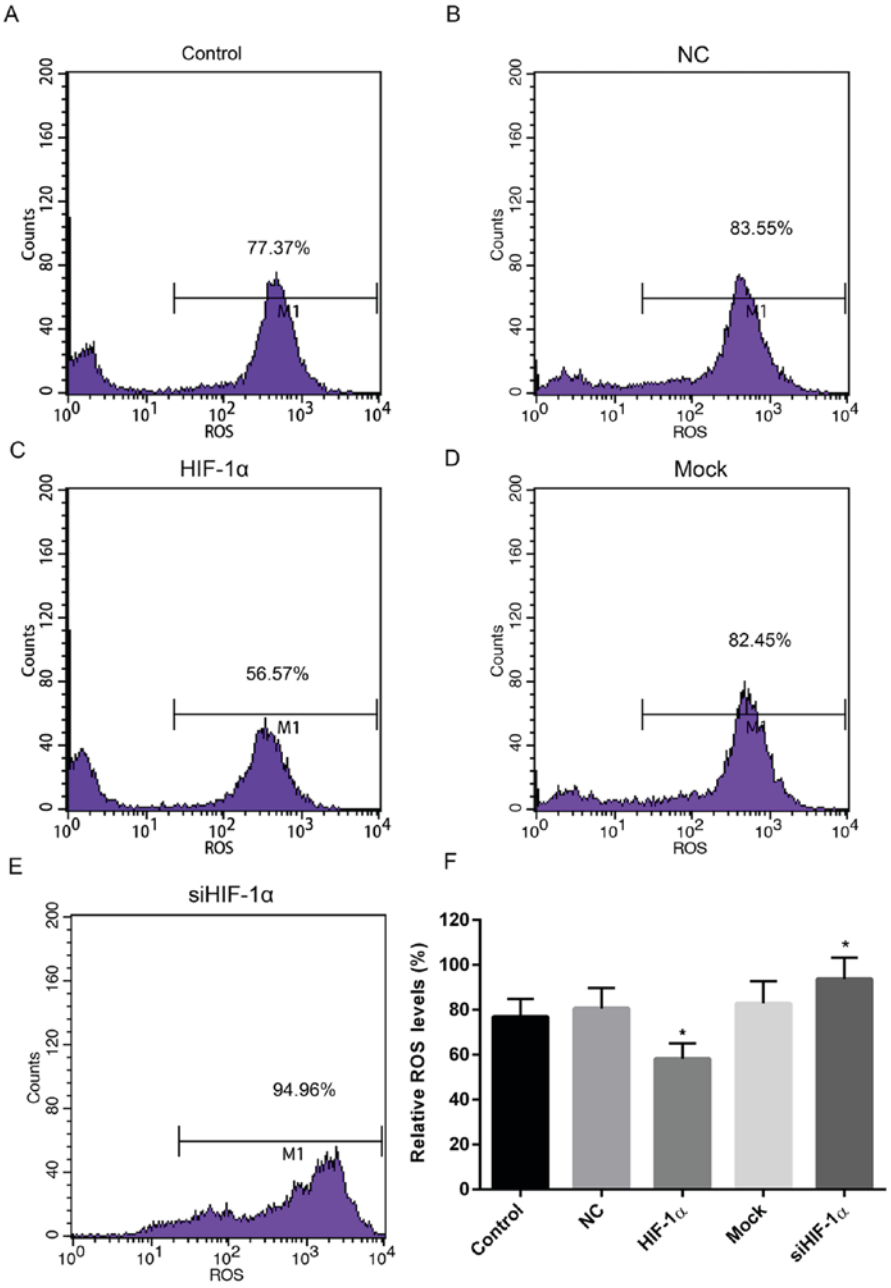

Figure 4. HIF-1α overexpression and knockdown affected the ROS levels. Flow cytometry was performed on the (A) control, (B) NC, (C) recombinant HIF-1α, (D) Mock and (E) siHIF-1α groups in order to determine (F) the relative ROS levels. The level of ROS in cells treated with recombinant HIF-1α was markedly decreased when compared with the control and NC groups. However, the relative ROS level in cells with silenced HIF-1α was increased when compared with the control and mock groups. *P<0.05 vs. control. HIF-1α, hypoxia inducible factor-1α; ROS, reactive oxygen species; NC, negative control; si-, small interfering RNA.

Overexpression or downregulation of HIF-1α suppresses or stimulates apoptosis, respectively. The effect of overexpression or downregulation of HIF-1α on apoptosis was investigated and the results revealed that the apoptosis rate of cells with HIF-1α overexpression was 2.33%, which was slightly lower than that of the control (3.87%) and NC (3.93%) groups (Fig. 5A-C and F). However, the rate of apoptosis for cells treated with HIF-1α siRNA was 14.03%, which was significantly higher than that of control and mock (4.32%) groups (Fig. 5D-F).

Up- or downregulated HIF-1α alters the expression of apoptosis-associated genes. To investigate the effect of HIF-1α on the expression of genes associated with apoptosis, the present study further detected the expression of AIF, Bax, Bcl-2 and caspase-3. The expression levels of AIF, Bax and caspase-3 mRNA and protein decreased in cells with overexpressed HIF-1α, compared with the control and NC groups (Fig. 6). By contrast, they were increased in cells with downregulated HIF-1α, when compared with the control and mock groups. However, unlike Bax, the Bcl-2 mRNA and protein levels were increased in cells treated with recombinant HIF-1α and decreased in cells treated with HIF-1α siRNA when compared with the control (Fig. 6).

Overexpression or down-regulation of HIF-1α increases or decreases the expression of Foxo1 and osteoblast markers. To

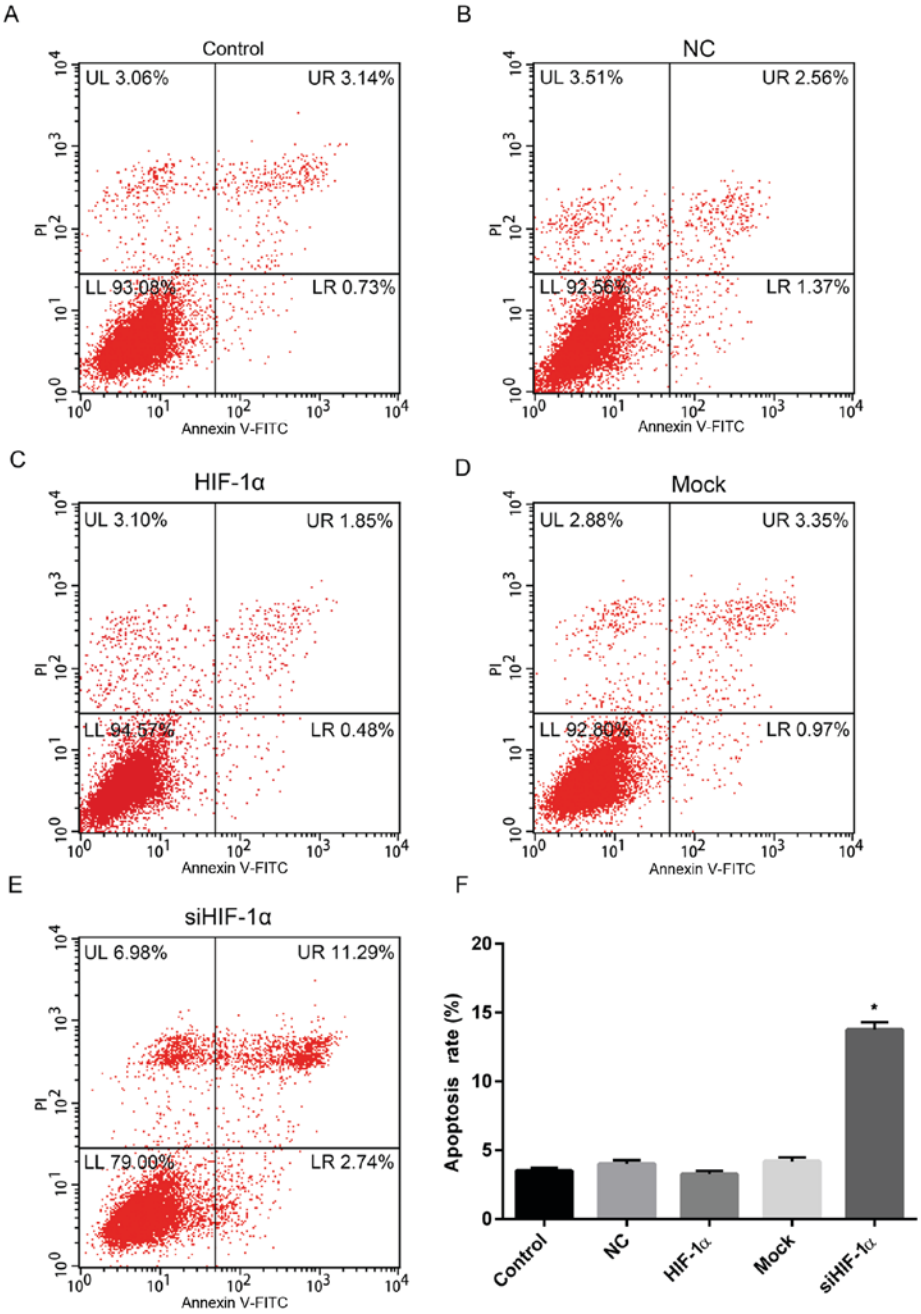

Figure 5. HIF-1α overexpression and knockdown influenced osteoblast apoptosis. The rate of apoptosis in the cells of the (A) control, (B) NC, (C) recombinant HIF-1α, (D) Mock and (E) siHIF-1α groups was evaluated. (F) HIF-1α overexpression was slightly decreased when compared with the control and NC groups. By contrast, silencing HIF-1α resulted in a significantly increased rate of osteoblast apoptosis. *P<0.05 vs. control. HIF-1α, hypoxia inducible factor-1α; NC, negative control; si-, small interfering RNA.

explore the function of HIF-1α in the regulation of the expression of Foxo1 and osteoblast markers including Runx2, ALP and osteocalcin, the present study determined their expression following the upregulation or silencing of HIF-1α. The results demonstrated that the protein levels of Foxo1, Runx2, ALP and osteocalcin were significantly elevated in cells treated with recombinant HIF-1α when compared with those of the control and NC groups (Fig. 7A and B). However, in cells treated with HIF-1α siRNA, the protein levels of Foxo1, Runx2, ALP and osteocalcin were markedly decreased compared with the control and mock groups (Fig. 7A and B). Similarly, the mRNA expression levels of Foxo1, Runx2, ALP and osteocalcin in cells overexpressing HIF-1α were ~1.5, 2, 2.2 and 2.7-fold greater of that of the control and NC groups, respectively. Furthermore, the expression levels of Foxo1, Runx2, ALP and osteocalcin mRNA in cells treated with HIF-1α siRNA were significantly suppressed when compared with the control and mock groups (Fig. 7C). To further confirm these altered osteoblast marker expressions, the expression of the osteogenic marker Runx2 was evaluated by immunofluorescence and the greatest Runx2 expression levels were observed in the HIF-1α overexpression group, while the lowest levels were seen in cells with HIF-1α silencing (Fig. 7D). In addition, to confirm the role of Foxo1 in HIF-1α-induced osteoblast proliferation, further blotting experiments were performed. The results revealed that Runx2 and ALP expression induced by HIF-1α were markedly reversed

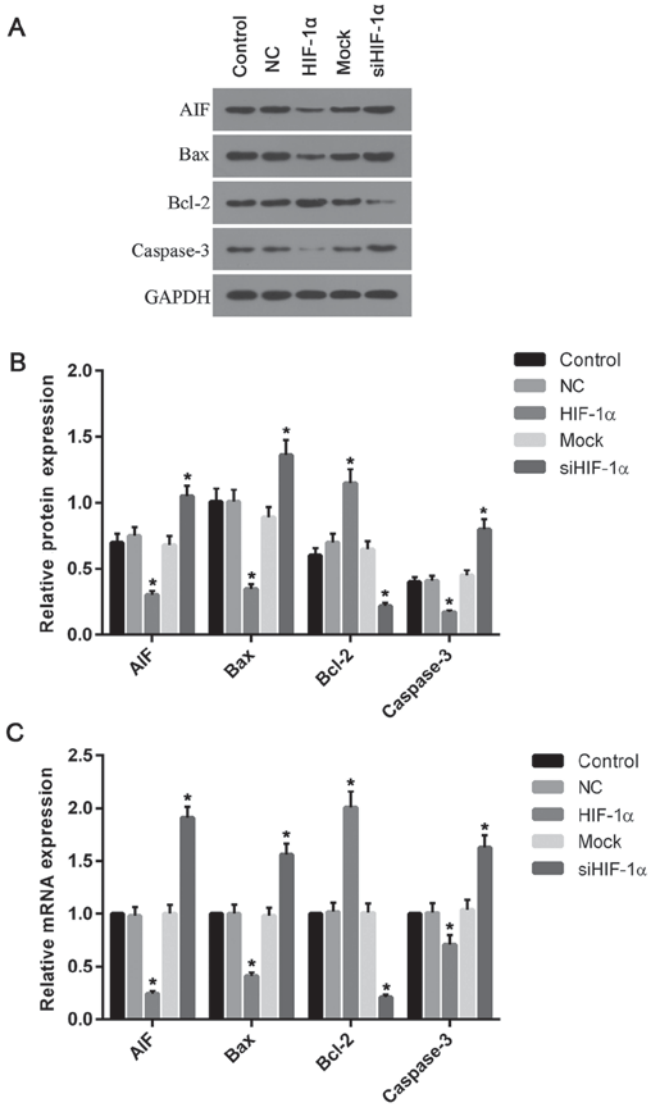

Figure 6. Expression of apoptosis associated genes was altered by the overexpression and silencing of HIF-1α. (A) Western blotting was performed to analyze the proteins levels of AIF, Bax, Bcl-2 and caspase-3. (B) The protein levels of AIF, Bax and caspase-3 were decreased in cells overexpressing HIF-1α compared with the control and NC groups, while the level of Bcl-2 was increased when compared with control. (C) Similarly, the relative mRNA levels of AIF, Bax and caspase-3 were decreased in cells with HIF-1α overexpression compared with the control and NC groups; however, the levels of Bcl-2 mRNA were elevated when compared with control. Silencing of HIF-1α had the opposite effects on protein and mRNA levels, with Bcl-2 levels significantly decreased and AIF, Bax and caspase-3 levels significantly increased. *P<0.05 vs. control. HIF-1α, hypoxia inducible factor-1α; NC, negative control; siRNA, small interfering RNA; AIF, apoptosis-inducing factor; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

by Foxo1 siRNA, while osteocalcin was not affected by Foxo1 siRNA (Fig. 7E). Thus, it was proposed that HIF-1α-induced expression of Runx2 and ALP may be completely dependent on the expression levels of Foxo1, and in turn, osteocalcin may be partially dependent on Foxo1, though to a much lesser degree.

Discussion

HIF-1α serves a pivotal role in the stimulation of bone formation via the regulation of several key factors such as Runx2 (12). The Foxo subfamily regulates the expression of genes associated with a variety of physiological and pathological processes (14); however, they also have a role in the proliferation, differentiation and apoptosis of osteoblasts, which to date has been quite well studied. Previous studies have demonstrated that Foxo1 can stimulate the growth of osteoblasts by increasing the expression of Runx2 (17). Therefore, the present study investigated whether HIF-1α affects the expression of Runx2 by regulating Foxo1. The results revealed that the interactions between HIF-1α and Foxo1 serve a key role in the proliferation, differentiation and apoptosis of osteoblasts.

HIF-1α is an important transcription factor involved in cell metabolism, with roles such as promoting glycolysis and inhibiting mitochondrial respiration (25). HIF-1α upregulates pyruvate dehydrogenase kinase, inhibits pyruvate dehydrogenase activity and blocks pyruvate entry into tricarboxylic acid (TCA) cycle, thereby inhibiting mitochondrial oxidative phosphorylation (25-27). As mitochondrial respiration is the primary source of ROS, it was hypothesized that HIF-1α may reduce ROS production. The results demonstrated that ROS levels were decreased in cells with overexpressed HIF-1α, which is consistent with the authors' hypothesis. In addition, ROS levels were markedly increased in cells treated with HIF-1α siRNA compared with normal osteoblasts. These results suggested that the underlying mechanism may involve the suppression effect of HIF-1α on ROS in osteoblasts.

In the process of bone development and regeneration, angiogenesis is closely associated with bone neoplasm (28). Previous studies had observed that HIF-1α can promote the proliferation and migration of vascular endothelial cells, and increase the permeability of vascular endothelial cells (29,30), which provides nutrition for the growth of cells and the establishment of capillaries, and also promotes the development of bone marrow-derived endothelial progenitor cells that transfer to the site of hypoxic injury (30,31). Thus, the present study measured the cell viabilities and rates of apoptosis in osteoblasts with HIF-1α overexpression or knockdown. The results revealed that the cell viabilities and proliferation were increased in cells with overexpression, and decreased in cells with downregulated HIF-1α. Furthermore, apoptosis was significantly increased in cells with silenced HIF-1α; however, the apoptosis rate in cells with overexpressed HIF-1α was marginally decreased compared with normal cells. Consistent with these results, the expression levels of the proapoptotic genes AIF, Bax and caspase-3 were increased, while the anti-apoptotic gene Bcl-2 was decreased in cells treated with HIF-1α siRNA. These results and those of previous reports indicate that the inhibition of HIF-1α function suppresses osteoblast proliferation (12).

The Foxo family mainly includes Foxo1, Foxo3 and Foxo4, and is a group comprised of multifunctional transcription factors involved in the cell cycle, apoptosis and ROS metabolism (14). In addition, previous investigations revealed that Foxo1 is closely associated with the proliferation, differentiation and apoptosis of osteoblasts (16,18,32,33). Therefore, the present study detected the expression levels of Foxo1 in order to determine the associations between HIF-1α and Foxo1. The transcriptional and translational levels of Foxo1 were increased in cells with HIF-1α overexpression. Previous studies have demonstrated that Foxo1 can upregulate the ROS reducing agent manganese peroxidase, Catalase and sestrin 3, which produce superoxide oxidation, antioxidant protein (overoxidized peroxiredoxins)

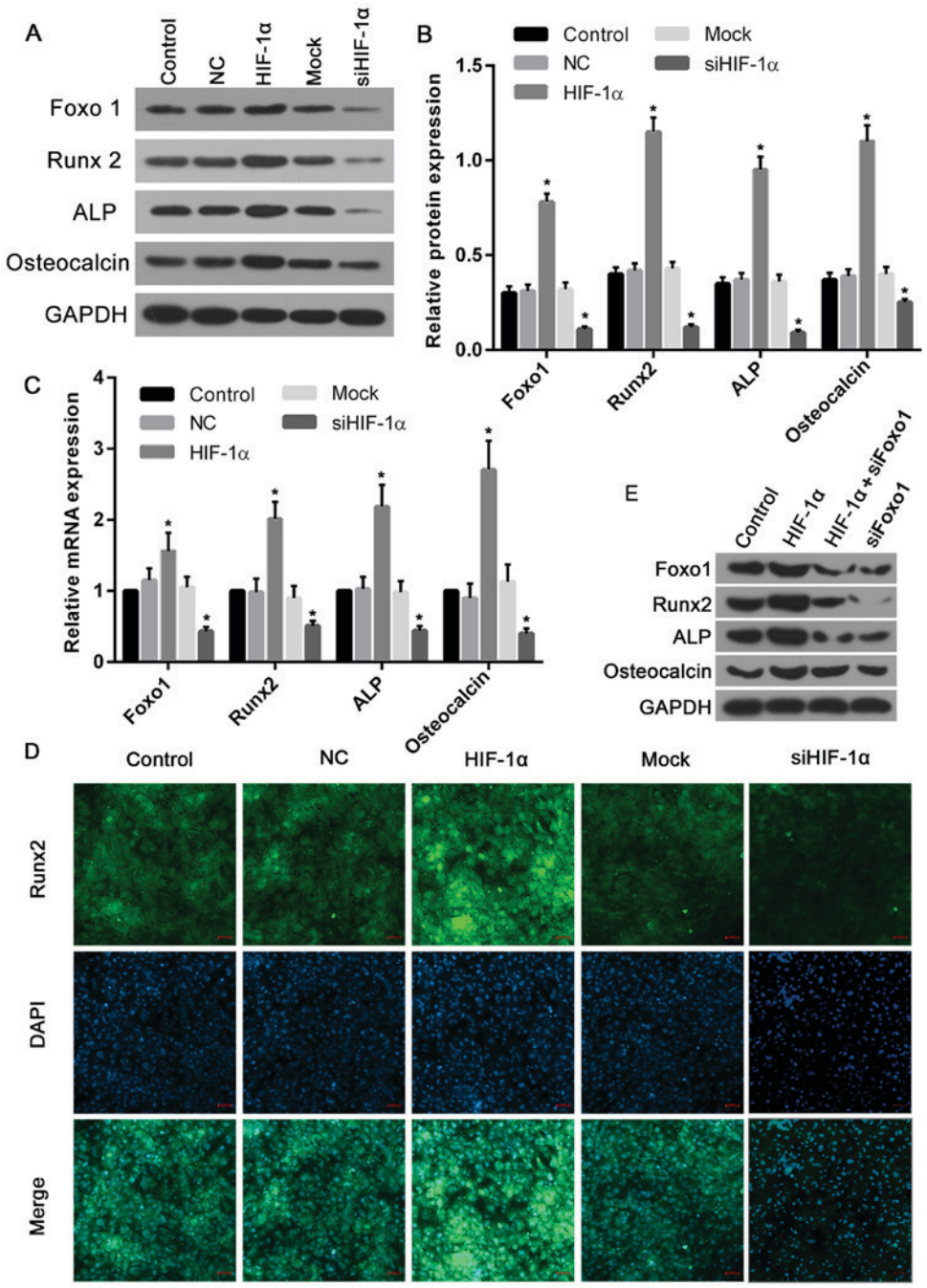

Figure 7. HIF-1α overexpression increased, and knockdown decreased, the expression levels of Foxo1 and osteoblast markers. (A) Western blotting was performed to analyze the proteins levels of Foxo1, Runx2, ALP and osteocalcin. (B) Their protein expression levels significantly increased in cells overexpressing HIF-1α when compared with the control and NC groups. However, the protein levels significantly decreased in cells treated with HIF-1α siRNA, compared with the control and mock groups. (C) Similarly, the mRNA expression levels of Foxo, Runx2, ALP and osteocalcin in cells with HIF-1α overexpression were elevated, while the mRNA levels in cells treated with HIF-1α siRNA were significantly inhibited. (D) Immunofluorescence analysis confirmed that the highest expression of Runx2 was observed in cells overexpressing HIF-1α, while the lowest expression of Runx2 was exhibited by cells with HIF-1α silencing (scale bars, 50 μm). (E) Runx2 and ALP protein expression induced by HIF1α were markedly decreased by Foxo1 siRNA, while only a slight reduction in osteocalcin was produced by Foxo1 siRNA. *P<0.05 vs. control. HIF-1α, hypoxia inducible factor-1α; NC, negative control; si-/siRNA, small interfering RNA; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; Foxo1, forkhead box class O1.

degradation of ROS (34,35). Therefore, it was assumed in the present study that the increased ROS levels in cells with HIF-1α silencing were associated with the downregulation of Foxo1 induced by knockdown HIF-1α. Notably, the complete knockdown of Foxo1 *in vivo* has previously been observed to induce the incomplete development of the embryonic vascular system, which in turn leads to the apoptosis of embryonic cells and thus, the termination of the pregnancy (36). Furthermore, Foxo1 serves a pivotal protective role in endoplasmic reticulum stress-, hypoxia- and tumor necrosis factor-induced apoptosis in a variety of cell lines (37-39). Therefore, the interactions between HIF-1α and Foxo1 may be an important factor for the regulation of osteoblast apoptosis.

To date, HIF-1 has been associated with the regulation of a variety of genes including VEGF, bone morphogenetic protein and osteocalcin, which in turn are closely associated

with angiogenesis and bone formation (30,31). Several studies have reported that the deletion of HIF-1α results in the down-regulation of osteoblast markers, including Runx2, ALP and osteocalcin (12,40,41). However, the mechanism by which HIF-1α regulates these genes remains unclear. In addition, a number of investigations have revealed that knockout of Foxo1 markedly reduced the expression of Runx2, ALP and osteocalcin, resulting in the reduction of culture calcification even with exposure to osteogenic stimulants (17,19,32). Thus, it was hypothesized in the present study that there may be a close association between HIF-1α and Foxo1 in the regulation of the expression of these genes. Therefore, the expression of these genes was further investigated. The results revealed that Runx2 and ALP expression induced by HIF1α were markedly reduced by Foxo1 siRNA; however, osteocalcin was not notably affected by Foxo1 siRNA. It is therefore a possibility that the HIF1α-induced expression of Runx2 and ALP may be completely dependent upon the expression levels of Foxo1, and osteocalcin may be partially dependent on Foxo1. The results of the present study were consistent with the authors' hypothesis and with the results of previous studies (17,18,32). Notably, the mRNA and protein levels of Runx2, ALP and osteocalcin had similar expression profiles as those of HIF-1α and Foxo1. Silencing HIF-1α resulted in the decreased expression of Runx2, ALP and osteocalcin, while overexpression of HIF-1α led to an increased expression of Runx2, ALP and osteocalcin. The accumulation of HIF-1α protein has been associated with Runx2 in ATDC5 chondrocytes and HEK293 cells (42). In addition, Runx2 can promote the nuclear translocation of HIF-1α in HEK293 cells (42). Runx2 can also stabilize the structure of HIF-1α by suppressing the ubiquitination of HIF-1α (40,42). In fact, there are only two specific transcripts in osteoblasts, one encoding Runx2 and the other encoding osteocalcin, in which osteocalcin is an inhibitor of osteoclast function and is expressed only when osteoblasts are completely differentiated (43,44). Furthermore, Runx2 is required for the expression of osteoblast-specific proteins such as osteocalcin (44). Through the regulation of osteocalcin expression, Runx2 can promote bone formation in differentiated osteoblasts (45).

In conclusion, the results of the present study indicated that the dependent activation of Foxo1 by HIF-1α may be essential for osteoblast cell survival, differentiation and proliferation. The increased viabilities of osteoblasts derived from children's iliac cancellous bone with elevated HIF-1α and Foxo1 levels provides evidence for novel approaches that stimulate the development of osteoblasts by activating HIF-1α and Foxo1 in combination.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GX performed the experiments and wrote and revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the institutional review board of The Children's Hospital (Zhejiang, China).

Consent for publication

Written informed consent was obtained from the parents of each participant.

Competing interests

The author declares that he has no competing interests.

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