HIF-1α-induced upregulation of lncRNA UCA1 promotes cell growth in osteosarcoma by inactivating the PTEN/AKT signaling pathway

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Received July 17, 2017; Accepted December 12, 2017

DOI: 10.3892/or.2018.6182

Abstract. Increasing evidence indicates that long non-coding RNAs (lncRNAs) play an important role in multiple biological processes including cell growth, differentiation, proliferation and invasion. Urothelial carcinoma associated 1 (UCA1) is a highly conserved nuclear ncRNA and a key regulator of cell proliferation and apoptosis in several types of cancers. However, its role in osteosarcoma progression is not well known. In the present study, we aimed to determine the biological role of UCA1 in osteosarcoma progression. RT-qPCR analysis showed that UCA1 expression was significantly increased in osteosarcoma cell lines and promoted cell growth in osteosarcoma. We then sought to determine the mechanism underlying the upregulation of UCA1 in osteosarcoma. Luciferase reporter assay and chromatin immunoprecipitation assay suggested that lncRNA UCA1 was induced by HIF-1α and HIF-1α interacts with the HIF-1α response element in the promoter region of UCA1. In addition, the gain- and loss-of-functional assay showed that HIF-1α promoted osteosarcoma cell growth through inducing the UCA1 expression level. More importantly, Cignal Signal Transduction Reporter Array and western blot assay showed that lncRNA UCA1 inactivated the PTEN/AKT signaling pathway. Finally, we observed that HIF-1α induced cell growth through the UCA1/PTEN/AKT signaling pathway. To conclude, our integrated approach demonstrates that UCA1 confers a tumor promoter function by promoting cell proliferation and silencing of the PTEN/AKT signaling pathway in osteosarcoma. Thus, UCA1 can serve as a promising therapeutic target for osteosarcoma patients.

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Key words: osteosarcoma, lncRNA UCA1, HIF-1α, PTEN

Introduction

Osteosarcoma is the most common primary bone malignancy in children and young adults, and accounts for approximately 60% of all malignant bone tumors diagnosed in the first two decades of life (1). Currently, pulmonary metastasis is the most common cause of osteosarcoma-related death (2). Despite advanced strategies such as surgery, adjuvant chemotherapy, and radiotherapy, the prognosis of osteosarcoma still remains poor, and the survival of osteosarcoma patients has reached a plateau (3-5). Genetic changes as well as dysfunction of oncogenes or tumor suppressors have been demonstrated to be tightly associated with the development and progression of osteosarcoma (6,7). Hence, identification of new molecules involved in tumor progression is of crucial importance to reduce the morbidity and mortality of this devastating disease.

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor normally regulated by the oxygen concentration but is often overexpressed in solid tumors such as cancers of the colon, breast, pancreas, kidney, prostate and bladder (8,9). Many tumor promoter genes are transactivated by HIF-1α, however, its interaction with other clusters of genes are not well known, such as long non-coding RNAs (lncRNAs). lncRNAs are most commonly defined as RNA transcripts of more than 200 nucleotides (nt) and located in nuclear or cytosolic fractions with no protein-coding capacity (10). Recent research suggests that lncRNAs can regulate gene expression at the transcriptional or post-transcriptional level, and may facilitate the diagnosis and prognosis of human cancers (11-13). A previous study indicated that many lncRNAs participate in osteosarcoma progression, including MALAT1, H19, TUG1, HIF3PUT and LOC285194 (14). However, only a few lncRNAs have been functionally identified and validated to be potential regulators of osteosarcoma, and more research is needed to clarify the role of other lncRNAs.

Urothelial cancer associated 1 (UCA1) gene is a lncRNA located at 19p13.12, which was initially discovered and investigated in bladder cancer, which has oncogenic roles in tumor proliferation and metastasis (15,16). Subsequently, it was found to be upregulated in other cancers, such as prostate and breast cancer, and has a potential oncogenic role (17,18). It is reported that some transcription factors such as Ets-2, TGF-β1

and C/EBPα-p30 protein bind the core promoter of UCA1 to enhance its expression (19). Previously, Li *et al* demonstrated that UCA1 promotes osteosarcoma progression and correlates with poor prognosis (20). However, the specific role and underlying mechanism of UCA1 in regards to proliferation and apoptosis in osteosarcoma remain unknown.

In the present study, we aimed to determine the expression level of UCA1 in osteosarcoma samples and cell lines. In addition, we further investigated the effect of UCA1 on osteosarcoma cell proliferation and apoptosis, and the underlying regulatory mechanism. The aim of the present study was to clarify i) the expression and role of UCA1 in osteosarcoma; ii) the mechanism underlying the UCA1 overexpression in osteosarcoma cells; and iii) the potential downstream target and pathway of UCA1 involved in proliferation and apoptosis in osteosarcoma.

Materials and methods

Cell culture. Human osteosarcoma cell lines MG-63, SAOS-2, U-2OS, HOS, SW1353 and one osteoblastic cell line (hFOB1.19) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All osteosarcoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 g/ml streptomycin (Life Technologies, Grand Island, NY, USA) at 37˚C in 5% CO_2 and 95% air. Osteoblastic hFOB cells were grown in DMEM/F12 1:1 medium with 10% FBS, 2.5 mM L-glutamine and 0.3 mg/ml G418 at 37˚C in 5% CO_2 and 95% air. The cell lines passed the DNA profiling test [short tandem repeat (STR)].

RNA oligoribonucleotides and cell transfection. RNA interference was conducted using synthetic small interfering RNA (siRNA) oligo (RiboBio Co., Guangzhou, China). Two synthetic siRNA oligos against UCA1 and a negative control sequence are as follows: si-UCA1-1: (sense) 5'-TGGTAATGT ATCATCGGCTTAGTTCAAGAGACTAAGCCGATGATA CATTACCTTTTTTC-3', (antisense) 5'-TCGAGAAAAAAG GTAATGTATCATCGGCTTAGTCTCTTGAACTAAGCC GATGATACATTACCA-3'; si-UCA1-2: (sense) 5'-GATCCG GCTAATATGCCTGATTACTTTCAAGAGAAGTAATCA GGCATATTAGCTTTTTTGGAAA-3', (antisense) 5'-AGC TTTTCCAAAAAAGCTAATATGCCTGATTACTTCTCT TGAAAGTAATCAGGCATATTAGCCG-3'; siRNA-NC: (sense) 5'-TTTCTCCGAACGTGTCACGTTTCAAGAGAA CGTGACACGTTCGGAGAATTTTTTC-3', (antisense) 5'-TCGAGAAAAAATTCTCCGAACGTGTCACGTTCTC TTGAAACGTGACACGTTCGGAGAAA-3'. UCA1 complementary DNA (p-UCA1) fragment, HIF-1α expressing vector (p-HIF-1α), PTEN expressing vector (p-PTEN) and control vector were purchased from RiboBio. Osteosarcoma cells were plated in 24-well plates at $1x10^5$/well. Forty-eight hours after plating, 100 nM of RNA oligoribonucleotides were transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA extraction, reverse transcription and RT-qPCR. Total RNA was isolated from primary osteosarcoma cell lines using TRIzol reagent (Invitrogen). Then, the cDNA was synthesized from 200 ng extracted total RNA using the PrimeScript RT reagent kit (Takara Bio Company, Shiga, Japan) and amplified by RT-qPCR with an SYBR-Green kit (Takara Bio Co., Ltd., Dalian, China) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the housekeeping gene GAPDH as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantification of gene expression levels. All the premier sequences were synthesized by RiboBio, and the premier sequences were as follows: UCA1 (forward) 5'-CTCTCCTATCTCCCTTCACTG A-3', (reverse) 5'-CTTTGGGTTGAGGTTCCTGT-3'; HIF-1α (forward) 5'-TCTAGACTCGAGTACAAGGCAGCAGAA AC-3', (reverse) 5'-TCTAGAGTTTGTGCAGTATTGTAG CC-3'; GAPDH (forward) 5'-AGTGGCAAAGTGGAGATT-3', (reverse) 5'-GTGGAGTCATACTGGAACA-3'. Each experiment was performed in triplicate.

Cell proliferation assay. Cell growth was quantified using the Cell Counting Kit-8 (CCK-8; Beyotime Corporation, Shanghai, China). Briefly, 100 μl of cells from the different transfection groups were seeded onto a 96-well plate at a concentration of 2,000 cells/well and were incubated at 37˚C. At different time points, the optical density was measured at 450 nm using a microtiter plate reader, and the rate of cell survival was expressed as the absorbance. The results represent the mean of three replicates under the same conditions.

Cell cycle assay. Cells were washed in PBS and fixed in 70% ethanol at 4˚C for 2 h. DNA staining was carried out with 10 mg propidium iodide/ml PBS and 2.5 μg DNase-free RNase/ml PBS for at least 30 min before flow cytometry in a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Cell cycle profiles were generated from flow cytometry analysis with Modifit software (BD Biosciences, San Jose, CA, USA).

Dual-luciferase reporter assay. Using MG-63 genomic DNA, the identified UCA1 promoter DNA region was amplified, and the PCR products were cloned into the pGEM-T Easy vector system (Promega, Madison, WI, USA). Then, the UCA1 promoter DNA region was incorporated into the pGL4 luciferase expression vector (Promega). Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection, and the ratio of Firefly/*Renilla* luciferase activity was determined.

Chromatin immunoprecipitation (ChIP). ChIP was performed using the EZ ChIP™ Chromatin Immunoprecipitation kit (Millipore, Bedford, MA, USA), according to the manufacturer's protocol. Briefly, cross-linked chromatin was sonicated into 200-1,000 bp fragments. The chromatin located on the promoter of lncRNA UCA1 was immunoprecipitated using anti-HIF-1α antibodies (#3434T, 1:1,000; Cell Signaling Technology, Beverly, MA, USA). An isotype-matched IgG was used as a negative control, and the total RNA that immunoprecipitated by the HIF-1α antibody served as a positive control. RT-qPCR was conducted to detect the relative enrichment of the lncRNA UCA1 promoter.

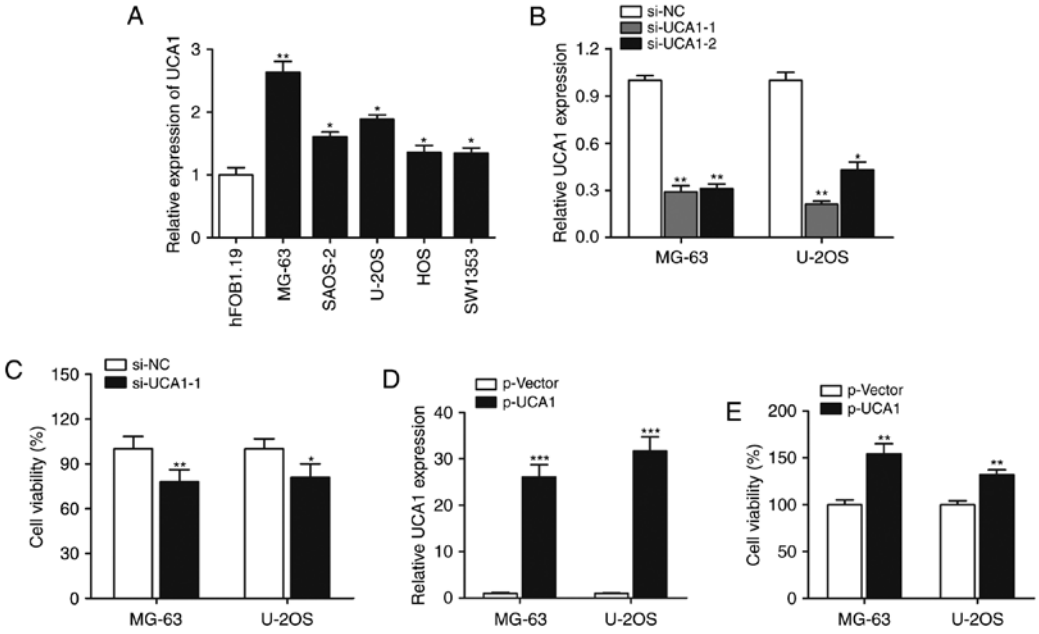

Figure 1. lncRNA UCA1 is upregulated in osteosarcoma cells and promotes cell growth. (A) RT-qPCR showed that lncRNA UCA1 expression was significantly increased in all five osteosarcoma cell lines when compared with that in the normal osteoblastic cells. (B) lncRNA UCA1 was silenced by the transfection with si-UCA1-1 or si-UCA1-2. (C) CCK-8 assay showed that knockdown of UCA1 significantly suppressed the cell proliferation rate. (D) UCA1 was overexpressed by the transfection of p-UCA1. (E) Overexpression of UCA1 promoted the cell growth of osteosarcoma cells. *P<0.05; **P<0.01; ***P<0.001.

Western blotting and antibodies. The primary antibodies were: rabbit anti-human HIF-1α antibody (#3434T, 1:1,000), PTEN antibody (#9559T, 1:1,000), rabbit anti-human phospho-AKT antibody (#9271T, 1:1,000), and rabbit anti-human β-actin antibody (#4970T, 1:1,000) (all from Cell Signaling Technology). Horseradish peroxidase-conjugated (HRP) anti-rabbit antibodies (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the secondary antibodies. The AKT inhibitor wortmannin was purchased from Sigma-Aldrich. The concentration used was 50 μM and the cells were treated for 12 h before further experiments. Cell lysates in 1X SDS loading buffer (60 mM Tris-HCl, pH 6.8; 2% SDS; 20% glycerol; 0.25% bromophenol blue; and 1.25% 2-mercaptoethanol) were incubated at 100˚C for 10 min to facilitate sample loading for conventional western blot analysis. The relative protein levels were quantified using densitometry with a Gel-Pro Analyzer (Media Cybernetics, Rockville, MD, USA).

Signal transduction reporter array. Cignal Signal Transduction Reporter Array (Qiagen, Valencia, CA, USA) was used to simultaneously investigate alterations in the activities of 50 canonical signalling pathways in response to UCA1 knockdown. Cells were transfected with antisense oligonucleotides-targeting UCA1 for 24 h and were subsequently transfected with a mixture of a transcription factor-responsive firefly luciferase reporter and a constitutively expressing *Renilla* construct. The relative activity of each pathway was determined by luciferase/*Renilla* and normalized to the untreated controls. Experiments were performed in triplicates.

Statistical analysis. Kolmogorov-Smirnov test was used to determine the normality of the distribution of data in each group. Data are presented as median (interquartile range). Differences in cell growth curves and cell cytotoxicity curves were determined by repeated measures analysis of variance. The differences in lncRNA or mRNA expression level between different groups were analyzed by the Mann-Whitney U-test or Kruskal-Wallis test. Count data were described as frequency and examined using Fisher's exact test. All differences were regarded as statistically significant at P<0.05. Statistical analyses were performed with GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA).

Results

lncRNA UCA1 is upregulated in osteosarcoma cells and promotes cell growth. We firstly performed RT-qPCR to determine the expression of lncRNA UCA1, and the results showed that expression of lncRNA UCA1 was significantly increased in all the five osteosarcoma cell lines when compared with the expression level in the normal hFOB1.19 cells (Fig. 1A). The MG-63 and U-2OS cell lines were selected for subsequent experiments. We investigated the functional role of UCA1 in cell growth. As shown in Fig. 1B, UCA1 was silenced by si-UCA1-1 or si-UCA1-2, and si-UCA1-1 was chosen for further gain- and loss-of-function assays. CCK-8 assay showed that knockdown of UCA1 significantly suppressed the cell proliferation rate (Fig. 1C). When UCA1 was overexpressed by transfection with p-UCA1 (Fig. 1D), cell growth was significantly promoted (Fig. 1E), suggesting that lncRNA UCA1 positively regulates osteosarcoma cell growth.

lncRNA UCA1 is induced by HIF-1α and HIF-1α interacts with the HIF-1α response element in the promoter region of UCA1. In order to determine the mechanism underlying the upregulation of lncRNA UCA1 in osteosarcoma cells, we focused on transcription factors that potentially bind to the UCA1 promoter. It has been reported that HIF-1α

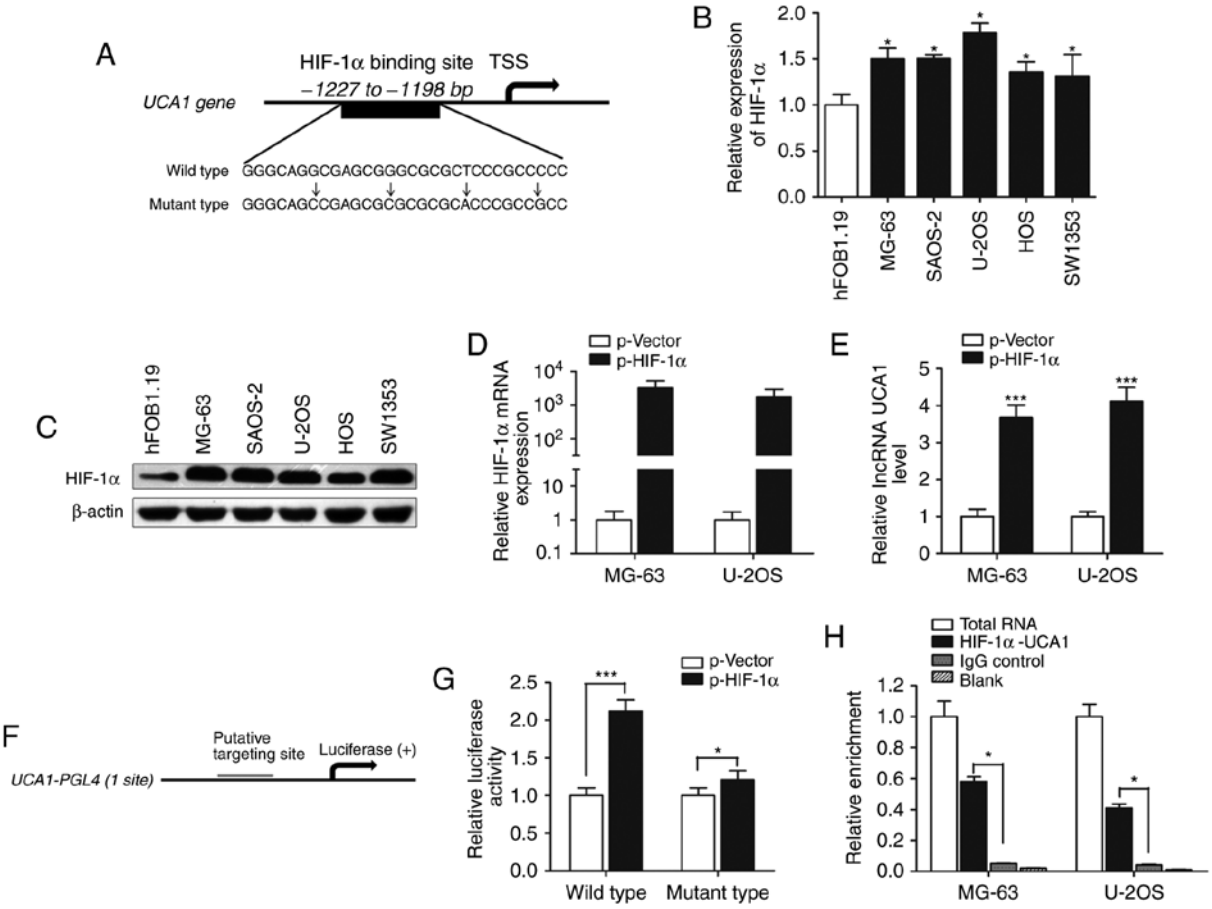

Figure 2. lncRNA UCA1 is induced by HIF-1α and HIF-1α interacts with the HIF-1α response element in the promoter region of UCA1. (A) The potential binding site of HIF-1α at the promoter region of lncRNA UCA1, and the description of wild-type HIF-1α and mutant-type HIF-1α. (B) RT-qPCR showed that HIF-1α mRNA was significantly increased in the osteosarcoma cell lines when compared with that noted in the osteoblastic cells. (C) Western blot analysis indicated that HIF-1α protein was markedly upregulated in the osteosarcoma cell lines compared with the level noted in the osteoblastic cells. (D) HIF-1α was significantly elevated by the transfection of HIF-1α overexpression vector. (E) lncRNA UCA1 was significantly upregulated by HIF-1α. (F) Potential HIF-1α binding site in the promoter region of UCA1 used for construction of the luciferase vector containing the binding region. (G) Luciferase activity was significantly increased in the HIF-1α-transfected cells compared with the control vector in MG-63 cells. (H) HIF-1α binding at the promoter regions of UCA1 was assessed by ChIP analysis. Shown are representative images of three independent experiments. *P<0.05; ***P<0.001.

is a positive regulator of osteosarcoma progression under a hypoxic condition. Thus, we aimed to ascertain whether HIF-1α regulates UCA1 expression. Based on computer algorithms PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), and GeneCards (http://www.genecards.org/cgi-bin/carddisp.pl?), we analyzed the promoter region of UCA1 and detected the presence of HIF-1α-binding sites (Fig. 2A). We then determined the expression of HIF-1α and found that both HIF-1α mRNA and protein expression levels were upregulated in osteosarcoma cells compared with the level noted in the osteoblastic cell line hFOB (Fig. 2B and C). lncRNA UCA1 expression was significantly increased after HIF-1α was overexpressed in MG-63 and U2OS cells (Fig. 2D and E).

To investigate the direct binding of HIF-1α to the UCA1 promoter, we cloned the promoter region (~1.5 kb) of UCA1 into luciferase reporter plasmid (pGL4 basic, Fig. 2F). As shown in Fig. 2G, luciferase activity was significantly increased in wild-type HIF-1α-transfected cells compared with the control vector in the MG-63 cells (P<0.001), while mutant HIF-1α had less impact on the promoter activity of UCA1 (P<0.05). In addition, ChIP experiments showed that HIF-1α immunoprecipitation was observed at the promoter of UCA1 in osteosarcoma cell lines (Fig. 2H). Taken together, these results demonstrate that HIF-1α interacts with the HIF-1α response element in the UCA1 promoter, thus inducing its transcription.

HIF-1α promotes cell growth of osteosarcoma by inducing lncRNA UCA1 expression. We then determined the effect of HIF-1α on osteosarcoma cell growth. CCK-8 assay indicated that enhanced expression of HIF-1α promoted cell growth (Fig. 3A), however, when HIF-1α was inhibited by its specific inhibitor IDF-11774, the cell growth was significantly suppressed (Fig. 3B). To further determine whether HIF-1α regulates cell growth by promoting lncRNA UCA1 expression, we transfected si-UCA1-1 into HIF-1α-overexpressing osteosarcoma cells. Our results indicated that si-UCA1-1 significantly abrogated the HIF-1α-induced promotion of cell growth (Fig. 3C). Similarly, overexpression of UCA1 by p-UCA1 partially reversed the HIF-1α inhibitor IDF-11774-induced suppression of cell growth in the MG-63 and U-2OS cells (Fig. 3D). We also detected the expression of proliferation marker Ki-67, and found that p-UCA1 significantly reversed the suppression of Ki-67 expression

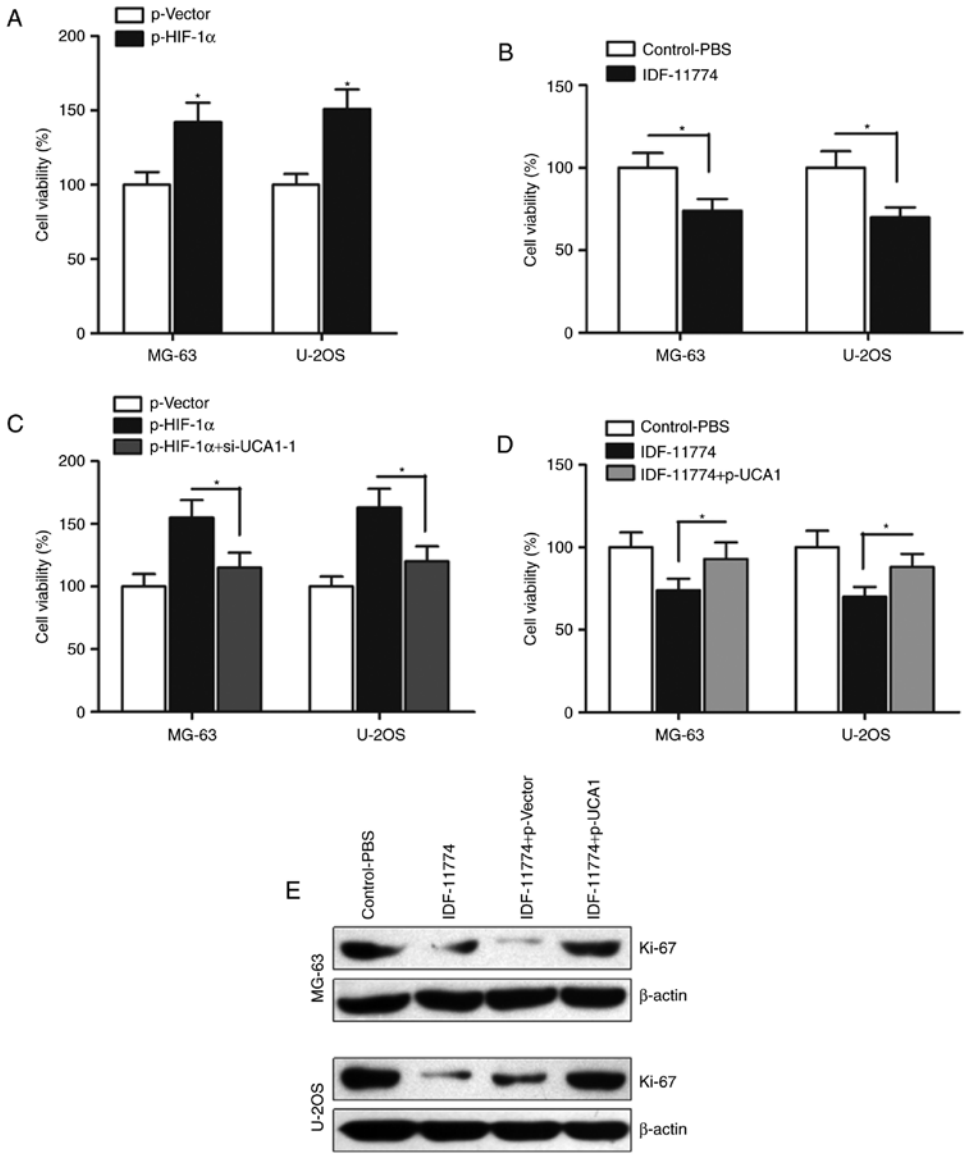

Figure 3. HIF-1α promotes the cell growth of osteosarcoma by inducing lncRNA UCA1 expression. (A) p-HIF-1α promoted the cell growth of MG-63 and U-2OS cells. (B) HIF-1α inhibitor IDF-11774 suppressed osteosarcoma cell growth. (C) Co-transfection with si-UCA1-1 significantly abrogated the HIF-1α-induced promotion of cell growth. (D) Overexpression of UCA1 by p-UCA1 partially reversed the HIF-1α inhibitor IDF-11774-induced suppression of cell growth in both MG-63 and U-2OS cells. (E) Western blot showed that p-UCA1 significantly reversed the suppression of Ki-67 expression induced by IDF-11774. *P<0.05.

induced by IDF-11774 (Fig. 3E). Collectively, HIF-1α regulates cell growth by influencing the function of lncRNA UCA1 in osteosarcoma.

lncRNA UCA1 regulates cell growth through inactivation of the PTEN/AKT signaling pathway. To investigate the molecular mechanisms underlying how lncRNA UCA1 contributes to osteosarcoma cell growth, we used Cignal Signal Transduction Reporter Array to simultaneously investigate the activities of 50 canonical signaling pathways upon UCA1 overexpression in MG-63 cells. This assay involved a mixture of a pathway-specific transcription factor-responsive firefly luciferase reporter, which contains a specific transcription factor-responsive element in the promoter (TRE), and a constitutively expressed *Renilla* luciferase reporter, which were co-transfected to monitor alterations in the activity of that signaling pathway. Notably, we identified PTEN/AKT signaling as one of the most significantly repressed pathways upon UCA1 overexpression (Fig. 4A). PTEN/AKT signaling pathway participates in the regulation of proliferation and cell cycle in tumors, and it is well accepted that there are functional interactions between HIF-1α and the PTEN/AKT signaling pathway (21). Herein, we sought to determine whether the PTEN/AKT pathway is responsible for the lncRNA UCA1-induced promotion of cell growth. The PTEN and phosph-AKT (phosphorylation site is Thr308) blot was then immunostained using the total extract and the western blot experiments showed that lncRNA UCA1 suppressed PTEN expression and promoted phosph-AKT expression. However, no change in total AKT (t-AKT) protein level was found (Fig. 4B). In addition, CCK-8 assay showed that transfection with p-PTEN or treatment with AKT inhibitor wortmannin potently abolished p-UCA1-induced promotion of cell growth in osteosarcoma cells (Fig. 4C). Cell proliferation

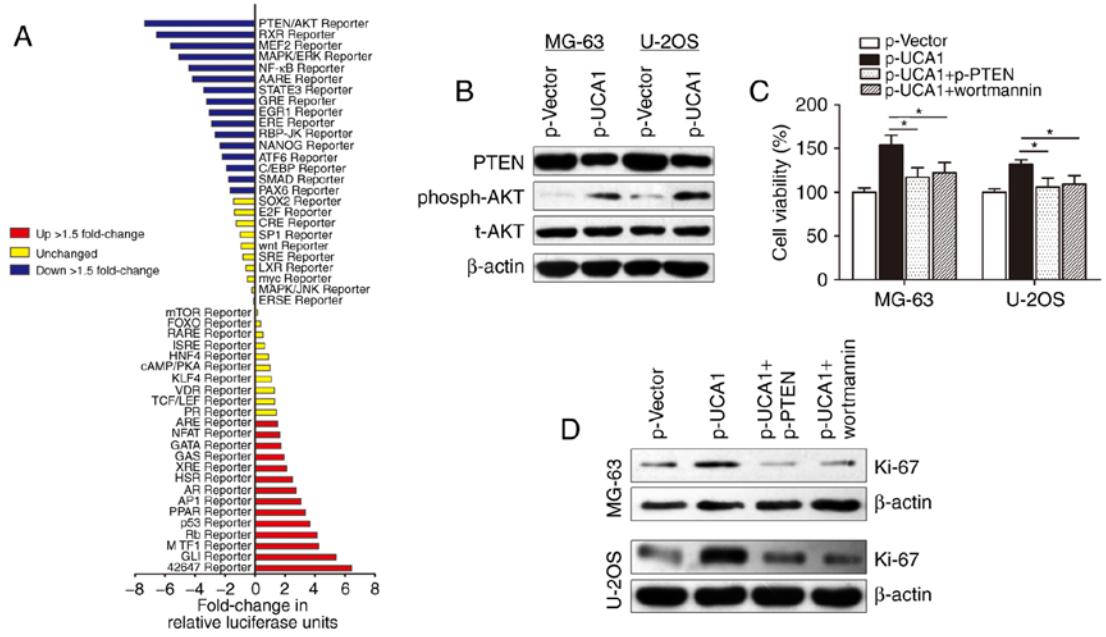

Figure 4. lncRNA UCA1 regulates cell growth through inactivation of the PTEN/AKT signaling pathway. (A) Histogram shows the fold changes for the activities of different signaling pathways, as indicated by reporter activity. (B) Western blot experiments showed that lncRNA UCA1 suppressed PTEN expression and promoted p-AKT expression; however, no change in the total AKT (t-AKT) protein level was identified. (C) CCK-8 assay showed that transfection with p-PTEN or treatment with AKT inhibitor wortmannin potently abolished p-UCA1-induced promotion of cell growth in the osteosarcoma cells. (D) The p-UCA1-induced upregulation of Ki-67 was reversed by p-PTEN or wortmannin treatment. *P<0.05,

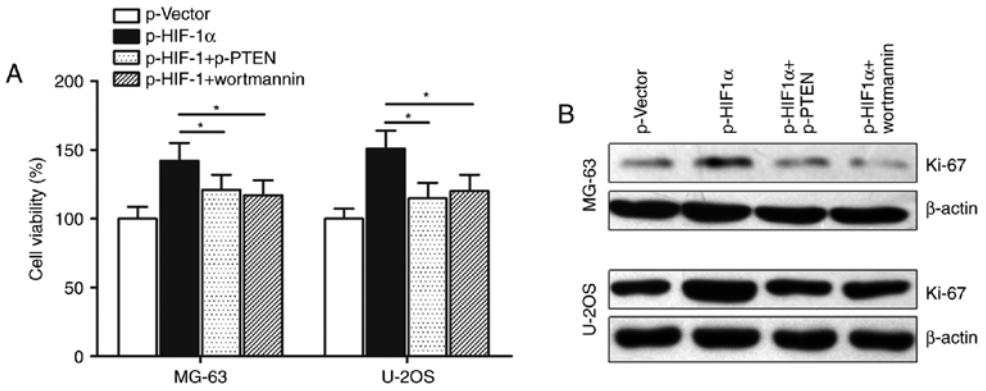

Figure 5. HIF-1α induces cell growth mediated by the UCA1/PTEN/AKT signaling pathway. (A) HIF-1α-induced promotion of cell growth was abrogated by pPTEN or AKT inhibitor wortmannin in the MG-63 and U-2OS cells. (B) Western blot assays showed that HIF-1α-induced upregulation of Ki-67 was almost abolished by p-PTEN and wortmannin in the MG-63 and U-2OS cells. *P<0.05

marker Ki-67 was also reversed by p-PTEN or wortmannin, suggesting that lncRNA UCA1 may regulate osteosarcoma cell growth via the PTEN/AKT pathway (Fig. 4D).

HIF-1α induces cell growth with cell cycle arrest via the UCA1/PTEN/AKT signaling pathway. The biologic consequences of UCA1 and PTEN/AKT pathway in HIF-1α regulation of cell growth were then examined. We validated that HIF-1α can promote cell growth by targeting UCA1, and then we determined the interaction between HIF-1α and the PTEN/AKT pathway. As shown in Fig. 5A, HIF-1α-induced promotion of cell growth was abrogated by pPTEN or AKT inhibitor wortmannin in the MG-63 and U-2OS cells. Similarly, western blot assays showed that HIF-1α-induced upregulation of Ki-67 was almost abolished by p-PTEN and wortmannin in the MG-63 and U-2OS cells (Fig. 5B).

We then determined whether the HIF-1α/UCA1 pathway regulates cell growth by inducing cell cycle arrest. Our cell cycle assay indicated that knockdown of HIF-1α significantly increased the percentage of cells in the G0/G1 phase (Fig. 6A). More importantly, si-UCA1 also caused cell cycle arrest in a the G0/G1 phase (Fig. 6B), suggesting that HIF-1α-induced UCA1 regulates cell growth through the PTEN/AKT pathway with G0/G1 cell cycle arrest. In contast, overexpression of HIF-1α or UCA1 downregulated the proportion of cells arrested at the G0/G1 phase (Fig. 6C and D).

Discussion

Recent advances in the analysis of the non-protein coding region of the human genome has allowed the discovery of extensive transcription of large RNA transcripts that lack protein-coding

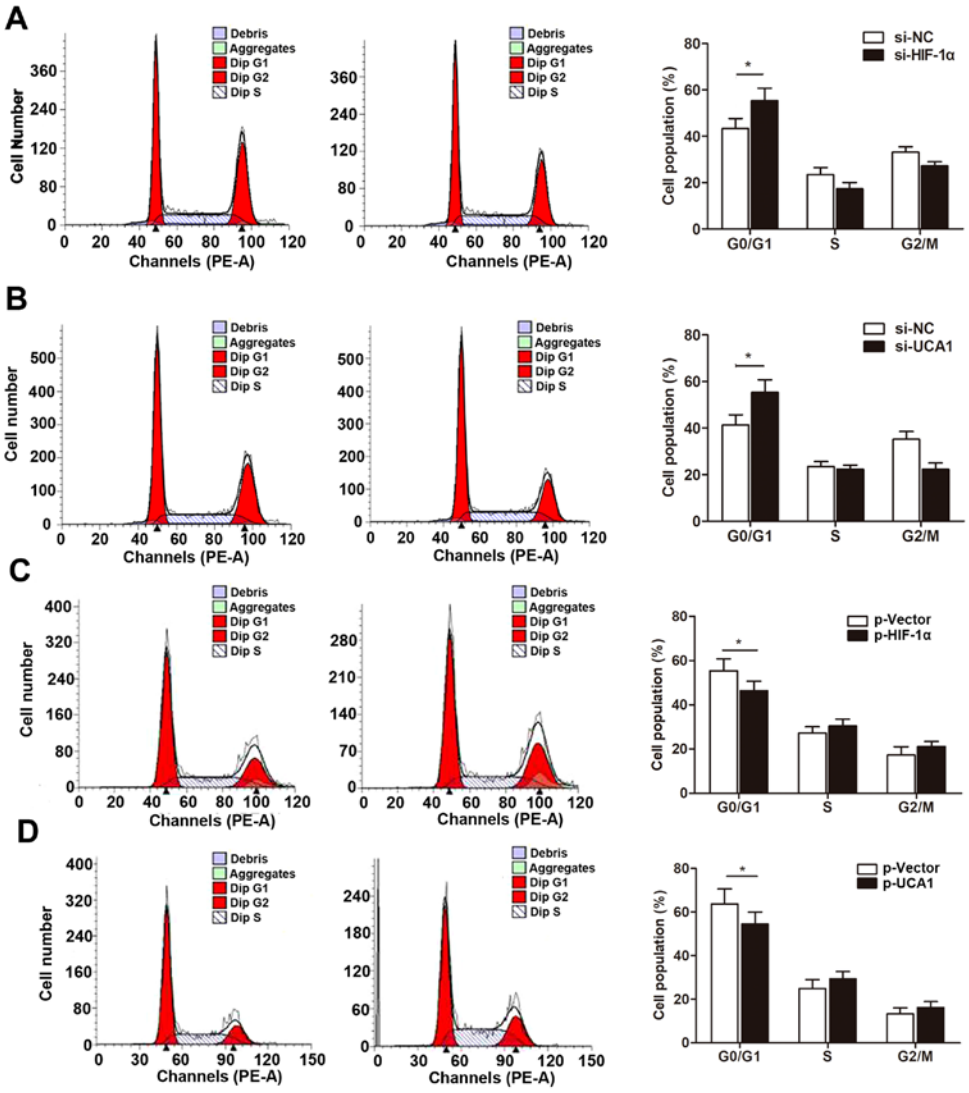

Figure 6. The HIF-1α/UCA1 pathway induces cell cycle arrest in the G0/G1 phase. (A and B) Knockdown of HIF-1α (A) or UCA1 (B) significantly increased the percentage of cells in the G0/G1 phase. (C and D) Overexpression of HIF-1α (C) or UCA1 (D) suppressed the percentage of cells in the G0/G1 phase. *P<0.05.

function, termed non-coding RNAs (22). It has become evident that lncRNAs may be an important class of genes involved in carcinogenesis (23). Currently, rapid tumor growth and pulmonary metastasis are the major reasons for the death of patients with osteosarcoma, revealing that effective prognostic factors and therapeutic targets could help improve treatment strategies to overcome metastatic osteosarcoma. Therefore, it stands to reason that defining the molecular mechanisms whereby lncRNAs have an impact on cancer progression may provide novel opportunities to treat osteosarcoma. Here in the present study, we found that lncRNA UCA1 was significantly upregulated in osteosarcoma cells compared with normal osteoblastic cells by Hiseq screening and RT-qPCR validation. Enhanced UCA1 expression was activated by the transcription factor HIF-1α which promoted the viability of osteosarcoma cells. We also identified that the HIF-1α-induced UCA1 promotion of cell growth was inactivated by the PTEN/AKT signaling pathway.

lncRNA UCA1 was first reported to be overexpressed and valuable as a prognostic marker for bladder cancer but has also been linked to several other human tumor entities (16). Numerous studies indicate that UCA1 plays critical roles in the development and progression of cancers, such as breast (24) and colorectal cancer (25), and esophageal squamous cell carcinoma (26). Currently, there are two studies that have focused on the role of UCA1 in osteosarcoma. Wen *et al* found that UCA1 was significantly increased in osteosarcoma specimens including primary tissues and serum samples when compared with controls, and it could be a specific and non-invasive candidate biomarker for the diagnosis and prognosis of osteosarcoma (27). Li *et al* demonstrated that enhanced expression of UCA1 was correlated with the poor prognosis of osteosarcoma patients and promoted proliferation and metastasis of osteosarcoma cells (20). Our data suggest that UCA1 was upregulated in osteosarcoma cells and promoted cell growth, which is consistent with the two previous reports. However, the underlying mechanism of why UCA1 is upregulated and how UCA1 regulates osteosarcoma progression remains unknown.

We firstly investigated the reason for high UCA1 expression in osetosarcoma. Bioinformatic databases including

PROMO and GeneCards were used to screen for potential proto-oncogenic transcription factors. Finally, we focused on the HIF-1α transcription factor due to a previous study involving HIF-1α regulation in osteosarcoma (28). As expected, we identified the presence of the HIF-1α-binding sites on UCA1 promoter region, and HIF-1α presented a relatively higher score than other regulators according to analysis of bioinformatic databases. Increased HIF-1α levels have been found in many tumor types, accompanied by increased expression of HIF-1 target genes, including but not limited to VEGFA, PGK1, ANGPTL4 and HK2 (29). HIF-1α overexpression has been correlated with a high risk of metastasis and high mortality in many human cancers, including osteosarcoma (30). We also found that HIF-1α was overexpressed in osteosarcoma cells and UCA1 was markedly upregulated after transfection of the HIF-1α-expressing vector. Following luciferase reporter assay and ChIP assay, both suggest that HIF-1α could interact with the promoter region of lncRNA UCA1. In addition, functional biological assays indicated that HIF-1α can enhance cell growth by targeting UCA1. It must be said that the positive regulation of HIF-1α on lncRNA UCA1 expression is under normoxic conditions. Further research is required to verify whether this interaction also applies under a hypoxic condition. Collectively, the integrated approach suggests that HIF-1α activates UCA1 translational expression in osteosarcoma under normoxic conditions.

In addition, we sought to determine the underlying regulatory mechanisms by which UCA1 exerts its function in osteosarcoma. To reveal whether lncRNA UCA1 participates in the regulation of cell proliferation via targeting the downstream pathway, we performed Cignal Signal Transduction Reporter Array. This array involved a mixture of a pathway-specific transcription factor-responsive firefly luciferase reporter, which contains a specific transcription factor-responsive element in the promoter, and a constitutively expressed *Renilla* luciferase reporter, which were co-transfected to monitor alterations in the activity of this signaling pathway. This high-throughput dual-luciferase assay led us to identify the PTEN/AKT pathway as one putatively affected by lncRNA UCA1. The western blot experiments showed that lncRNA UCA1 suppressed PTEN expression. Additionally, the phosphorylation of AKT, which was under the regulation of PTEN, was altered accordingly after the overexpression or inhibition of UCA1.

PTEN, a well-known tumor suppressor, has been found to play an important role in the development and progression of various human cancers (31,32). It is a major negative regulator of the PI3K/Akt signaling pathway (33). PI3K/PTEN balance is involved in the expression of long-term potentiation (LTP) and the regulation of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor densities. Herein, the phosphatase and tensin homologue on PTEN/Akt signaling pathway participates in the regulatory process of synaptic plasticity associated with glutamate receptors, and PTEN/AKT signaling is frequently activated in various cancers (34). lncRNAs were reported to be involved in the regulation of the PTEN/AKT pathway in various cancers. Guo *et al* demonstrated that lncRNA AFAP1-AS1 promotes the cell proliferation of gastric cancer cells via the PTEN/p-AKT pathway (35); Liao *et al* found that lncRNA CASC2 interacts with miR-181a to modulate glioma growth and resistance to TMZ through the PTEN/AKT pathway (36); Yang *et al* suggested that MEG3 regulates the growth of testicular germ cell tumors through the PTEN/PI3K/AKT pathway (37). However, the interaction of lncRNA and this signaling pathway in osteosarcoma is not well known. The present study demonstrated that lncRNA UCA1 regulates osteosarcoma cell growth by suppressing PTEN and activating the p-AKT protein level, indicating that the interaction between UCA1 and the PTEN/AKT pathway may exert important regulatory function.

After having established the interaction between HIF-α and lncRNA UCA1 and the subsequent downstream PTEN/AKT pathway, we then sought to identify whether HIF-α promoted cell proliferation by inducing UCA1 and suppressing the PTEN/AKT pathway. Gain- and loss-of-function assays showed that HIF-1α-induced promotion of cell growth was abrogated by pPTEN or AKT inhibitor wortmannin in the MG-63 and U-2OS cells. Notably, the degree of overexpression of UAC1 and/or HIF-α were not related to that of cell growth. It is reasonable that the cell growth was regulated by different pathways and different factors, and the interval of cell growth changes may be limited due to the characteristics of the cell lines. Moreover, knockdown of HIF-1α and UCA1 induced cell cycle arrest in the G0/G1 phase, which further validated the co-regulation of HIF-1α and UCA1 on the PTEN/AKT signaling pathway. One of the limitations of the present study is that no *in-vivo* experiments were performed to support our *in-vitro* findings. We will extend our study in the future to validate the data *in vivo*.

In conclusion, our integrated approach reveals that UCA1 is upregulated in osteosarcoma cells. Moreover, it promotes cell growth and caused cell cycle arrest through inactivation of the PTEN/AKT signaling pathway. Hence, UCA1 may be a potential prognostic marker and therapeutic target for osteosarcoma patients.

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