

## Original Article

# FoxC1 promotes osteosarcoma cell proliferation and metastasis through the activation of EZH2

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**Abstract:** The underlying mechanism of osteosarcoma proliferation, migration and invasion remains to be elucidated. Here, we set out to investigate the role of FoxC1 in the regulation of osteosarcoma cell proliferation and metastasis and to assess its role as potential therapeutic target. The evidence was as follows: we found that FoxC1 was markedly up-regulated in both osteosarcoma samples and osteosarcoma cell lines, compared to adjacent non-tumor tissues and osteoblast cell line hFOB1.19, respectively. In addition, we found that in the osteosarcoma cell lines Saos-2 and U2OS, siRNAs targeting FoxC1 remarkably reduced the endogenous expression of FoxC1 and, concomitantly, inhibited cancer cell proliferation and invasion. Using qChIP and a luciferase assay, we found that FoxC1 directly targets EZH2. Consistently with this notion, we found that the mRNA and protein expression of EZH2 were down-regulated upon FoxC1 knockdown in Saos-2 and U2OS cells. Finally, the silencing of EZH2 by siRNA could markedly reversed the inhibitory effect of FoxC1 knockdown on osteosarcoma cell growth and invasion.

**Keywords:** FoxC1, osteosarcoma, proliferation, metastasis, EZH2

## Introduction

Osteosarcoma, one of the most prevalent malignant bone tumor, usually arise from the metaphysis of long bones, and has a high potential of lung metastasis [1, 2]. The 5-year survival rate still remains poor, although surgical resection combined with chemotherapy and radiotherapy were used for treatment [3]. Therefore, for better prognosis and reduce mortality associated with OS, investigations for potential therapeutic targets and identification the osteosarcoma metastasis molecular mechanisms are in emergency needed [4, 5].

Enhancer of zestehomlog 2 (EZH2), a core subunit of the polycomb repressive complex 2 (PRC2), usually function as the trimethylation of histone 3 lysine 27 (H3K27) to silence the expression of target genes [6]. EZH2 was found to be broadly overexpressed in many cancer types, such as osteosarcoma [7], breast cancer [8] and colorectal cancer [9]. As reported, the expression of EZH2 in osteosarcoma patient biopsy specimens was higher than normal bone tissues, but the mechanism was still unclear [10].

FoxC1 transcription factor is a member of the forkhead box (Fox) family, which contains a characteristic DNA-binding forkhead domain. The overexpression of FoxC1 promotes several human cancers progression, such as breast cancer [11], endometrial cancer [12], hepatocellular carcinoma [13], pancreatic cancer and tongue cancer [14]. In gastric cancer patients, the overexpression of FoxC1 was correlated with poor prognosis [15]. By regulating the target genes, FoxC1 could participate in carcinogenesis, including proliferation, differentiation, invasion and metastasis.

But until now, to our knowledge, whether FoxC1 could take part in the osteosarcoma was still unclear. Here, we investigate the high expression of FoxC1 in osteosarcoma cell lines and human osteosarcoma samples, compared with the human osteoblast cell line and normal bone tissues. Additionally, we found that exogenous over expression of FoxC1 promotes osteosarcoma cell growth and invasion, and we further investigate the mechanism of FoxC1 in triggering proliferation and metastasis. EZH2 was identified as a direct target of FoxC1.

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## Materials and methods

### Cell lines

Human osteosarcoma cell lines, Saos-2, U2OS, and an osteoblast cell line, hFOB1.19, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in 6-well plates at 37°C containing 5% CO<sub>2</sub> humidified atmosphere, in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Invitrogen, San Diego, USA).

### Patients

20 osteosarcoma tissues and their pairs of adjacent non tumor tissues were collected at the department of Orthopaedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. The study was approved by the ethics committee of Tongji Medical College, the informed consent was obtained from each patient. All tissues were obtained and stored at -80°C immediately after surgery, until they were used for mRNA isolation.

### Reagents and antibodies

Lipofectamine 2000, TRIZOL reagent, and MTT kit used were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). FoxP1 small interfering RNA (siRNAs) and FoxP1 plasmid were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Transwell kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Antibodies used are as follows: Rabbit anti-human FoxC1 polyclonal antibody (cat. no. ab16645), and Rabbit anti-human EZH2 polyclonal antibody (cat. no. ab3748) were purchased from Abcam (Cambridge, MA, USA). The secondary antibody was purchased from Santa Cruz. Enhanced chemiluminescence (ECL) kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA).

### RNA isolation and qRT-PCR

Total RNA from cell lysates was extracted using TRIZOL solution, according to the manufacturer's protocol. M-MLV Reverse Transcription kit was used to synthesize cDNA using 2 µg of

RNA. Oligo (deoxythymidine)<sub>20</sub> was used as primers. Quantitative real-time PCR was then performed by ABI 7500 Real-Time PCR detection system (Thermo Fisher Scientific, Inc.), using SYBR (Roche).

The amplification protocol were 95°C for 2 min, and 35 cycles of denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 30 sec, extension at 72°C for 30 sec. GAPDH was used as an internal reference. The relative expression was analyzed using 2<sup>-ΔΔCt</sup> methods. All the experiments were performed for three times.

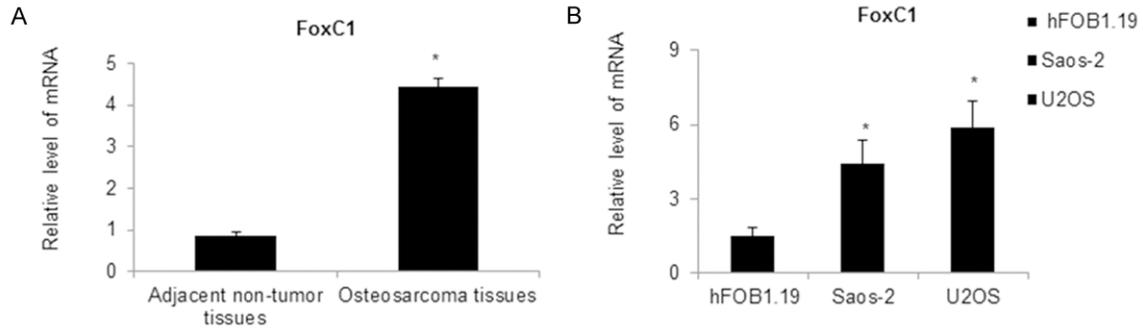
### Western blot assay

Western blot was used to detect the relative protein expression. Tissues or relative cells were lysed in SDS lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were collected and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime) and transferred onto polyvinylidene difluoride (PVDF) membranes, following by incubated with phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 hour at 4°C. The PVDF membrane was subsequently incubated with the primary antibody at 4°C overnight, followed by goat anti-rabbit or goat anti-mouse secondary antibodies (1:5,000) at room temperature for 1 hour. Finally, the enhanced chemiluminescence system was used to detect the immunoreactive bands. Image Pro Plus software (Media Cybernetics, Rockville, MD, USA) was used to measure the protein expression.

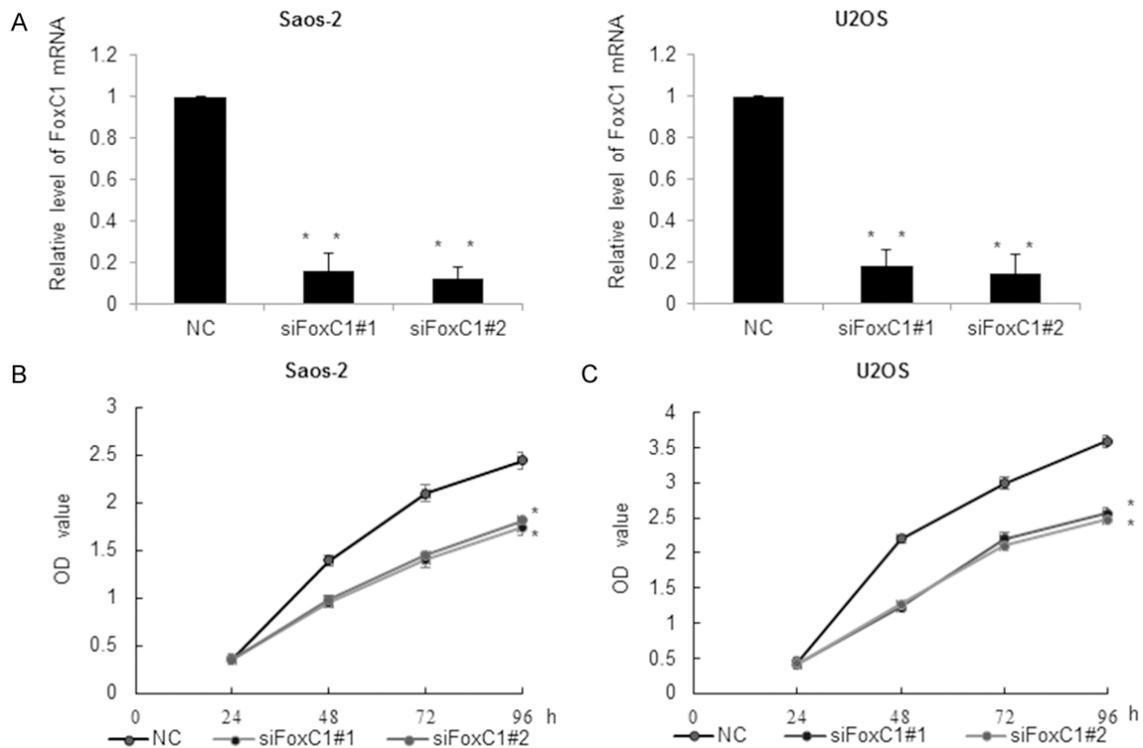
### MTT assay

MTT assay was performed to measure cell proliferation. The relative cells were placed in 96-well plates, each well containing 5000 cells, 24 h, 48 h, 72 h or 96 h after transfection, each well was replaced with 0.5 g/l MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in 100 µl fresh serum-free RPMI-1640 medium. The cells were further incubated for 4 hours at 37°C. 100 µl of acidic isopropanol was added to terminate the reaction. The contents of the plates were mixed for 5 min, and the absorbance was read on an ELISA plate reader (Biotek Instruments, Inc.) at 570 nm. Each assay was done in triplicate.

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**Figure 1.** FoxC1 is up-regulated in osteosarcoma tissues and cell lines. A: FoxC1 expression levels in 20 osteosarcoma tissues relative to nonmalignant (adjacent non-tumor) tissues assessed by qRT-PCR, data were presented as Mean  $\pm$  SD and the experiments were performed in triplicate. \* $P < 0.05$ . B: FoxC1 expression levels in osteosarcoma cell lines Saos-2, U2OS and the osteoblast cell line hFOB1.19 were assessed by qRT-PCR. Data were presented as Mean  $\pm$  SD, and the experiments were performed in triplicate. \* $P < 0.05$ .



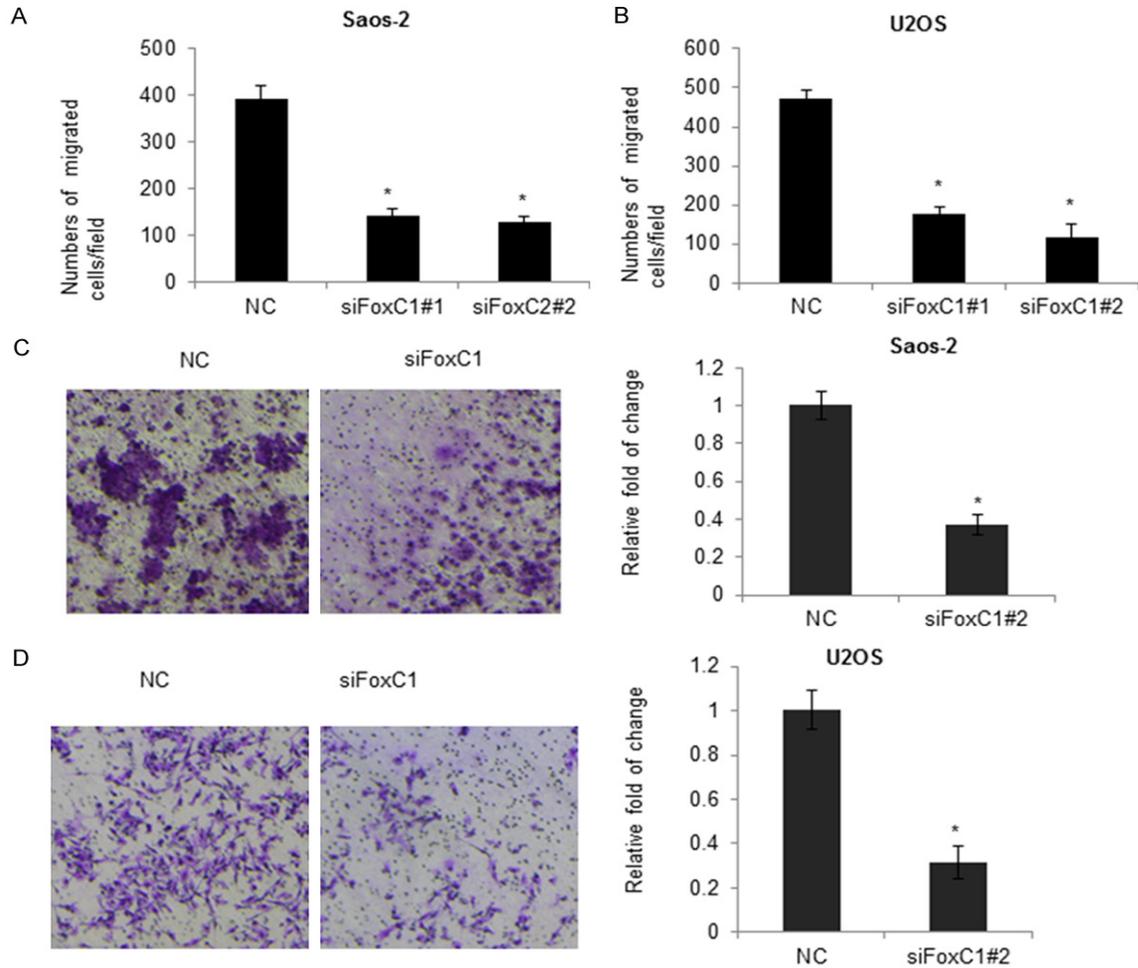
**Figure 2.** FoxC1 promotes osteosarcoma cell proliferation in vitro. A: Reverse transcription-quantitative polymerase chain reaction was used to determine the relative expression of FoxC1 in Saos-2 and U2OS cells transfected with control siRNA or siFoxC1. GAPDH served as an internal control. \*\* $P < 0.01$ . B: The MTT assay was performed in Saos-2 cell transfected with control siRNA or siFoxC1. 24 h, 48 h, 72 h or 96 h after transfection, the cell number was analyzed as the value of 570 nm absorbance. \* $P < 0.05$ . C: Similarly, MTT assay was performed in U2OS cells. \* $P < 0.05$ .

### Wound healing assay

Relative cells were seeded in 6-well plates and cultured to full confluence. A scratch “wound” of  $\sim 1$  mm width was made using a plastic scrib-

er. Cells were then washed with PBS to remove detached cells. Phase contrast micrographs were taken using the microscope (CX23; Olympus Corporation, Tokyo, Japan) immediately. Then the cells were cultured at  $37^{\circ}\text{C}$  with

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**Figure 3.** FoxC1 significantly promotes the osteosarcoma cells migratory and invasive capacities. A: Woundhealing ability was observed to analyze Saos-2 cells migration transfected with FoxC1 siRNA or NC. Numbers of migrated cells were counted at 0 hour and 24 hours after making the wound under a microscope. Of three experiments, \*P<0.05. B: U2OS migration analysis was performed using the wound healing assay. C: Saos-2 cells were transfected with FoxC1 siRNA or NC. Forty-eight hours later, the transwell assay was employed. The relative photos were shown. Data were expressed as mean  $\pm$  standard deviation. \*P<0.05 is accepted as statistically significant. D: Relative transwell assay was performed in U2OS cells.

5% CO<sub>2</sub> for 48 hours, the wound was taken photos again, Numbers of cells which have migrated were counted. For each sample, three representative fields were counted.

### Cell invasion assay

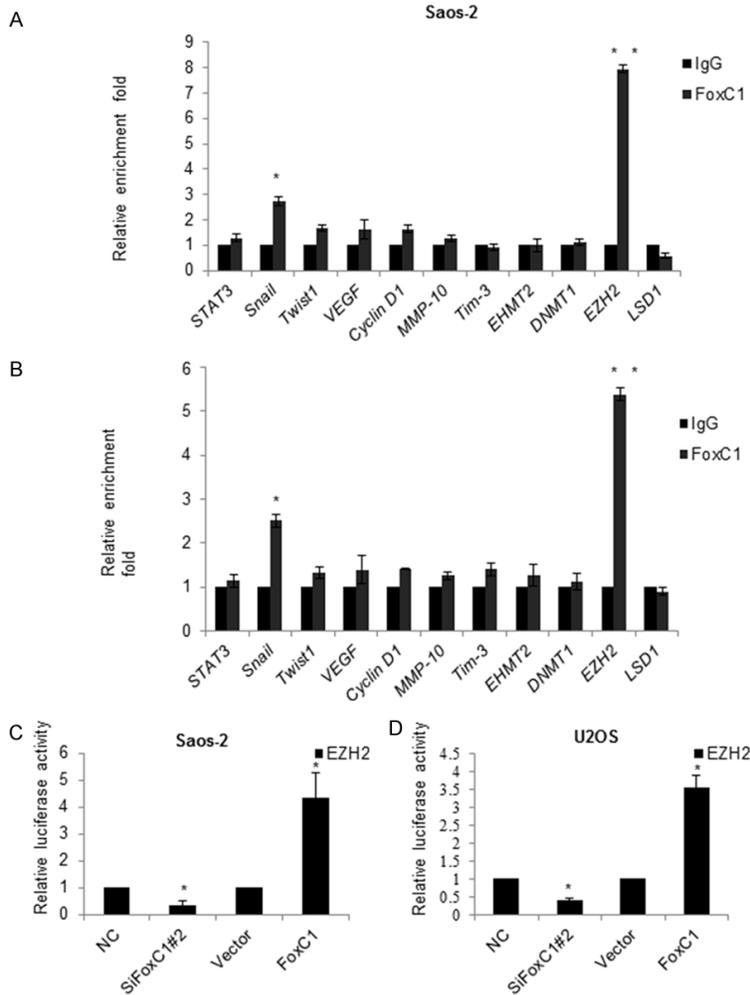
Invasion assay was performed using transwell membrane filter pre-coated with Matrigel. 50,000 cells were collected and re-suspended in serum-free RPMI-1640 media and placed in the upper chamber of each Transwell. 500  $\mu$ l RPMI-1640 with 10% FBS was placed into the lower chamber. After 24 h of incubation at 37°C in a humidified incubator, non-invading cells

were removed using a cotton-tipped swap. The remaining cells were fixed in 90% alcohol for 20 min and stained with crystal violet (Sigma, St Louis, MO, USA), and the cells on the underside of filters was observed using an inverted microscope (CX23; Olympus Corporation). Each experiment was repeated at least three times for each group.

### Luciferase reporter assay

For EZH2 reporter construction, the sequence of EZH2 promoter was obtained and ligated into the pGL3 basic vector (Promega) to generate pGL3-EZH2 promoter luciferase reporter

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**Figure 4.** EZH2 is a target gene of FoxC1 in osteosarcoma cells. A: Recruitment of FoxC1 on EZH2 promoters. qChIP experiments were performed in Saos-2 cells with indicated antibodies. Normal IgG was used as negative control, of three independent experiments. B: The relative qChIP experiments were performed in U2OS cells with FoxC1 antibody, or normal IgG. C: FoxC1 regulates EZH2 promoter driven luciferase activity. Saos-2 cells were transfected with promoter luciferase constructs together with FoxC1 over-expression or depletion constructs. Luciferase activity was measured and normalized to that of Renilla. Error bar represent as mean  $\pm$  S.D, of three independent experiments. D: The above Luciferase reporter experiments were performed in U2OS cells.

construct. The relative cells were transfected with pGL3-EZH2 luciferase reporter, the Renilla plasmid, which was used to normalize the control, and the indicated plasmid constructs. Lipofectamine LTX-Plus (Invitrogen) was used for the transfection. 24 hours after transfection, the firefly and Renilla luciferases were assayed using a Microplate reader (ELX-800, Biotek Instruments, Inc., Winooski, VT, USA). Each experiment was repeated in triplicate.

## ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's protocol (Corning Costar, Cambridge, MA, USA). 2  $\mu$ g of FoxP1 antibody or 2  $\mu$ g normal Rabbit IgG was used to incubate with the chromatin at 4°C overnight, followed by the addition of protein A Sepharose CL-4B beads for 2 h. The immune complexes were washed five times and the immunoprecipitated DNA was purified with the PCR purification kit (Qiagen). qChIP was analyzed using quantitative PCR.

## Statistical analysis

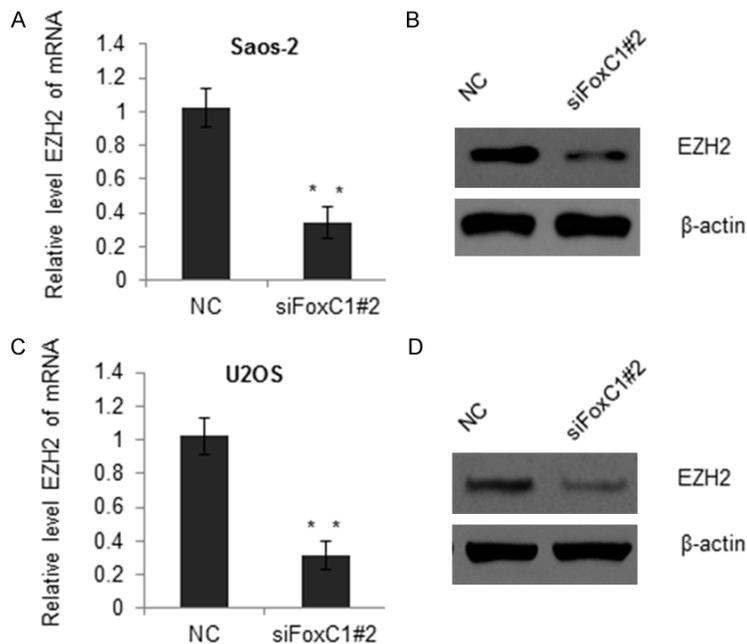
SPSS version 17.0 software (SPSS, Inc. Chicago, IL, USA) was used for statistical analysis. Data are shown as mean  $\pm$  standard deviation.  $P < 0.05$  was taken to indicate statistical significance. Differences between groups were analyzed using Student's t-test. One-way analysis of variance (ANOVA) was used if there were more than two groups.

## Results

### *FoxC1 is up-regulated in osteosarcoma tissues and cell lines*

To investigate the potential role of FoxC1 in osteosarcoma, qRT-PCR analysis was performed in the 20 osteosarcoma tissues and the adjacent non-tumor tissues. FoxC1 expression in the tumor samples was obviously higher than that in the non-tumor tissues. The difference was statistically significant ( $P < 0.05$ , **Figure 1A**). Following this, the expression levels of FoxC1 in osteosarcoma cell lines, Saos-2 and U2OS cells were investigated, compared with the osteoblast cell line hFOB1.19 (**Figure 1B**). The data revealed

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**Figure 5.** FoxC1 positively regulates the expression levels of EZH2 in osteosarcoma cells. A: RT-PCR was used to determine the relative expression of EZH2 in Saos-2 cells transfected with control siRNA or siFoxC1. Data were expressed as mean  $\pm$  standard deviation. \* $P < 0.05$  vs. the control. B: Western blot analysis was performed to examine the protein expression level of EZH2 in Saos-2 cells transfected with control siRNA or siFoxC1.  $\beta$ -actin was used as an internal reference. C: QRT-PCR analysis of EZH2 mRNA expression in the siFoxC1 or control siRNA treated U2OS cells. D: Western blot analysis of EZH2 protein expression in the siFoxC1 or control siRNA treated U2OS cells.

that FoxC1 is significantly upregulated in osteosarcoma cell lines.

### *FoxC1 promotes osteosarcoma cell proliferation in vitro*

Saos-2 and U2OS cells were separately transfected with two different FoxC1 siRNAs at a concentration of 50 nM. qRT-PCR was carried out to detect the expression level of FoxC1 72 hours after transfection. As shown, the expression level of FoxC1 was significantly downregulated in both two cell lines after treatment with siFoxC1, compared with the control siRNAs, respectively ( $P < 0.01$ , **Figure 2A**). The MTT assay was used to evaluate cell viability. The results indicated that siFoxC1 significantly suppressed the proliferation in the Saos-2 cells (**Figure 2B**) and U2OS cells (**Figure 2C**) transfected with siFoxC1 compared with the control siRNA.

### *FoxC1 significantly promotes the osteosarcoma cells migratory and invasive capacities*

Wound healing assay was first performed to detect the osteosarcoma cells migratory.

The migratory potential of the Saos-2 cells treated with siFoxC1 was significantly decreased when compared with that of the control siRNAs (**Figure 3A**). The similarly tendency was also observed in the U2OS cells (**Figure 3B**). Furthermore, transwell assay was used to measure the invasive capacities of Saos-2 cells (**Figure 3C**) and U2OS cells (**Figure 3D**). The results showed that relative cells treated with the siFoxC1 displayed significantly lower invasive capacity, compared with the control groups.

### *EZH2 is a target gene of FoxC1 in osteosarcoma cells*

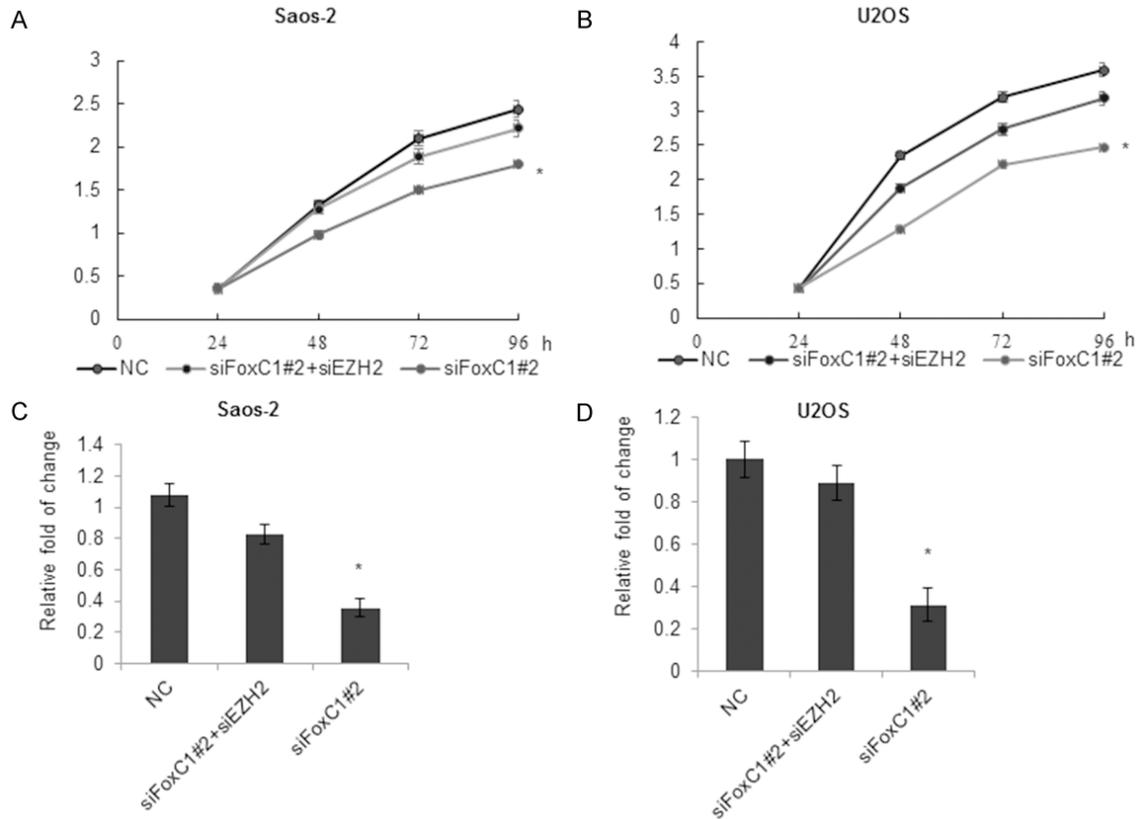
The promotion of cell growth and migration by FoxC1 is derived from its target gene. In order to explore the mechanisms of FoxC1 regulation in osteosarcoma, qChIP assay

was performed in Saos-2 cells (**Figure 4A**) and U2OS cells (**Figure 4B**) to search the potential targets of FoxC1. Several key genes in different signaling pathway were chosen, compared with the normal IgG, FoxC1 has obviously enrichment on the promoter of EZH2, so it was considered as potential target genes for FoxC1. To further support the argument, reporter activity assay was performed in Saos-2 cells (**Figure 4C**) or U2OS cells (**Figure 4D**) with EZH2 promoter-driven luciferase reporter under FoxC1 overexpression or depletion. As demonstrated, the overexpression or silencing of FoxC1 also led to a significant effect on activation or repression of the EZH2 reporter activity.

### *FoxC1 positively regulates the expression levels of EZH2 in osteosarcoma cells*

In addition, to verify that FoxC1 could activate the endogenous expression of EZH2 from the

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**Figure 6.** FoxC1 promotes the proliferation and invasion of osteosarcoma cells via targeting EZH2. A: The MTT assay was performed in Saos-2 cells transfected with control siRNA or siFoxC1 or co-transfected with siEZH2. 24 h, 48 h, 72 h or 96 h after transfection, the cell numbers were analyzed. B: The relative MTT assay was performed in U2OS cells. C: Transwell assay to determine the invasive capacity of Saos-2 cells transfected with siFoxC1 or control siRNA, or co-transfected with siEZH2. Data were expressed as mean  $\pm$  standard deviation.  $P > 0.05$  is not considered as a statistically significant difference. D: The relative transwell assay was performed in U2OS cells.

transcriptional level, qRT-PCR and western blot were performed to detect the effect of FoxC1 on EZH2 mRNA and protein expression in the relative cell lines, as shown in **Figure 5A** and **5B**, in Saos-2 cells, the knockdown of FoxC1 could remarkably reduce the EZH2 expression from both mRNA and protein level. Similarly, in the U2OS cells, RT-qPCR indicated that transfection with siFoxC1 led to a significant decrease in EZH2 mRNA level (**Figure 5C**). The reduced protein levels of EZH2 were then determined by western blot analysis (**Figure 5D**).

### *FoxC1 promotes the proliferation and invasion of osteosarcoma cells via targeting EZH2*

To reveal whether EZH2 is involved in FoxC1-mediated OS cell proliferation and invasion, the effects of FoxC1 and EZH2 were investigated. Saos-2 cells (**Figure 6A**) or U2OS cells (**Figure 6B**) were transfected with siFoxC1 or control

siRNA, or co-transfected with siEZH2. The suppressive effect of FoxC1 knockdown on Saos-2 cells or U2OS cells proliferation was markedly reversed by the EZH2 knockdown.

Similar to the cell proliferation data, it was identified that the suppressive effect of FoxC1 knockdown on Saos-2 cells (**Figure 6C**) and U2OS cells (**Figure 6D**) invasion was reversed by the downregulation of EZH2. The aforementioned data suggested that FoxC1 increased osteosarcoma cells invasion, to a certain extent, via targeting EZH2.

### **Discussion**

It has been well studied by many groups that the overexpression of oncogenes or inactivation of tumor suppressors is strongly associated with osteosarcoma. Immuno-histochemical assay revealed that EZH2 was overexpressed

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in osteosarcoma cells, but the reason was still unclear. Some miRNAs, such as miR-26a and miR-101 have been reported to inhibit osteosarcoma cell migration and invasion by down-regulation of EZH2 expression [7, 16], but the activation of EZH2 in osteosarcoma was not understood.

FoxC1 participated in the development of different kinds of cancer. As reported, by inducing matrix metalloprotease 7 (MMP7) expression, FoxC1 could promote breast cancer invasion [17]. FoxC1 could also regulate the epithelial-mesenchymal transition (EMT) and contributes to cell migration in hepatocellular carcinoma [18]. However, little is known concerning the role of FoxC1 in osteosarcoma. We provide several levels of evidence to identify FoxC1 functions in regulation of osteosarcoma phenotypes. The aforementioned findings suggest that FoxC1 is able to inhibit osteosarcoma cell proliferation and invasion, to a certain extent, via targeting EZH2.

In conclusion, this study is the first in vitro study to provide new insights into the role of FoxC1 in osteosarcoma. FoxC1 promotes in cellular proliferation and invasion of osteosarcoma by activating the EZH2 expression level. Our study demonstrated a novel regulatory mechanism by which FoxC1 acts as an oncogene in osteosarcoma.

This study helps us to further understand the FoxC1 regulation mechanism in osteosarcoma, further, in vivo studies are being conducted. We are trying to provide a new gene therapeutic target for osteosarcoma patient's treatment.

### Disclosure of conflict of interest

None.

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