ORIGINAL ARTICLES

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# Long non-coding RNA Sox4 promotes proliferation and migration by activating Wnt/β-catenin signaling pathway in osteosarcoma

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Osteosarcoma is a bone tumor without effective treatment in the world. Recently, long non-coding RNAs (lncRNAs) are considered as essential regulators in cancer progression. LncSox4 plays crucial roles in liver tumor-initiating cells self-renewal and tumor initiation. However, the effect of lncSox4 in osteosarcoma remains largely unclear. Quantitative real-time PCR (qRT-PCR) and Northern blot were performed to detect lncSox4 expressions in osteosarcoma. The functions of lncSox4 in osteosarcoma were determined using cell viability and migration assays. In addition, the proteins associated with lncSox4 were further evaluated by western blot. We found that lncSox4 was expressed highly in U-20S and Mg63 cells and osteosarcoma tumor tissues (all $P < 0.001$). LncSox4 silencing attenuated but lncSox4 overexpression promoted cell viability (all $P < 0.001$) and migration ($P < 0.01$) in the Mg63 cells. Next, we found lncSox4 regulation of osteosarcoma is involved in β-catenin, and overexpression of lncSox4 could stable β-catenin expression. Further, Wnt agonist CID11210285 completely abolished the decrease of Mg63 cells viability induced by lncSox4 silencing when cells cultured for 3 and 4 days (both $P < 0.01$), while Wnt inhibitor IWP-3 abolished the increase of Mg63 cells viability induced by overexpression of lncSox4 after treatment for 2 ($P < 0.01$), 3 ($P < 0.001$) and 4 ($P < 0.01$) days. Our study offers evidence for the first time that lncSox4 plays a positive role in osteosarcoma development and progression, and could act as a potential prognostic and therapy biomarker.

## 1. Introduction

Osteosarcoma is a malignant bone tumor which is detrimental in adolescents and children (Yang et al. 2016). It is located on the bone surface or within the bone, and eventually exhibits a high propensity for lung metastases (Angulo et al. 2017; Messerschmitt et al. 2009). Despite clinical therapy has been greatly progressed recent decades, the development of advanced treatments is still urgently needed.

Long noncoding RNAs (lncRNAs) are a family of noncoding RNAs with approximately more than 200 nts (Isin and Dalay 2015). It participates in the regulation universal bio-functions including DNA synthesis, gene expression, transcription and post-transcription modification, RNA interference, as well as defiance virus DNA (Khandelwal et al. 2017; Kolarz and Majdan 2017). lncRNAs are dysregulated in a variety of malignancies including osteosarcoma (Chandra Gupta and Nandan Tripathi 2017; Chen et al. 2017). In terms of osteosarcoma, several lncRNAs have been reported as oncogenes by controlling osteosarcoma cells growth, proliferation and migration (Chen et al. 2017; Li et al. 2016a, 2016b). For instance, lncRNA TUG1, ZEB1-AS1 and PVT1 enhanced the growth and migration of osteosarcoma cells through interplaying with miRNAs (Liu et al. 2017a; Wang et al. 2017b; Zhou et al. 2016). LncRNA BCAR4 or EWSAT1 strengthened the development of osteosarcoma by activating GLI family zinc finger 2 (GLI2) or suppressing maternally expressed 3 (MEG3) expression, respectively (Chen et al. 2016a; Sun et al. 2016). LncRNAs play pivotal roles in the development of osteosarcoma and may be regarded as a candidate target for the treatment of this cancer.

The Wnt/β-catenin signaling pathway is an evolutionary conserved pathway that plays a crucial role in embryonic development and determination of cell fate (Vallee and Lecarpentier 2016). The activated Wnt can induce β-catenin to translocate from cytosol into the nucleus, then target transcription factors T-cell factor/lymphoid enhancer-binding factor (LEF/TCF) and ultimately result in the transcription of its downstream Wnt-responsive genes including cyclin D1 (CCND1), c-MYC, PPAR(Angers and Moon 2009). Its dysregulation is involved in various pathological processes including tumor genesis (Zhao et al. 2016).

LncSox4 has been reported as a vital regulator in liver tumor-initiating cells self-renewal through activating expression of Sox4 (Chen et al. 2016b). However, its roles in malignant bone tumor remains largely underexplored. In this study, we found that lncSox4 is highly expressed in osteosarcoma cell lines and tumor tissues. Ectopic expression of lncSox4 participates in modulation of proliferation and migration of Mg63 cells through targeting the Wnt/β-catenin pathway.

## 2. Investigations and results

### 2.1. *LncSox4 is highly expressed in osteosarcoma*

LncSox4 is highly expressed and participates in the modulation of self-renewal and tumor initiation in hepatocellular carcinoma (Chen et al. 2016b). To see whether it is also dysregulated in osteosarcoma, we measured the expression level of lncSox4 in osteosarcoma cell lines and tumor tissues. qRT-PCR detection showed that the mRNA level of lncSox4 was significantly higher in U-20S and Mg63 cell lines compared with 293T cells (both $P < 0.001$, Fig. 1A). Similar results were achieved by Northern blot assay. LncSox4 were abundantly expressed in U-20S and Mg63 cells while less in 293T cells (Fig. 1B). Remarkably, we also found that lncSox4 mRNA level was strongly upregulated in human osteosarcoma tissues compared with normal human tissues (Fig. 1C, both $P < 0.001$). Together, the results showed that lncSox4 is highly expressed in osteosarcoma.

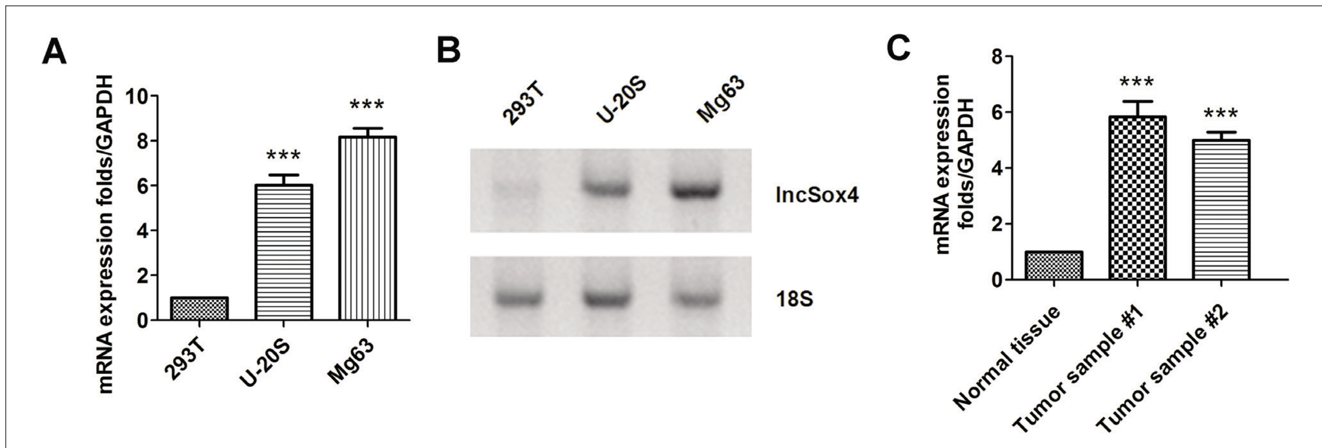


Fig. 1: Expression of lncSox4 in osteosarcoma cell lines and tumor tissues. A, The mRNA level of lncSox4 in HepG2, U-20S and Mg63 osteosarcoma cells and 293T were detected by qRT-PCR. $^{***}P < 0.001$ compared with 293T. B, The expression of lncSox4 in U-20S, Mg63 osteosarcoma cells and 293T were measured by northern blot. C, The mRNA expression of lncSox4 in two osteosarcoma tissues and normal tissue were detected by qRT-PCR. $^{***}P < 0.001$ compared with normal tissue.

Fig. 2: The influence of lncSox4 on the proliferation and migration in Mg63 cells. A, The inhibited expression of lncSox4 treated with silncSox4 1# and silncSox4 2# and up-regulated expression of lncSox4 treated with oelncSox4 determined by qRT-PCR. B, The effects of lncSox4 silencing or overexpression on the number of Ki-67$^+$-positive Mg63 was detected by FACS. C, The roles lncSox4 of silencing or overexpression on the viability of Mg63 was measured by MTT assay. D, The functions of silencing or overexpression of lncSox4 on the migration ability of Mg63 were determined by transwell migration assay. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

### 2.2. *LncSox4 promotes proliferation and migration in Mg63 cells*

LncSox4 level was downregulated or overexpressed in Mg63 cells by transfection with silncSox4 and oelncSox4, respectively. LncSox4 expression was evaluated using qRT-PCR analysis, and the result showed that mRNA level of lncSox4 were significantly downregulated by silncSox4 1# and silncSox4 2# (both $P < 0.001$), while significantly unregulated by oelncSox4 compared with negative control transfection (Fig. 2A, $P < 0.01$). Next, we identified the growth and proliferation capacity of Mg63 cells which were detected by fluorescence activated cell sorter (FACS) and MTT assay after silencing or overexpression of lncSox4 level. As shown in Fig. 2B and C, after Mg63 cells were cultured with silncSox4 1# or silncSox4 2#, the proliferation (both $P < 0.001$) and viability (both $P < 0.001$) of Mg63 cells were inhibited compared with negative control, while was enhanced by overexpression of lncSox4 (proliferation, $P < 0.01$; cell viability, $P < 0.001$). Further, the effect of lncSox4 on migration of Mg63 cells was analyzed by transwell assay. Results showed that lncSox4 silencing by silncSox4 1# or silncSox4 2# significantly attenuated the migration potential of Mg63 cells ($P < 0.01$), while overexpression of lncSox4 resulted in a reverse impact ($P < 0.01$, Fig. 2D). These data suggest that lncSox4 plays a crucial role in the modulation of Mg63 cells proliferation and migration.

### 2.3. *LncSox4 enhances the proliferative and migrative potential of Mg63 cells via activating Wnt/β-catenin pathway*

Sox4 is highly expressed in liver cancer and acts as a key modulator in response to lncSox4 regulation (Chen et al. 2016b). We checked whether Sox4 is the downregulator of lncSox4 in Mg63 cells, to reveal the possibly mechanism(s) by which lncSox4 affected Mg63 cell proliferation and migration. The result showed that there were no influences on the Sox4 mRNA level either under lncSox4 silencing or overexpression conditions ($P > 0.05$, Fig. 3A). Next, we screened the roles of lncSox4 in the classic tumor-related pathway using qRT-PCR and western blot

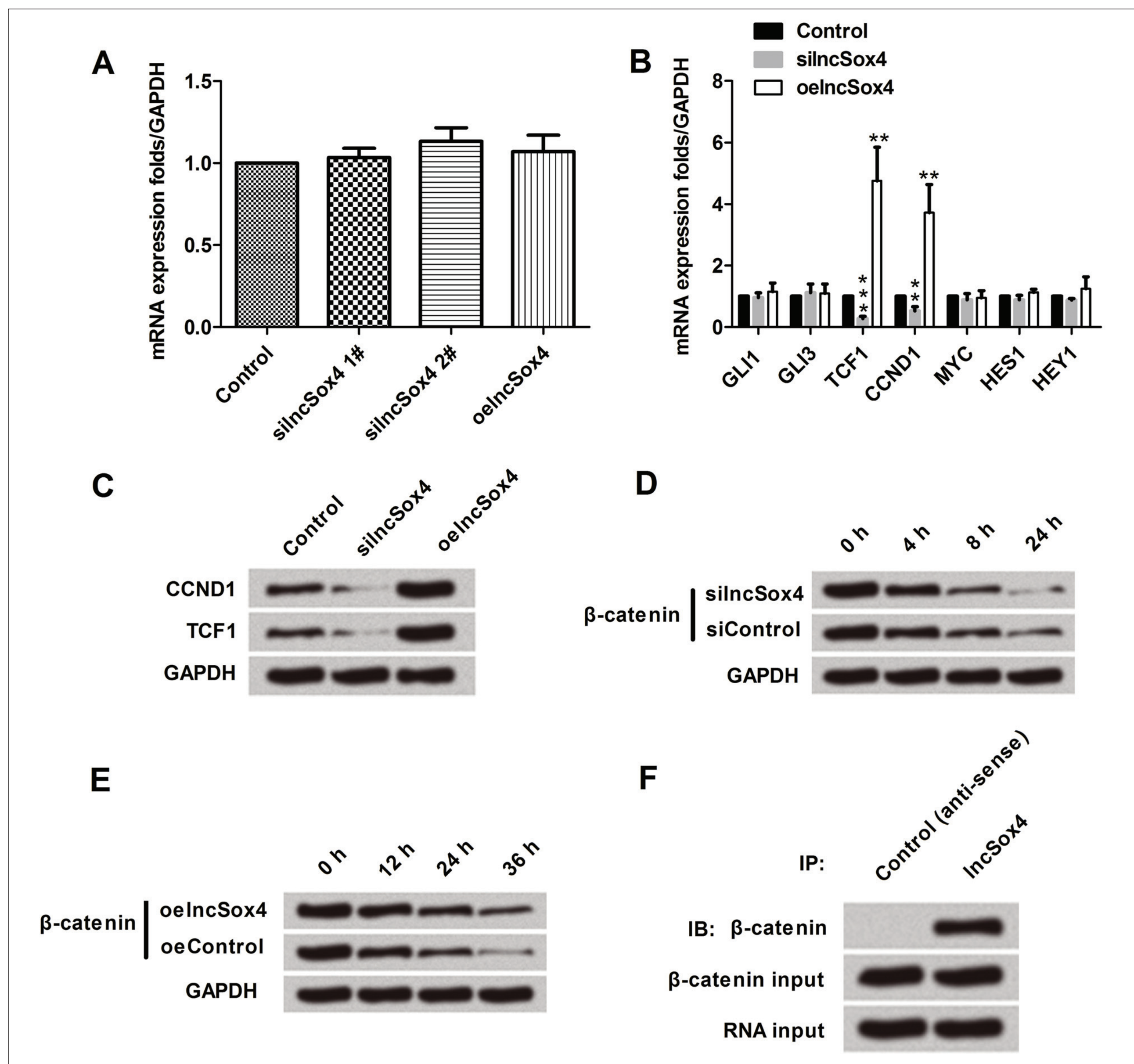


Fig. 3: Promoting role of lncSox4 in Mg63 cells proliferation and migration is via affecting Wnt/β-catenin pathway. A, The mRNA level of Sox4 gene in Mg63 cells treated with silncSox4 or oelncSox4 was detected by qRT-PCR. B, The mRNA expressions of a series of tumor related proteins induced by silncSox4 or oelncSox4 in Mg63 cells were measured by qRT-PCR. C, The expression of CCND1 and TCF1 in Mg63 cells treated with silncSox4 or oelncSox4 were detected by western blot. D, The expression of β-catenin in Mg63 cells treated with silncSox4 or its control (siControl) under CHX treatment for 0 h, 4 h, 8 h and 24 h, respectively, was determined by western blot. E, The expression of β-catenin in Mg63 cells treated with oelncSox4 or its control (oeControl) under treatment for 0 h, 12 h, 24 h and 36 h, respectively, was determined by western blot. F, The interaction of lncSox4 and β-catenin was detected by RNA pull-down assay which immunoprecipitated with lncSox4 and checked with β-catenin antibody. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

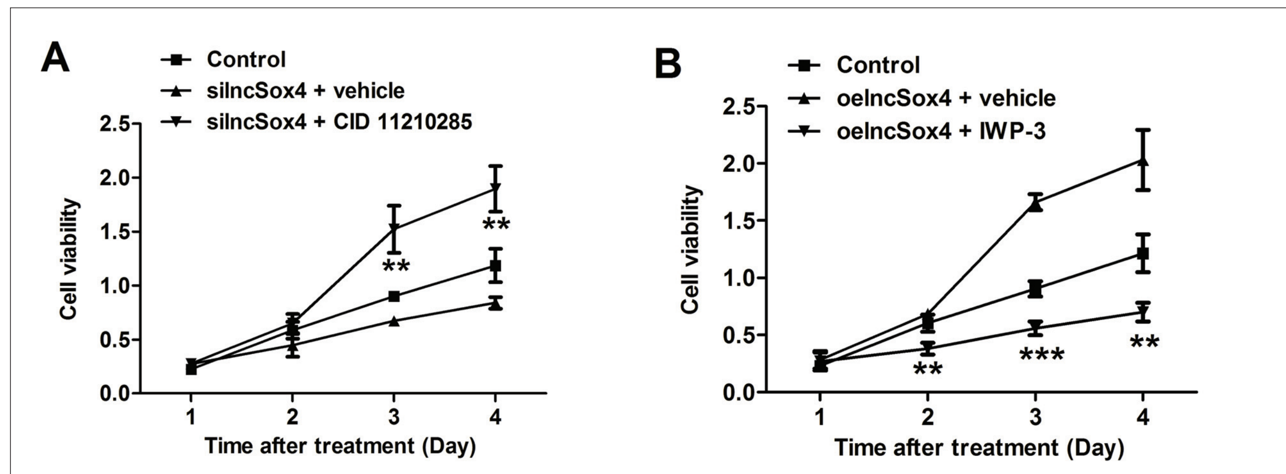


Fig. 4: Wnt/β-catenin signaling pathway is linked with lncSox4-modulated proliferation in Mg63 cells. A, The effect of Wnt agonist CID11210285 on the suppressed cell viability induced by silncSox4 was measured by MTT assay. B, The influence of Wnt inhibitor IWP-3 on the promotion of cell viability induced by oelncSox4 was detected by MTT assay. $^{**}P < 0.01$ compared with silncSox4 + vehicle; $^{***}P < 0.001$ compared with oelncSox4 + vehicle.

methods. As shown in Fig. 3B, silencing of lncSox4 decreased the mRNA level of TCF1 ($P < 0.001$) and CCND1 ($P < 0.01$), while overexpression of lncSox4 enhanced their mRNA level (both $P < 0.01$). The protein levels of TCF1 and CCND1 were promoted by oelncSox4 but reduced by silncSox4 (Fig. 3C). The expression levels of β-catenin under CHX treatment for 0 h, 4 h, 8 h and 24 h were degraded by silncSox4 treatment (Fig. 3D). However, the expression level of β-catenin treated by CHX for 0 h, 12 h, 24 h and 36 h was stable despite overexpression of lncSox4 (Fig. 3D). Further, the interactions between lncSox4 and β-catenin were proved by RNA pull-down experiment. The results showed that the direct combination of lncSox4 and β-catenin protected β-catenin from degradation (Fig. 3F). These data suggest that lncSox4 enhanced tumor progression by activating Wnt/β-catenin pathway in osteosarcoma.

Further, the importance of Wnt/β-catenin pathway in Mg63 cells proliferation was determined by MTT assay. The results showed that the decrease of Mg63 cells viability induced by lncSox4 silencing was completely abolished by the Wnt agonist CID11210285 when the cells were cultured for 3 and 4 days ($P < 0.01$, Fig. 4A). Moreover, the enhanced Mg63 cell viability induced by overexpression of lncSox4 was abolished by the Wnt inhibitor IWP-3 after treatment for 2 ($P < 0.01$), 3 ($P < 0.001$) and 4 ($P < 0.01$) days (Fig. 4B). Above all, lncSox4 enhanced proliferation through promoting Wnt/β-catenin pathway in osteosarcoma.

## 3. Discussion

As one of most common primary malignancies in the world, osteosarcoma is the second cause of cancer leading to the death of children (Allison et al. 2012). Despite the recent advances in identification of multiple crucial oncogenes and tumor suppressors, the underlying pathogenesis mechanism is still unclear (Jaffe et al. 2013; Kansara et al. 2014). The survival of osteosarcoma patients with metastasis remains poor as effective therapeutic biomarkers are lacking (Savage and Mirabello 2011). Thus, it is important to explore novel prognostic and diagnostic targets for osteosarcoma patients. In the present study, we probed into the expression, function, and underlying molecular mechanisms of lncSox4 in osteosarcoma.

Multiple studies have suggested that lncRNAs play vital roles in the progression of osteosarcoma. For instance, lncRNA SATB2-AS1, lncRNA BCAR4, lncRNA PANDA, lncRNA EWSAT1, lncRNA ANRIL and lncRNA HNF1A-AS1 enhanced proliferation, migration and invasion of osteosarcoma in response to various pathological conditions (Gong et al. 2017; Kotake et al. 2017; Liu et al. 2017b; Sun et al. 2016; Wei et al. 2016; Zhao et al. 2016); lncRNA TUG1, lncRNA ZEB1-AS1, lncRN PVT1 and lncRNA LINC00161 participate in strengthening proliferative and invasive capacity by cross-talk with miRNAs (Liu et al. 2017a; Wang et al. 2017b; Wang et al. 2016b; Zhou et al. 2016). Recently, a new long noncoding RNA termed lncSox4 has been found to participate in enhancing liver tumor-initiating cells self-renewal and liver tumorigenesis by activating the Stat3-Sox4 signaling pathway (Chen et al. 2016b). However, whether lncSox4 is involved in the modulation of osteosarcoma is still uncovered. In the current study, we firstly investigated if the level of lncSox4 was highly expressed in both osteosarcoma cells lines and tumor tissues. Further, to explore the function of lncSox4 on cancer progression in osteosarcoma, we detected the roles of lncSox4 in proliferation and migration of Mg63 cells by silencing or overexpression of lncSox4. Our study confirmed that lncSox4 plays a promoting role in growth and migration of osteosarcoma cells, as evidenced by the improved proliferation and migration of Mg63 cells.

Next, we detected the underlying molecular mechanisms of lncSox4 in osteosarcoma. As lncSox4 has been proved to be associated with Sox4 in liver tumor-initiating cells self-renewal, Sox4 is known to play an important role in osteosarcoma by enhancing cell growth and metastasis of osteosarcoma cells (Liu et al. 2015; Luo et al. 2014). However, we found that there was no alteration of Sox4 expression upon either down- or upregulating lncSox4 level. The hedgehog-GLI signaling pathway is well known to contribute to the proliferation and survival of osteosarcoma cells (Shahi et al. 2014; Yoon et al. 2015). CCND1 is suggested to act as a tumor metastasis enhancer in osteosarcoma (Wang et al. 2016a; Yoon et al. 2015); Notch signaling and hes family bHLH transcription factor 1 (HES1), hes related family bHLH transcription factor with YRPW motif 1 (HEY1) expression is involved in enhancing metastasis and invasiveness in osteosarcoma cells (Dailey et al. 2013; Ongaro et al. 2016). A combination of c-MYC overexpression and Rb loss is linked with the transformation of human mesenchyme stem cells to osteosarcoma like tumors (Wang et al. 2017a). Although no reports suggested that TCF1 participates in progression of osteosarcoma, it is a key downstream effector of Wnt/β-catenin and plays crucial roles in various cancers and is associated to chondrosarcoma prognosis (Hrckulak et al. 2016; Xu et al. 2016). Therefore, we screened these classic tumor-oncogene related signaling pathway proteins. We found that there was no change of mRNA level of GLI1, GLI3, MYC, HES1 and HEY1 in response to either lncSox4 down- or upregulation. However, the expressions of TCF1 and CCND1 were decreased by lncSox4 silencing but increased by lncSox4 overexpression. Both TCF1 and CCND1 are tightly associated with the Wnt/β-catenin pathway (Angers and Moon 2009). Previous studies have shown that the Wnt/β-catenin pathway is a key pathway in the regulation of osteosarcoma (He and Zhang 2015; Li et al. 2016c; Zhao et al. 2016). For example, lncRNA HNF1A-AS1 can enhance proliferation and metastasis of osteosarcoma cells by initiation of the Wnt/β-catenin pathway

(Zhao et al. 2016); lncRNA HOTTIP enhanced chemoresistance of osteosarcoma cells via the Wnt/β-catenin pathway (Li et al. 2016c). In addition, viability of osteosarcoma cells was suppressed by baicalein through targeting the Wnt/β-catenin pathway (He and Zhang 2015). Our results confirmed that Wnt/β-catenin is an essential regulator in human osteosarcoma cells. We found that lncSox4 silencing promoted β-catenin protein degradation while lncSox4 overexpression stabled β-catenin expression, and the direct interaction between lncSox and β-catenin protected β-catenin from degradation. Furthermore, we found that Wnt was involved in proliferation and growth regulated by lncSox4. These finding suggested that upregulated lncSox4 promotes proliferation and migration of osteosarcoma cells by activating the Wnt/β-catenin pathway.

Collectively, our studies provided solid evidence that lncSox4 was highly expressed during osteosarcoma, and it promoted proliferation and migration of osteosarcoma cells. Besides, it acted as an oncogene for osteosarcoma, at least in part via activation of the Wnt/β-catenin pathway. This study suggests that lncSox4 could be an effective therapeutic target and a novel potential biomarker for osteosarcoma.

## 4. Experimental

### *4.1. Human osteosarcoma specimens*

A total of 58 osteosarcoma and 21 normal bone tissue samples were obtained between August 2014 and August 2016 from The First Affiliated Hospital of Xinxiang Medical College. There were 38 males and 20 females with the mean age of 12.3 years, including 32 cases with lung metastasis. All the osteosarcoma tissues were randomly divided into two groups. All cases were confirmed as osteosarcoma by clinical, radiological and histological diagnosis. None of patients had received radiation therapy, chemotherapy or other anti-cancer therapy. All the tissues were snap frozen and stored in liquid nitrogen until analyzed. All the clinical information of the patients was collected. Prior written and informed consent were obtained from each patient or legal guardian and the study was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University.

### *4.2. Cell culture*

The human embryonic kidney cell lines 293T, and osteosarcoma cell lines, Mg63 and U-20S were all obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle Media (DMEM, GIBCO, Grand Island, NY, USA) with 10% (v/v) fetal bovine serum (FBS, GIBCO) and 100 mg/ml penicillin/streptomycin (GIBCO) at 37 °C in 5% $CO_2$, then were subcultured at 80–90% confluence. Cells used in this study were subjected to no more than 20 cell passages.

### *4.3. Cell transfection*

For lncSox4 silencing, cells were treated with pRS-silncSox4 1# or 2#, which were two siRNAs of lncSox4 with different sequences (Shanghai GeneChem Company, Shanghai, China), pRS-si-NC (negative control) and then selected with puromycin (1.5 μg/ml, Invitrogen, Carlsbad, CA, USA) for 2 weeks. For lncSox4 overexpressing, cells were transfected with pcDNA3-lncSox4 (Invitrogen) or pcDNA3-NC (negative control) vectors. Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. After transfection, Mg63 cells were treated with a protein synthesis inhibitor-cycloheximide (CHX, 5 μg/ml, Sigma-Aldrich, St Louis, MO) for 0 h, 4 h, 8 h, 12 h, 24 h and 36 h to sensitize cells for protein detection. In addition, after transfection, cells viability were checked after treatment with the Wnt agonist CID 11210285 (500 nM, Sigma-Aldrich) or the Wnt inhibitor IWP3 (1 μM, Sigma-Aldrich) for 1, 2, 3 and 4 days which were dissolved in DMSO.

### *4.4. MTT assay*

The viability of cells were checked with a 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay according to instruction's protocol (Sigma-Aldrich). Briefly, after incubation with MTT (0.5 mg/ml) at 37 °C for 4 h, the supernatant media were carefully discarded and 150 μl DMSO was added to each well. Finally, purple crystals of formazan were dissolved by vigorously shaking of microplates and absorbance (OD) values were checked at 570 nm by spectrophotometry with a microplate reader apparatus (Eliza MAT 2000, DRG Instruments, GmbH). All samples were measured in duplicate. Each experiment was performed independently three times.

### *4.5. Ki-67⁺ cells detection and flow cytometry*

Cells were planted in 6-well plates ($1 \times 10^6$ cells/well), then trypsinized and washed twice with phosphate-buffered saline (PBS). The cells were fixed in 4% paraformaldehyde for 30 min at 4 ˚C. Next, cells were incubated with 1% RNase at 37 ˚C for 30 min and stained with 50 μg/ml propidium iodide (PI; Sigma-Aldrich) for 1 h at 4˚C. Cell suspensions were incubated with antibodies to Ki-67 (1 μg/$1 \times 10^6$ cells, ab15580, Abcam, Cambridge, UK) for 30 min at 37 ˚C and incubated with FITC-labeled secondary antibodies (Jackson Laboratory, Bar Harbor, ME, USA) for 30 min at 37 ˚C. The Ki-$67^+$ cells were distinguished by flow cytometer and the rate of Ki-$67^+$/10,000 cells was calculated.

### *4.6. Cell migration assay*

Transwell migration assays were performed using 24-well transwell chambers (8-μm pore size) (Corning Incorporated, Corning, NY, USA). After trypsinization, cells were suspended in 100 μl DMEM medium without serum and plated onto the upper chamber. Culture medium with 10% FBS (600 μl) was added to the lower chamber. After incubation for 18 h, non-migrated cells on the upper membrane surface were removed using cotton swab; migrated cells were fixed in 4% paraformaldehyde and stained with hematoxylin. The numbers of cells that pass through the membrane were quantified by microscope. This assay was performed in triplicate.

### *4.7. Quantitative real-time PCR (qRT-PCR)*

Total RNA isolated from transfected cells were extracted using Trizol reagent (Invitrogen). After treatment with DNaseI, the cDNA were reverse transcribed with 1 μg RNA as template and oligo (dT) as a primer using a RT kit from Takara (Shiga, Japan). Quantitative real-time PCR of the lncSox4 mRNA was performed using SYBR® Premix Ex Taq TM II (TaKaRa) through 35 PCR cycles (95°C for 10 s, 60°C for 30 s) in a Bio-Rad Hard Shell® 96 microplate on Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). To obtain the fold change in mRNA, data were analyzed using the $2^{-\Delta CT}$ method. The final gene expression levels were normalized to the internal control GAPDH mRNA. The expression data were analyzed with the comparative Ct method.

### *4.8. Western blot*

Total proteins were extracted by RIPA lysis buffer (Beyotime Biotechnology, Nanjing, China) supplemented with protease inhibitors (Roche, Basel, Switzerland) and protein concentration were quantified using the BCA™ Protein Assay (Pierce, Appleton, WI, USA). Then proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfected to polyvinylidene difluoride (PVDF, EMD Millipore, Billerica, MA, USA) membranes. After incubation in the 5% blocking buffer for 2 h at room temperature, membranes were incubated with primary rabbit anti-β-catenin (ab16051, Abcam), rabbit anti-cyclin D1 (ab134175, Abcam), rabbit anti-TCF1 (2206, Cell signaling technology, Beverly, USA) or mouse anti-GAPDH (ab8245, Abcam) antibodies (1:1000 dilution) at 4 °C overnight, respectively, followed by incubation with secondary anti-mouse or anti-rabbit IgG antibodies (1:5000; both from Santa Cruz Biotechnology Inc., Dallas, TX, USA) marked by horseradish peroxidase for 1 h at room temperature. The PVDF membranes were transferred into the Bio-Rad ChemiDoc™ XRS system and added with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). The signals were captured and the intensities of the bands were quantified using Image Lab™ Software (Bio-Rad).

### *4.9. Northern blot*

After extraction of total RNA, lncSox4 and 18S fragments were cloned into PCDNA3 plasmid. Northern probes were obtained using Biotin RNA Labeling Mix (catalogue 11685597910, Roche) and T7 RNA polymerase (catalogue 10881767001, Roche) was used for *in vitro* transcription. The RNA samples for northern blot were separated by formaldehyde gel, followed membrane transferring. Then membranes were incubated with a proper amount of probes dissolved in hydration buffer. The nucleic acid signal was detected using Chemiluminescent Nucleic Acid Detection Module (catalogue 89880, Thermo Scientific, Carlsbad, USA).

### *4.10. RNA pull-down assay*

LncSox4 and its antisense RNA were bio-labeled by T7/SP6 RNA polymerase (Roche Diagnostics, Indianapolis, IN, USA) using the Biotin RNA Labeling Mix (Roche) and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Next, total protein (1 mg) extracted from Mg63 cells were incubated with 50 pmol of biotinylated lncSox4 or its antisense for 1 h at 4 °C. Next, the complexes were mixed with dynabeads Myone Streptavidin T1 beads (Invitrogen) for 1 h according to manufacturer's protocol. Finally, the proteins binding to the streptavidin-coupled dynabeads were detected by SDS-PAGE and the presence of β-catenin was detected by a conventional western blot analysis.

### *4.11. Statistical analysis*

Data were analyzed by SPSS 16.0 (SPSS, Chicago, IL, USA) and *P*-values were calculated using a one-way analysis of variance (ANOVA) or two-way ANOVA. A value of $P < 0.05$ was considered statistically significant. The results were presented as mean±standard deviation (SD). All experiments were repeated independently for three times.

Conflicts of interest: None declared.

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