JBUON 2017; 22(1): 258-264
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

SOX5 promotes epithelial-mesenchymal transition in osteosarcoma via regulation of Snail

Daiyang Zhang[1, 2], Shiqing Liu[1]

[1]Department of Orthopedics, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, 430060, China; [2] Department of Orthopedics, The Central Hospital of Enshi Autonomous Prefecture, Enshi Clinical College of Wuhan University, Enshi, 445000, China

Summary

Purpose: *SOX5 plays important roles in various kinds of cancers. However, the expression and roles of SOX5 in osteosarcoma (OS) have not been investigated well. In the present study we aimed to investigate the mechanism of SOX5 in OS.*

Methods: *OS and adjacent non-cancerous specimens were obtained from patients with OS. PCR was applied to detect SOX5 mRNA. Then human OS cell lines (U2OS, SoSP-M, SoSP-9607, and MG-63) and one immortalized normal osteoblast hFOB1.19 were investigated. SOX5 knocking with shRNA in U2OS and SOX5 upregulation with recombinant plasmid in MG-63 were applied. Real-time cell monitoring system and invasion assay were used, and Western blot assay was performed to detect the protein level of E-cadherin, N-cadherin, Vimentin and Snail, where Glyceraldehyde3-phosphate dehydrogenase (GAPDH) was presented as control. P<0.05 was considered as statistically significant.*

Results: *Significant upregulation of SOX5 in OS tissues and cell lines was identified. The gain- and loss-of-function studies suggested that OS cell migration and invasion were promoted significantly by SOX5. Additionally, SOX5 promoted epithelial–mesenchymal transition (EMT) by regulation of Snail.*

Conclusion: *SOX5 is a novel regulator of EMT in OS, and is a potential target for OS.*

Key words: *SOX5, osteosarcoma, epithelial–mesenchymal transition, Snail*

Introduction

OS, as a common primary bone-forming malignant mesenchymal tumor [1,2], is characterized by high metastatic potential (especially to the lung) [3]. Although neoadjuvant chemotherapy and surgery have been used for the treatment of OS patients with metastatic disease, 5-year survival is still <20% [4]. Therefore, novel therapeutic strategies should be developed for the management of this malignancy. Generally, a primary tumor is not fatal to the organism, while cancer with invasion and metastasis can lead to disease progression and death [5]. Metastasis is the cause >90% deaths in OS, however, the mechanism of metastasis remains poorly understood.

EMT is a developmental process, in which epithelial cells lose polarity and develop a cell-cell adhesion. EMT is involved in the initiation of metastasis. Moreover, EMT usually causes 'cadherin switching' (upregulation of mesenchymal cadherins and downregulation of E-cadherin) [6] and the accumulation of β-catenin [7]. Cancer cells with EMT may develop migratory and in-

Correspondence to : Shiqing Liu, MD. Department of Orthopedics, Renmin Hospital of Wuhan University, Zhang Zhidong Road, Wuhan, 430060, China. Tel : +86 27 8806 3953, Fax : +86 27 8804 2292, E-mail : liushiqing1357@sina.com.
Received : 19/08/2016; Accepted : 09/09/2016

vasive properties, and ultimately develop cancer stem cell (CSC) properties [8]. In addition, specific transcription factors have been identified to accomplish the phenotypic changes associated with EMT, such as Snail, Slug, Twist1, ZEB1 and ZEB2 [9]. Overexpression of Snail occurs in human solid tumors, including sarcomas, gliomas, neuroblastomas, and melanomas [10]. Furthermore, overexpression of Snail in cancer cells influences cell survival, angiogenesis, and chemoresistance *in vitro*, and promotes metastatic ability *in vivo* [11]. Additionally, downregulation of E-cadherin and induction of EMT are promoted by exogenous overexpression of Snail, and subsequently cancer cells developed invasive and metastatic characteristics. However, the molecular mechanism for the upregulation of Snail in cancer cells is less clear.

Sex determining region Y-box protein 5 (SOX5), a transcription factor, serves important role in the regulation of embryonic development and in the determination of the cell fate [12]. SOX5 was determined to be involved in human glioma, seminoma, and nasopharyngeal carcinomas [13]. To the best of our knowledge, SOX5 expression has not been investigated well in human OS. In this study we aimed to investigate the mechanisms of SOX5 in OS.

Methods

OS cell lines

Human OS cell lines U2OS, SoSP-M, SoSP-9607, MG-63 (American Type Culture Collection) with different metastatic potential and one immortalized normal osteoblast hFOB1.19 were used in this study. Cells (Shanghai Cell Bank, Chinese Academy of Sciences) were cultured in DMEM (Gibco, Inc., USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Inc., USA) in a humidified incubator with 5% CO_2 at 37°C.

Tissue specimens

The Institutional Ethics Committee of Renmin Hospital of Wuhan University approved our project for human tissue usage. Written informed consent has been provided by the patients. Pathological examination was applied to confirm OS. The OS and adjacent non-cancerous samples were obtained from patients who underwent surgical resection. These tissue samples were immediately frozen at -80°C until RNA extraction.

RNA extraction and real-time PCR assays

Trizol Reagent (Invitrogen, Inc.) was applied for total RNA extraction from cultured cells. Reverse transcription was conducted with Prime Script TM Master Mix (Takara, Tokyo, Japan) according to the manufacturer's instructions. Then, qRT-PCR was performed with SYBR Premix EX Taq TM II (Takara) according to its product manual on the real-time PCR detection system Bio-Rad IQ5 (Bio-Rad, California, USA). GAPDH was used as reference. Relative gene expression was normalized to GAPDH and reported as $2^{-\Delta Ct}$ [Δ Ct = Ct (SOX5)-Ct (GAPDH)]. All measurements were performed in triplicate. The primers for the genes (SOX5, GAPDH) were synthesized as follows:

5'-CTTCATGCCTGACTGATAT-3' (forward) and 5'-GACGTCTCAGGACTTACG-3' (reverse) for SOX5;

5'-CTGCAGGTCTCATCATGGA-3' (forward) and 5'-ACCTGTAGACCTCGGCACTG-3' (reverse) for GAPDH.

Western blot analysis

Mixture of ProteoJET Mammalian Cell Lysis Reagent (Thermo Scientific, Waltham, USA), phenylmethanesulfonyl fluoride (Roche, Inc., Basel, Switzerland) and Phos STOP (Roche, Inc., Basel, Switzerland) were added in cultured cells (about 1×10^6) to obtain lysates. Samples were separated by 10% SDS-PAGE to obtain about 50μg protein in each sample. Following being blocked in nonfat milk (5%), protein was incubated with rabbit antibody against human SOX5, p-RAF, RAF, p-ERK1/2 (1:1000, Abcam, Cambridge, UK), p-RAF, N-cadherin, E-cadherin, Snail, E-cadherin or GAPDH (1:1000, Cell Signaling Technology, Boston, MA, USA), then incubated with goat anti-mouse or anti-rabbit IgG (1:10,000 for both; Jackson ImmunoResearch Laboratories, Lancaster, USA). Then, enhanced chemiluminescence reagents were applied for detection (Thermo Fisher Scientific, Rockford, ILL, USA). Subsequently, 1-stepTM NBT/BCIP reagents (Thermo Fisher Scientific) were applied for visualization of the bands, and Alpha Imager (Alpha Innotech, San Leandro, CA, USA) was used for detection.

Plasmid, siRNA and transfection

The full-length cDNA of SOX5 (4,333bp, GenBank accession number NM_006940) from human liver cDNA library was amplified by PCR, and cloned into pcDNA3.1 (Thermo Scientific, Waltham, USA). siRNAs (synthesized by GenePharma, Suzhou, China) were applied to downregulate endogenous expression of SOX5 in OS cells. Si-NC served as negative control. All constructs were to be fully sequenced. The plasmid or siRNA were then transfected in cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Invasion assay

1.0×10^5 cells in medium supplemented with 1% FBS were seeded into 24 wells. Then, medium containing 10% serum as chemoattractant, was added to the wells. Following 48-hr incubation, wiping with a cotton swab was used to remove non-invading cells from the

upper surface. Then, the membranes were fixed at room temperature by using 4% formaldehyde for 15 min, and subsequently, invading cells were stained with Giemsa (Sigma, Munich, Germany) for 25 min, and the cell number was counted by microscope.

Measurements of cell movement

Cell-IQ cell culturing platform (Chip-Man Technologies, Tampere, Finland) controlled by Imagen software (Chip-Man Technologies, Tampere, Finland) was applied for measurement of cell movement with phase-contrast microscope (Nikon, Tokyo, Japan). Achromat phase-contrast objective with 10x magnification) and camera (Nikon, Tokyo, Japan). The equipment was set at 5 min intervals after 72-hr shooting. In addition, image software (Cell-IQ Imagen v2.9.5c, McMaster Biophotonics Facility, Hamilton, Ontario) was applied for analysis of time-lapse data and cell functions and morphological parameters were analyzed and quantified by Cell-IQ system. The distance of cell movement in the image field was measured as movement of each individual cell.

Statistics

Data were presented as mean±SE. Student's t-test and one way ANOVA were performed with SPSS 14.0 software. A p value <0.05 was considered as statistically significant.

Results

Overexpression of SOX5 in OS tissue and cell lines

qRT-PCR assays were performed to examine the expression of SOX5 in 49 pairs of matched OS samples. The results displayed significant upregulation of SOX5 ($p<0.01$; Figure 1A) in OS tissues compared with adjacent non-cancerous tissues. Subsequently, these OS specimens were randomly divided into two groups, including OS with pulmonary metastasis (PM) group and OS without PM group. Interestingly, SOX5 level in the OS specimens with PM group was significantly higher ($p<0.05$; Figure 1B) than that in OS specimens without PM group. In addition, SOX5 expression in cells was determined in 4 OS cell lines with different metastatic potentials (U2OS, SoSP-M, SoSP-9607, and MG-63) and the immortal normal osteoblast hFOB1.19. The results showed that

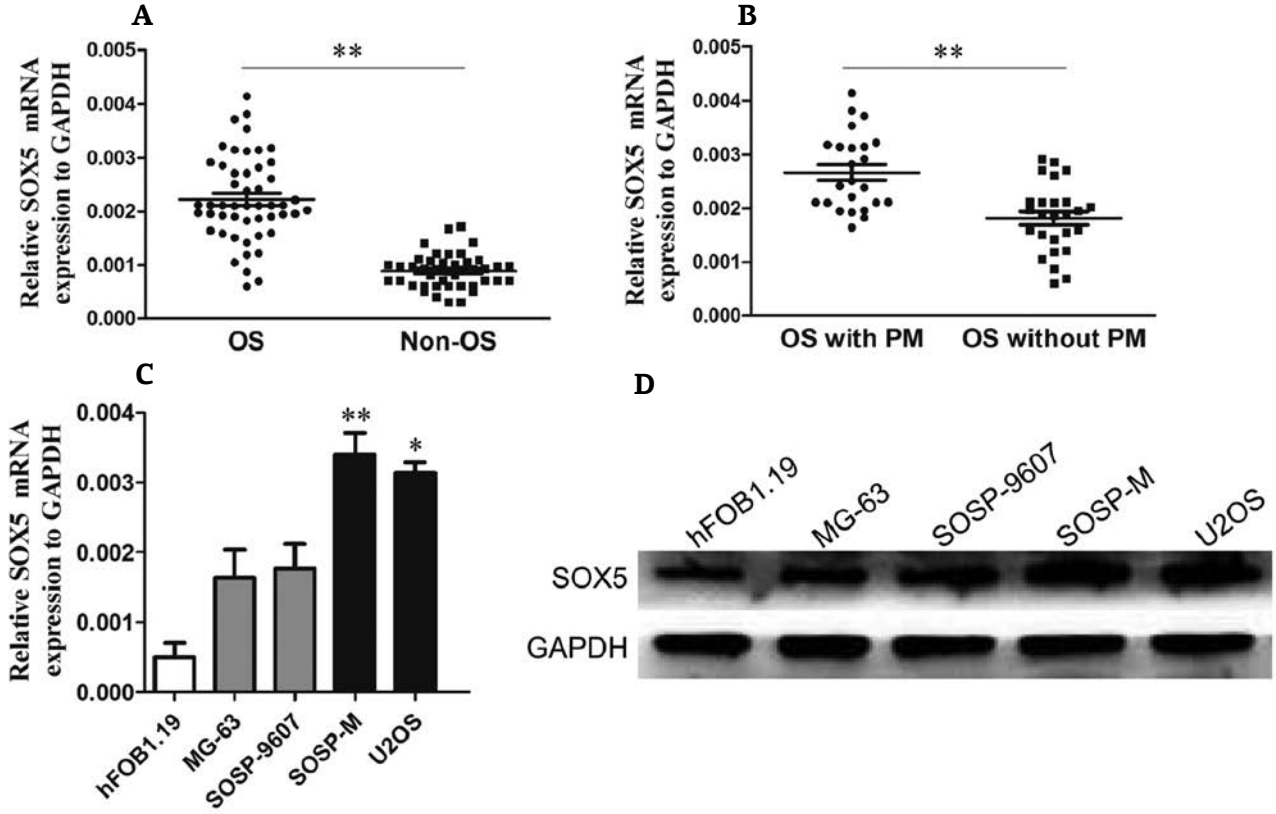

Figure 1. Overexpression of SOX5 in OS tissues and cell lines. **(A)** The SOX5 mRNA expression in 49 pairs of OS and the corresponding adjacent non-cancerous tissues. The results of **(A)** indicated that the expression of SOX5 in OS was significantly higher than that in Non-OS. **(B)** The mRNA expression of SOX5 in OS with PM and OS without PM. The results of **(B)** indicated that the expression of SOX5 in OS with PM was significantly higher than that in OS without PM. (**C,D)** The SOX5 mRNA expression in OS cell lines with different metastatic potentials as well as the normal human liver cell line. The results of **(C)** and **(D)** indicated that the expression of SOX5 was relatively higher in all four OS cell lines compared with that in hFO b1.19. *p<0.05, **p<0.01 versus the control.

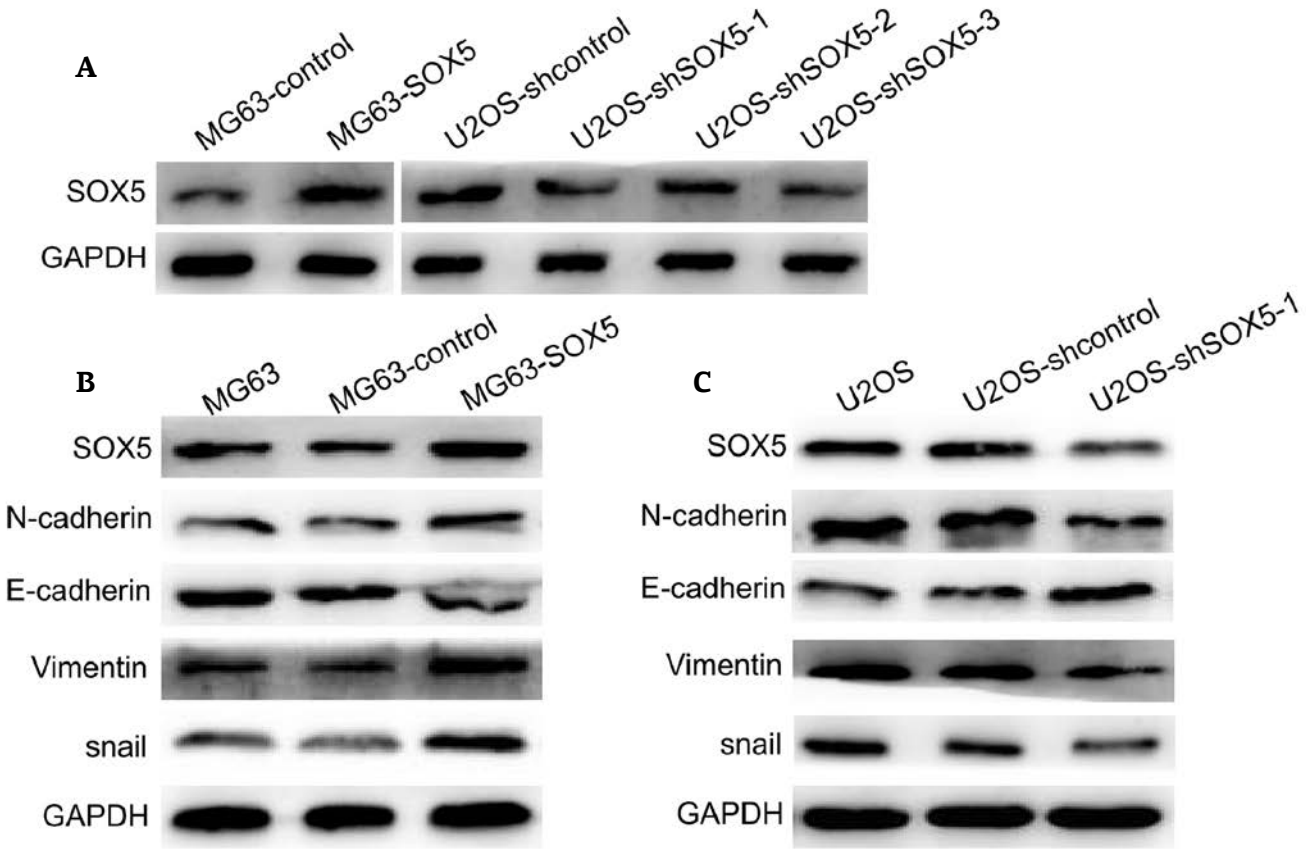

Figure 2. Regulation of SOX5 expression on EMT in OS cells. **(A)** Ectopic expression of SOX5 in MG63 and knockdown of endogenous SOX5 in U2OS demonstrated by Western blot assay. The results of **(A)** indicated that SOX5 was ectopically expressed in MG63 and U2O2 cells. Western blot assay indicated EMT markers in **(B)** MG63 cells ectopically expressing SOX5 and **(C)** U2OS cells with SOX5 depletion. The results of **(B)** indicated that expression of N-cadherin and vimentin increased, while expression of E-cadherin decreased. And, the ectopic expression of SOX5 resulted in a variation of Snail levels. Then the results of **(C)** indicated that compared with negative control cells, SOX5 silencing resulted in decrease of N-cadherin, vimentin and Snail, and increase of E-cadherin.

the expression of SOX5 was relatively higher in all 4 OS cell lines compared with hFOB1.19 cell line (Figures 1C,D). These findings suggested that overexpression of SOX5 in OS may facilitate the cells' metastatic pontential.

Promotion of OS cell migration and invasion by upregulation of SOX5 in vitro

To investigate whether SOX5 is involved in the regulation of OS cells migratory and invasive properties, the recombinant plasmid pcDNA3.1-SOX5 was transfected into MG-63 cells (these cells expressed a relative low SOX5), to upregulate SOX5 expression (Figure 2A). Consequently, SOX5 overexpression increased significantly the migratory ability of cells with pcDNA3.1-SOX5 ($p<0.05$, Figure 3A). Similarly, SOX5 overexpression markedly promoted cell invasion ($p<0.05$, Figure 3B). Therefore, these results determined that SOX5 promoted migratory and invasive abilities of OS cells *in vitro*.

Reduction of OS cell migration and invasion by SOX5 downregulation in vitro

Knockdown of SOX5 with specific shRNA (sh-1) was performed in U2OS cells. Then, the loss of function by sh-1 significantly reduced the migration capabilities ($p<0.05$, Figure 3A). Similarly, SOX5-repressed U2OS cells showed significant reduction of invasive capabilities ($p<0.05$, Figure 3B). The results of gain-of-function and loss-of-function assays suggested that that SOX5 plays key role in OS migration and invasion.

Induction of EMT by SOX5 in OS cells through modulation of Snail

Ectopic expression of SOX5 was performed in MG-63 cells, by transferring the recombinant plasmid pcDNA3.1 SOX5 into MG63 cells, and consequently expression of N-cadherin and vimentin (mesenchymal phenotype markers) increased significantly ($p<0.05$), while expression of E-cadherin (epithelial phenotype marker) was

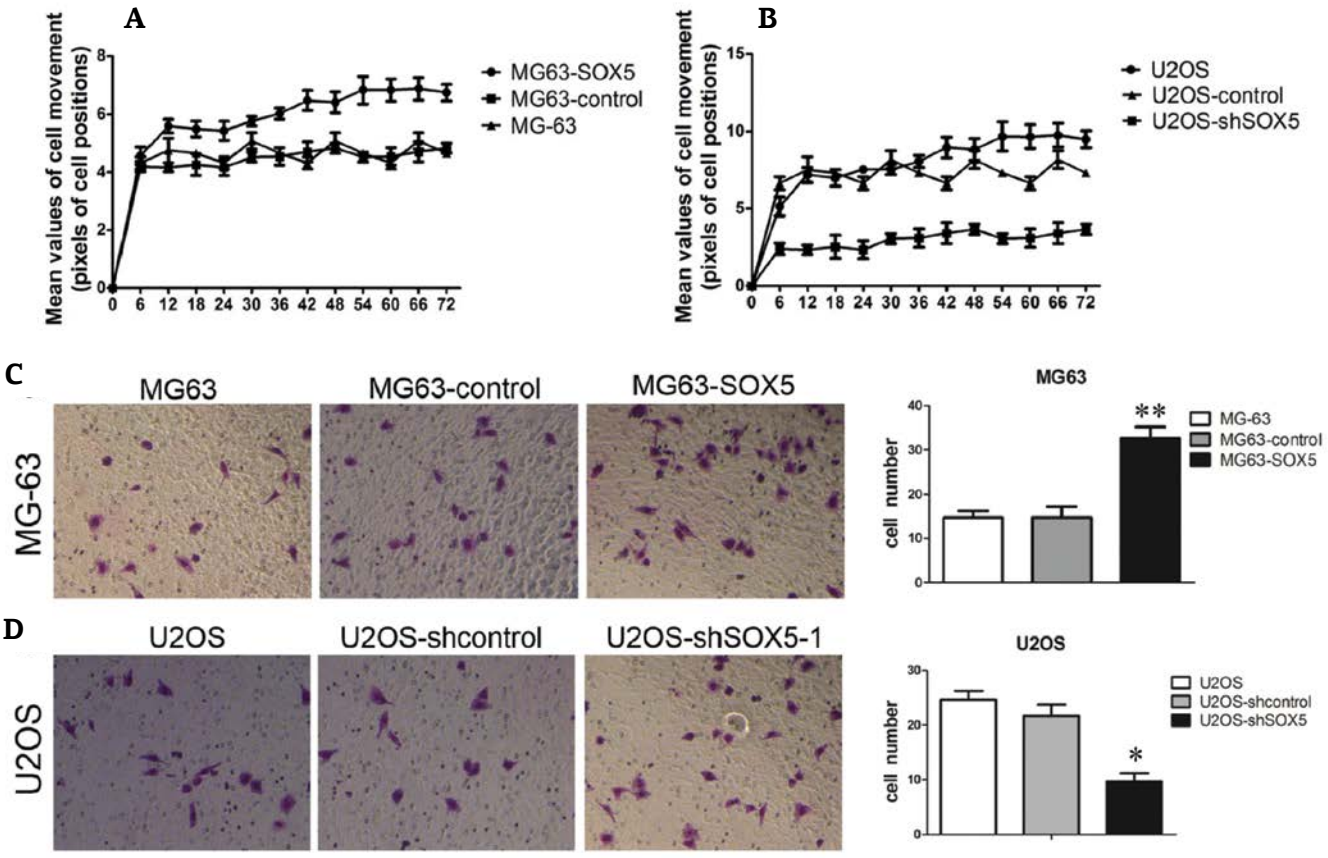

Figure 3. SOX5 regulates OS cell migration and invasion in vitro. **(A,B)** Cell movement evaluate the effect of SOX5 on cell motility. Then, the results in **(A)** indicated that SOX5 overexpression increased the migratory ability of MG63 cells, and **(B)** indicated that SOX5 overexpression increased the migratory ability of U2OS cells. **(C,D)** Trans-well assay to determine the effect of SOX5 on cell invasion capacity. And the results in **(C)** and **(D)** indicated that SOX5 overexpression promoted cell invasion. *p<0.05, **p<0.01 versus the control.

markedly decreased (p<0.05, Figure 2B). Interestingly, the ectopic expression of SOX5 resulted in a significant variation of Snail levels (p<0.05, Figure 2B). Consistent with these results, compared with negative control cells, Western blot analysis of U2OS cells with SOX5 silencing revealed that the relative protein expression of N-cadherin, vimentin and Snail were significantly decreased while the expression of E-cadherin was notably increased (Figure 2C). Therefore, SOX5 induced progression of EMT possibly through regulation of Snail.

Induction of EMT by SOX5 overexpression via Raf1/ERK1/2 pathway

Activation of ERK1/2 in MG-63 cells was significantly enhanced by SOX5 overexpression (p<0.05, Figure 4A). Raf1 was identified as the upstream activator of ERK signaling pathway, which was significantly upregulated by SOX5 overexpression (p<0.05, Figure 4A). Moreover, U0126 performed as inhibitor of ERK1/2. EMT markers were significantly inhibited by U0126 (p<0.05, Figure 4A), and the upregulation of Snail was also inhibited by SOX5 overexpression (p<0.05, Figure 4A). On the other hand, SOX5 silencing in U2OS cells could inactivate p-ERK1/2, p-Raf signalling pathways (Figure 4B). These results indicate that SOX5 activated Raf/ ERK1/2/Snail pathway to induce EMT.

Discussion

SOX family members (including SOX2 and SOX4) have been identified as important players in the occurrence and development of cancer [14,15]. SOX5 is a transcription factor of SOX family, which plays key roles in embryonic development and cell fate determination [16]. SOX5 is also involved in various cancers. For example, upregulation of SOX5 associated with the early development of distant metastasis has been determined in prostate cancer [17]. Then, the promotion of cell proliferation and invasion by SOX5 was identified in breast cancer, and the mechanism was involved in the induction of EMT [13]. Additionally, invasive tumor growth was regulated by SOX5 in

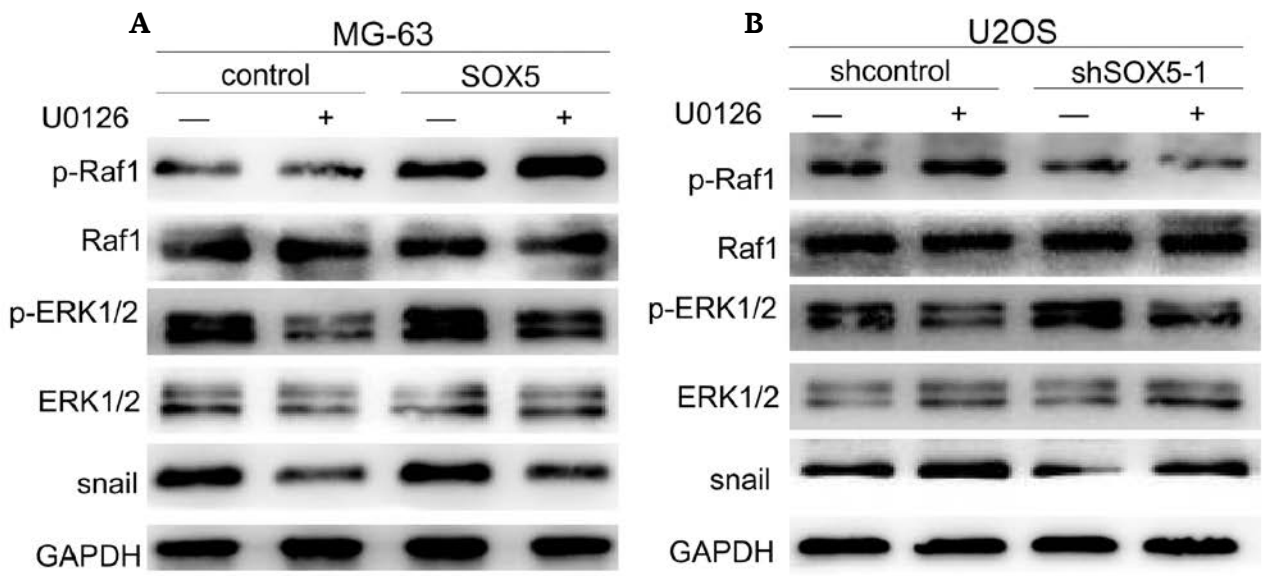

Figure 4. SOX5 regulates snail expression via Raf- ERK signalling pathway in OS cells. **(A,B)** Snail as well as ERK signalling pathway were assayed using Western blot in MG63 and U2OS with overexpression or depletion of SOX5 after treatment with U0126, an inhibitor of ERK1/2. The results of **(A)** indicated that ERK1/2 and Raf1 were upregulated in MG-63 cells by SOX5 overexpression. Then, U0102 performed as inhibitor of EMT markers. And the upregulation of Snail was also inhibited by SOX5 overexpression. The results in **(B)** indicated that SOX5 silencing in U2OS cells inactivated p-ERK1/2, p-Raf and snail signalling pathways.

testicular seminomas [13]. Moreover, our results showed overexpression of SOX5 in OS tissues and cell lines. Taken together, these suggest that SOX5 performed as an oncogene. Furthermore, gain or loss of function assay suggested that upregulation of SOX5 promoted cell migration and invasion of OS cells *in vitro*.

EMT enables epithelial cells to develop invasive mesenchymal phenotype. and EMT was identified as a key mechanism in tumor invasion and metastasis [18,19]. In EMT, epithelial functions (such as cell-cell contacts) were lost with the acquisition of mesenchymal functions, and cytoskeletal remodeling performed with the change of polarity. These processes led to increased cell motility and invasion [20].

We found that depletion of SOX5 induced decreased invasive capability of U2OS cell line, which reminded us that SOX5 might be involved in the regulation of EMT. Expectedly, SOX5 silencing resulted in the upregulation of the epithelial marker E-cadherin and downregulation of the mesenchymal markers N-cadherin and vimentin. In addition, ectopic expression of SOX5 was observed, which resulted in opposite results. Furthermore, SOX5 expression was higher in OS patients with PM compared with those without PM, which was explained by the mechanism of regulation of EMT by SOX5.

Snail plays key roles in promoting invasiveness and EMT progression [21]. Ectopic expression or dysregulated function of Snail contributed to tumor invasion and metastasis, which has been identified in several solid cancers, including esophageal squamous cell carcinoma, oral cancer, prostate cancer, and hepatocellular carcinoma [22]. In the present study, the upregulation or knockdown of SOX5 led to opposite expression of Snail.

SOX5 participated in many pathways, such as activation of NF-κB and AKT, and activation of MEK/ERK branch of the Ras downstream pathway [23]. Our study also confirmed the activation of Raf-ERK signaling pathway by SOX5, and further determined that SOX5 regulated the expression of Snail by Raf-ERK signaling pathway. Specifically, inactive ERK with U0126 suppressed EMT regulated by SOX5.

In conclusion, EMT was induced by SOX5 in OS cells by regulating Snail expression via Raf-ERK pathway. SOX5 performs as a metastasis-associated factor in OS, rendering it a potential target for OS therapy.

Acknowledgements

We thank Renmin Hospital of Wuhan University, Wuhan, Hubei Province for financial support.

Conflict of interests

The authors declare no confict of interests.

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