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Original Article

HoxB9 promotes the migration and invasion via TGF-β1/Smad2/Slug signaling pathway in oral squamous cell carcinoma

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Abstract: HoxB9, as a HOX family member, is known to play important roles in embryonic development. Recent studies have shown that HoxB9 is involved in cancer progression. However, little is known about the role of HoxB9 and the underlying mechanisms that suppress oral squamous cell carcinoma (OSCC) progression. In the present study, we used immunohistochemical staining to demonstrate that HoxB9 is over-expressed in OSCC cells and found that high levels of HoxB9 were significantly associated with shorter overall survival in patients with OSCC. Functional studies revealed that knocking down HoxB9 in OSCC cells using RNA interference decreased the migration and invasion of OSCC cells *in vitro*. Our mechanistic studies suggested that HoxB9 could stimulate the migration and invasion of OSCC cells by targeting EMT via the TGF-β1/Smad2/Slug signaling pathway. Collectively, these findings suggest the vital roles of HoxB9 in OSCC progression through its effects in promoting EMT.

Keywords: HoxB9, OSCC, EMT

Introduction

The identification of key molecular alterations in cancer has resulted in major advances in diagnosis and targeted therapies with validated biomarkers, heralding the advent of personalized medicine. However, oral squamous cell carcinoma (OSCC) lags behind in this regard because no consistent oncogenic drivers have been identified, and cetuximab is currently the only approved targeted therapeutic [1]. OSCC is the 6th most common cancer worldwide with an incidence exceeding 450,000 cases annually. Furthermore, survival rates have not significantly improved for several decades [2]. Thus, finding novel targets for therapeutic intervention as well as new biomarkers in OSCC is necessary and urgent.

HoxB9 is a member of the homeobox-containing (HOX) transcriptional factor family that includes 39 genes in humans and is classified into four different clusters: HOX A, B, C and D, all of which play important roles in embryonic development, especially in the patterning of the anterior-posterior axis [3, 4]. In addition to their critical roles in development, increasing evidence has demonstrated that HOX family genes are associated with cancer progression [5, 6]. HoxB9 was shown to induce angiogenesis, invasion and lung metastasis in breast cancer [7], was identified as an important prognostic factor for ovarian cancer [8] and pancreatic ductal adenocarcinoma [9], as well as for lung cancer patients [10]. These results strongly suggest that HoxB9 is involved in cancer progression and metastasis. Epithelial-mesenchymal transition (EMT) comprises a set of rapid changes in the cellular phenotype in which epithelial cells experience a molecular switch from a polarized, epithelial phenotype to a highly motile, non-polarized mesenchymal phenotype [11]. EMT is frequently observed at the invasive front of advanced tumors and is significantly correlated with metastasis in tumor progression [12, 13]; however, whether HoxB9 could influence EMT in OSCC remains unclear.

In this study, we over-expressed HoxB9 in OSCC cells and found that high levels of HoxB9 are significantly associated with shorter overall survival in patients with OSCC. In addition, we dis-

Tablel 1. Statistical analyses of factors associated with survival in OSCC patients withthe multivariate Cox proportional hazards model

	Overall survival		
Variables	RR	95% CI	*P*
HoxB9 expression			
Positive VS negative	2.472	1.367-4.469	0.003
Gender			
Male VS female	1.017	0.512-2.020	0.438
Age			
< 60 VS ≥ 60	1.008	0.983-1.033	0.941
Smoking			
Yes VS no	0.790	0.378-1.650	0.296
Drinking			
Yes VS no	0.960	0.422-1.962	0.990
Tumor stage			
3-4 VS 1-2	1.005	0.525-1.791	0.641
Lymph node metastasis			
+ VS -	1.676	1.230-2.282	0.001
Clinical stage			
3-4 VS 1-2	1.433	1.034-1.986	0.037
Histological type			
Poor VS well-moderate	1.312	0.986-1.744	0.062

CI, confidence interval.

covered that downregulation of HoxB9 could significantly reduce cell migration in OSCC cells. These results could be attributed to the functions of HoxB9 in regulating epithelial-mesenchymal transition (EMT) process via the TGF-β1/Smad2/Slug signaling pathway.

Materials and methods

Ethics and patient tumor sections

Acquisition of tumor samples from oral squamous cell carcinoma patients who were enrolled in the current study was approved by the Ethics Committee of Second Xiangya Hospital of Central South University, Changsha, China. The procedures for handling human materials were in accordance with the ethical standards of the 1975 Declaration of Helsinki, which was revised in 1983. Primary OSCC tissue specimens (n = 50) were obtained from patients who underwent surgery at the Department of Oral and Maxillofacial Surgery of Second Xiangya Hospital. Adjacent matched normal mucosa samples (n = 50) were obtained at least 3 cm from the tumor tissues in the same group of patients and were used as controls. These patients underwent surgery between July 2010 and April 2011, and the follow-up period used for survival analyses ended in May 2016. No patients involved in this investigation received chemotherapy prior to surgery. The clinicopathological characteristics of the patients are summarized in **Table 1**.

Immunohistochemistry

All tumors used for this investigation were reviewed by at least two pathologists to confirm the diagnosis. After the sections were deparaffinized in xylene, they were rehydrated using an alcohol gradient. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature (25°C). The sections were placed in a 95°C solution of 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. The primary antibody used for detecting HoxB9 was a rabbit anti-HoxB9 polyclonal antibody (Epitomics, Burlingame, CA, USA), which was applied overnight at 4°C at a 1:50 dilution. A PV9000 two-step method was used to develop the primary antibody with a polyclonal horseradish peroxidase (HRP)-conjugated antimouse/rabbit immunoglobulin (Ig)G (Zhongshan Jinqiao, Jiaxing, China). Detection was accomplished with a Dako Envision System (Dako, Glostrup, Denmark).

Cell lines, antibodies and reagent

The human OSCC cell lines Cal27 and SCC-25 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. When the cells reached 80-90% confluency, they were passed by dissociation with 0.25% trypsin-EDTA solution (Gibco) for 1-2 min. Chemical reagents for the experiments were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Two different HoxB9 siRNA sequences were purchased from Sigma-Aldrich. Primary antibody against human HoxB9 was purchased from Epitomics (Burlingame, CA, USA). E-cadherin, N-cadherin, vimentin, Snail, α-catenin and Slug were purchased from Cell Signaling Technology (Danvers, MA).

HoxB9 siRNA transfection

To further analyze the role of HoxB9 in OSCC malignancy, Cal27 and SCC-25 cells were trans-

fected with HoxB9 siRNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA). The HoxB9 siRNA sequence used is 5'-CCATTTCTGGGACGCTTAGCA-3', and the non-targeted control sequence is 5'-CTGAGCGTGGCTACTCCTTC-3'. After a 48-h transfection, the cells were harvested for Western blot analysis.

Wound healing assay

Cells were seeded in six-well plates to 100% confluency. After serum starving for 10 h, a wound was induced by scratching the cell cultures with a 5 μl pipette tip. Following three rinses with PBS to remove the detached cells, the adherent cells were cultured in medium without serum. Images of four random fields of each well were captured immediately and again after 3 h and 6 h using a microscope (Nikon Corporation, Tokyo, Japan) at ×10 magnification. The width of the wound at these specific locations was visualized on each plate to quantify the rate of cell migration.

Transwell invasion assays

Transwells (6.5 mm) with polycarbonate membrane inserts with an 8-μm pore (Corning, Albany, NY) were embedded with 120 μg of Matrigel (BD Biosciences, San Jose, CA, USA) and 100 μg of gelatin (Sigma-Aldrich, St Louis, MO, USA) in DMEM. Either Cal27 or SCC-25 cells (1×10^5 per well) in serum-free medium were added into the Matrigel-embedded inserts (the top chambers), and the inserts were placed into chambers containing 10% FBS media. After incubation for 36 h at 37°C, the remaining cells in the upper chamber were carefully removed with a cotton swab, and the cells that had invaded through the Matrigel were stained with hematoxylin, photographed and quantified.

Cell immunofluorescence

Cal27 or SCC-25 cells were seeded onto coverslips at a density of 10^5/mL and cultured in a 6-well plate for 24 h with the indicated treatment. After treatment, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 minutes. Then, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes and blocked with non-immune goat serum for 60 minutes at room temperature. Then, the cells were incubated at 4°C overnight with corresponding primary antibody at dilutions recommended by the manufacturers. After a PBS washout, PerCP-Cy5.5-conjugated secondary antibody (1:200, Jackson ImmunoResearch, USA) was used to detect the proteins, and DAPI was used for counterstaining nuclei. The coverslips were mounted on microscope slides with anti-fade mounting media (Molecular Probes, Carlsbad, CA, USA) and photographed on a fluorescence microscope (Leica).

Western blot analysis

Cal27 and SCC-25 cell lines were transfected with HoxB9 siRNA (100 nM, Sigma-Aldrich) using HiPerFect transfection reagent (Qiagen, Germantown, MD). Then, the cells were lysed, and after the protein concentration was measured by using the bicinchoninic acid (BCA) method, total protein was separated on a 12% gel by using SEMS polyacrylamide gel electrophoresis and transferred onto polyvinyldinefluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with specialized antibodies. Afterwards, the membranes were washed three times, incubated with secondary antibody for 1 h at room temperature and visualized with enhanced chemiluminescence.

Statistical analysis

HoxB9 expression differences among the various subgroups were determined using the Kruskal-Wallis rank sum test. Patient survival was calculated using Kaplan-Meier analysis, and comparisons were made using the log-rank test. Univariate and multivariate Cox analyses were used to examine the significance of other factors related to survival. Student's t-test was used for paired studies. The data were analyzed and visualized using GraphPad Prism 5.0. *, **, and *** indicated $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

HoxB9 expression is upregulated in oral squamous cell carcinoma (OSCC)

We examined HoxB9 expression in 50 oral squamous cell carcinoma tissue samples using

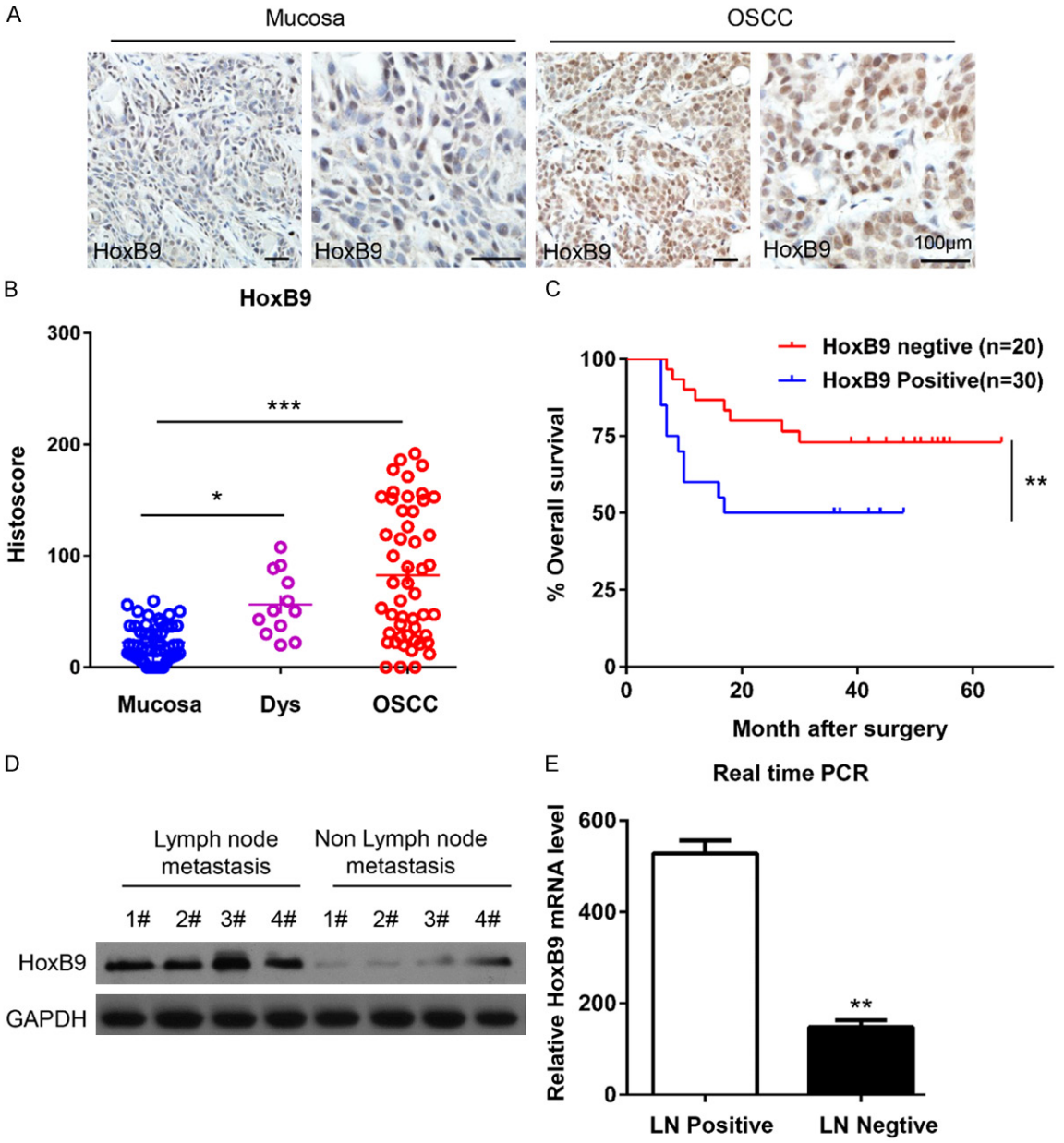

Figure 1. HoxB9 expression was up-regulated in oral squamous cell carcinoma (OSCC). A. Representative immunohistochemical staining of HoxB9 in human OSCC tissue (right panel) compared with normal mucosa (left panel; scale bars = 100 μm). B. Quantification of HoxB9 expression levels in human mucosa, dysplastic tissue and OSCC tissue (*P < 0.05; ***P < 0.001; one-way ANOVA with GraphPad Prism 5.0). C. Kaplan-Meier curve of overall survival of 50 patients with OSCC stratified by the expression level of HoxB9. The duration of survival was measured from the beginning of the treatment to the time of death or at the final follow-up (60 months). The cumulative survival for patients with HoxB9-positive OSCC was significantly lower than that for patients with HoxB9-negative OSCC (**P < 0.01; one-way ANOVA with GraphPad Prism 5.0). D. Western blot analysis of the protein expression of HoxB9 in patients with or without lymph node metastasis, GAPDH was used as a loading control. E. The relative mRNA levels of HoxB9 were detected by RT-PCR in patients with or without lymph node metastasis. The data are presented as the means ± SEM. One-way ANOVA with post-Dunnett analysis was performed using GraphPad Prism 5.0. **P < 0.01 versus the control group (n = 3).

immunohistochemical staining and compared that to paired normal mucosa samples. The results indicated that HoxB9 immunoreactivity was fairly weak in normal mucosa, whereas OSCC samples showed strong protein expression in the tumor cells, especially in the nucleus

Table 2. Association between the patient's clinicopathological characteristics and HoxB9 expression in 50 OSCC patients

Clinicopathological features	No.	HOXB9 Expression Negative (%)	Positive (%)	P
Gender				0.895
Male	37	15	22	
Female	13	5	8	
Age, years				0.904
< 60	32	13	19	
≥ 60	18	7	11	
Smoking				0.405
Yes	31	11	20	
No	19	9	10	
Drinking				0.815
Yes	21	8	13	
No	29	12	17	
Tumor stage				0.239
3-4	20	6	14	
1-2	30	14	16	
Clinical stage				0.028
3-4	27	7	20	
1-2	23	13	10	
Lymph node metastasis				0.006
+	19	3	16	
-	31	17	14	
Histological type				0.523
Poor	4	1	3	
Well-Moderate	46	19	27	

(**Figure 1A**). Through quantification, we found that HoxB9 expression in epithelial dysplasia and OSCC is strongly positive compared to that of normal oral mucosa ($P < 0.05$ and $P < 0.001$, respectively. **Figure 1B**). We plotted the overall survival for HoxB9 using Kaplan-Meier curves. Based on the follow-up data of the 50 OSCC patients, we analyzed whether HoxB9 expression affected overall survival (OS). The Kaplan-Meier survival curves showed that patients with more HoxB9-positive expression had a significantly poorer 5-year OS ($P < 0.01$; **Figure 1C**). What's more, the multivariate Cox regression analyses revealed that HoxB9 expression was an independent prognostic factor for poor OS ($P = 0.003$; **Table 2**). Interestingly, the relationship between HoxB9 expression and the clinicopathological characteristics was statistically analyzed and showed a direct association between HoxB9 expression and lymph node metastasis ($P < 0.001$; **Table 2**). To further explore whether HoxB9 is associated with OSCC progression, we compared HoxB9 expression to the different Grades, T categories and N categories of the HNSCC (Supplementary Figure 3A-C). Unfortunately, no differences were found among the different Grades or T categories. However, the difference between positive lymph node metastasis (N+) and negative lymph node metastasis (N-) reached statistical significance. Protein and mRNA expression levels of HoxB9 were examined in eight fresh tumor samples (four N+ and four N-) by Western blot and real-time PCR, respectively. Both HoxB9 protein levels and mRNA levels in N+ patients were generally much higher than those in N-patients (**Figure 1D** and **1E**).

Knockdown of HoxB9 decreases migration and invasion of OSCC cell lines

To determine whether the activity of HoxB9 is involved in invasion and/or migration of OSCC, we first detected the HoxB9 expression in OSCC cell lines. As shown in **Figure 2A**, HoxB9 expression was up-regulated in OSCC cell lines (Cal27, SCC25, FaDu, SCC4, and SCC23) compared with that in normal keratinocytes (OKC). The Cal27 and SCC25 cell lines were selected because they had the highest content of HoxB9. siRNAs were designed to knock down HoxB9 (**Figure 2B**). We employed wound healing assays to examine the cytological effect of HoxB9 on the migratory ability of OSCC cells and transwell invasion assays to determine the effect of HoxB9 downregulation on malignant progression and metastasis. The results show that knockdown of HoxB9 notably decreased the cell motility of the Cal27 (**Figure 2C**) and SCC-25 (Supplementary Figure 1B) cell lines, and the number of migratory cells was quite different between the respective control groups and the HoxB9 siRNA-treated groups for both the Cal27 (**Figure 2D**) and SCC-25 (Supplementary Figure 1C) cell lines.

HoxB9 regulates the transition between epithelial and mesenchymal phenotypes in OSCC cells

To further confirm the relationship between HoxB9 and EMT progression, we employed

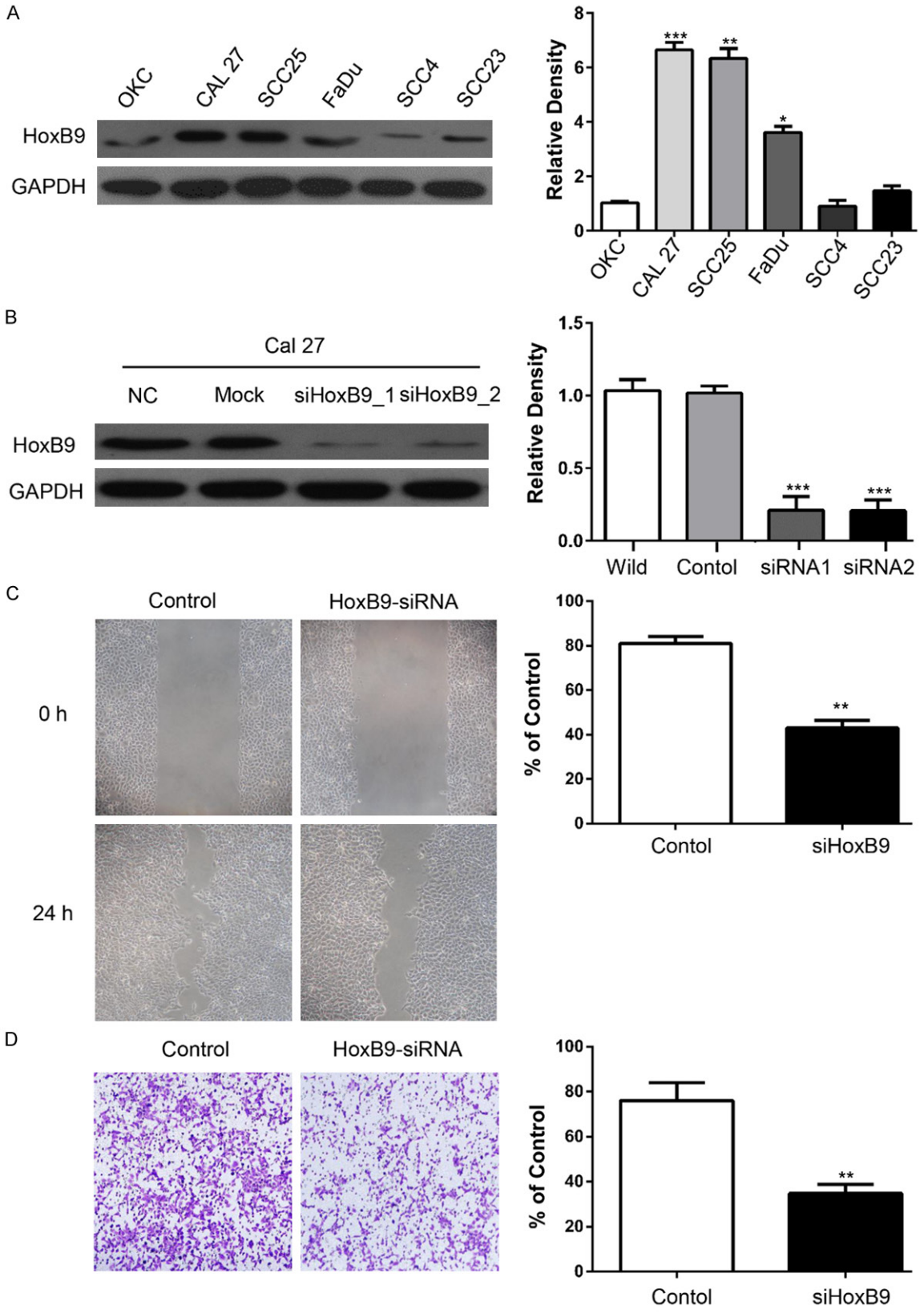

Figure 2. Knockdown of HoxB9 decreases the migration and invasion of OSCC cell lines. A. Western blot analysis was performed to assess the expression levels of HoxB9 in a normal keratinocyte cell line (OKC) and in OSCC cell lines. GAPDH served as a loading control, and the relative densities were calculated by using Image J. The data are

presented as the mean of three independent experiments. **P < 0.01, ***P < 0.001. B. Knockdown of HoxB9 by two different siRNAs in the Cal27 cell line with GAPDH as the loading control. The relative densities were calculated by using Image J, and the data are presented as the mean of three independent experiments. ***P < 0.001. C. Wound healing assay showed that knockdown of HoxB9 suppressed the cell motility of the Cal27 cell line, and quantification of the wound closure shows that the difference is statistically significant (mean ± SD; **P < 0.01, Student's *t*-test with GraphPad Prism 5.0); D. Transwell assay showed that the migration abilities of Cal27 cells were impaired after knocking down HoxB9 compared with those of the control group. Quantification of the cell numbers was performed by using the Image J "cell counter" module (mean ± SEM; **P < 0.01, Student's t-test with GraphPad Prism 5.0 (n = 3).

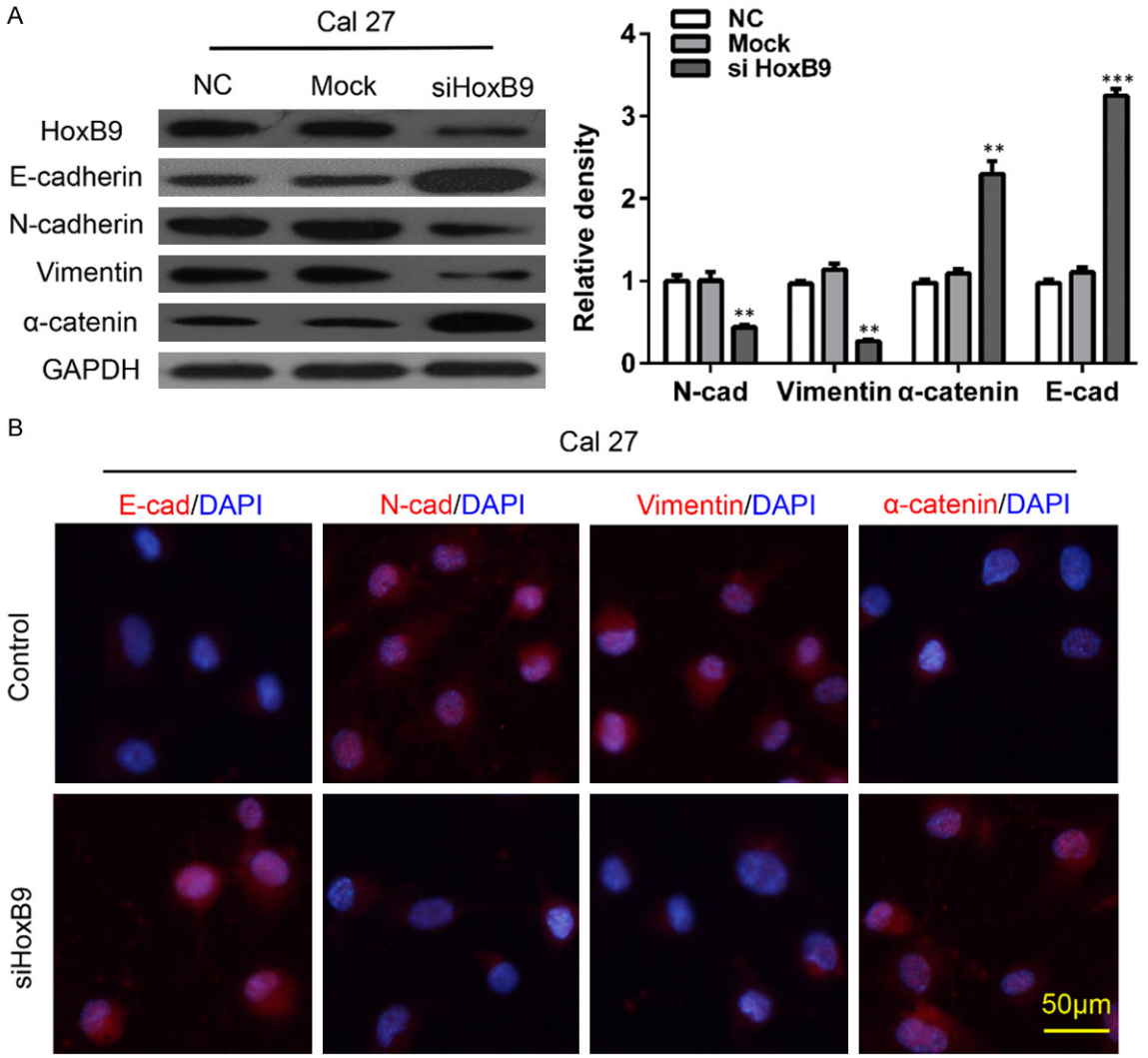

Figure 3. HoxB9 regulates the transition between epithelial and mesenchymal phenotypes in OSCC cells. A. Cal27 cells were treated with siRNA targeting HoxB9, and the E-cadherin, N-cadherin, vimentin and α-catenin levels were determined. GAPDH was used as an internal standard for protein loading. The values are presented as the means ± SEM. One-way ANOVA with post-Dunnett analysis was performed using GraphPad Prism 5.0. **P < 0.01 versus the control group (n = 3). B. Cal27 cells were treated with siRNA targeting HoxB9, and the representative immunofluorescence levels of E-cadherin, N-cadherin, vimentin and α-catenin were determined (Scale bars = 50 μm).

siRNA to knock down HoxB9 and detect alterations of the EMT as indicated by detecting putative EMT markers *in vitro* via Western blotting. After knockdown of HoxB9, the expression levels of E-cadherin and α-catenin were up-regulated, while N-cadherin and vimentin were downregulated in each of the HoxB9-silenced groups compared with the negative control

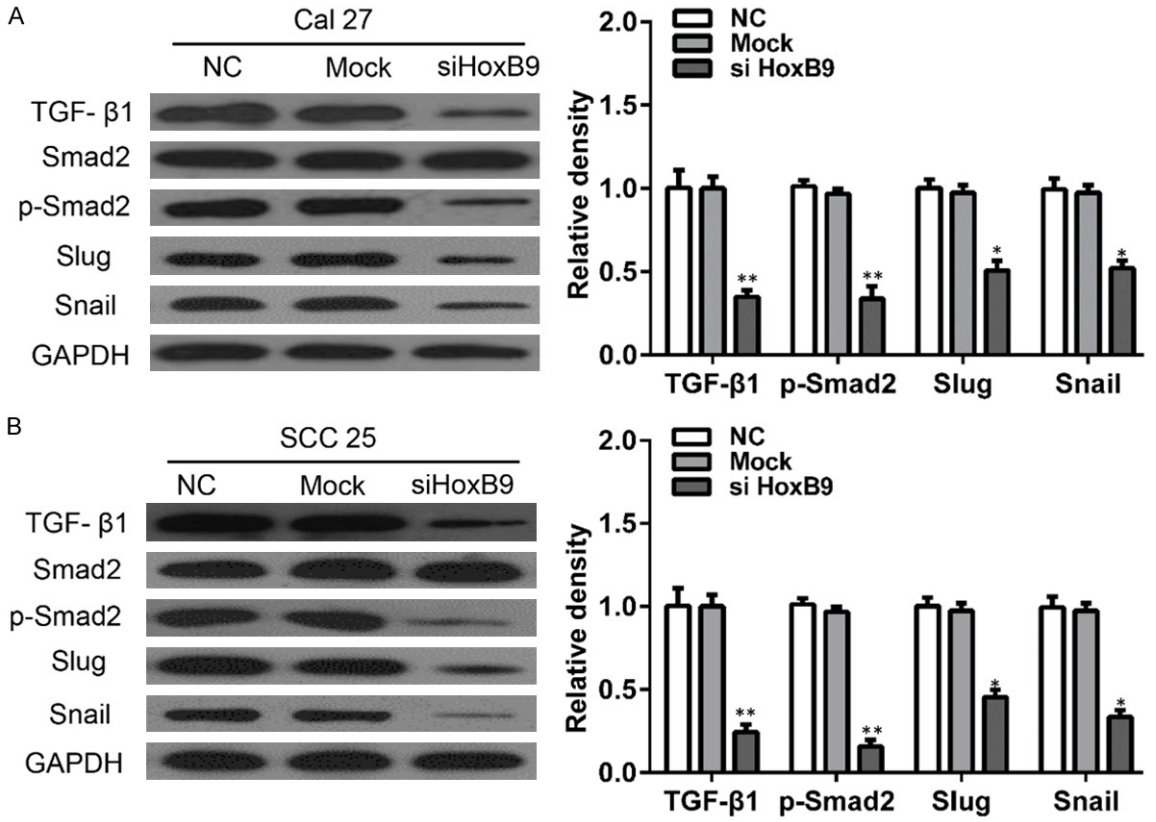

Figure 4. HoxB9 regulates TGF-β1 expression in OSCC cells. A. Cal27 cells were treated with siRNA targeting HoxB9, and the TGF-β1, Smad2, p-Smad2, Slug and Snail levels were determined. GAPDH was used as an internal standard for protein loading. B. SCC-25 cells were treated with siRNA targeting HoxB9, and the TGF-β1, Smad2, p-Smad2, Slug and Snail levels were determined. GAPDH was used as an internal standard for protein loading. The values are presented as the means ± SEM. One-way ANOVA with post-Dunnett analysis was performed using GraphPad Prism 5.0. *P < 0.05, **P < 0.01 versus the control group (n = 3).

groups (**Figure 3A** and Supplementary Figure 2A). Furthermore, we detected the morphological expression of the cell lines by using immunofluorescence. Similar results were observed in SCC-25 cell lines: vimentin and N-cadherin were decreased while E-cadherin and α-catenin were increased after knockdown of HoxB9 (**Figure 3B** and Supplementary Figure 2B). These results suggested that HoxB9 may play a role in the EMT of OSCC.

HoxB9s regulates the migration of OSCC cells by targeting EMT via the TGF-β1/Smad2/Slug signaling pathway

To further confirm the relationship between HoxB9 and EMT progression, we employed siRNA to knock down the HoxB9 and detect alterations of the EMT as indicated by detecting putative EMT markers *in vitro* via Western blotting. The members of multifunctional cytokine TGF-β1 superfamily exert important functions that control cell proliferation, apoptosis, differentiation, and aging. TGF-β1 is the most widely used inducer of EMT for *in vitro* studies and acts by inducing EMT via a Smad2-dependent pathway. Thus, we explored the possibility that TGF-β1 acts downstream of HoxB9 to control EMT in OSCC cells. Knockdown of HoxB9 in Cal27 cells significantly reduced TGF-β1 protein expression (**Figure 4A**), which was consistent with the results in SCC-25 cells (**Figure 4B**). To investigate the involvement of TGF-β1-dependent signaling in HoxB9-induced EMT, we examined the expression of phospho-Smad2. Consistent with the observed TGF-β1 activation, phospho-Smad2 expression was downregulated in Cal27 and SCC-25 cells with HoxB9 knockdown. Previous studies have shown that TGF-β1 promotes invasion by inducing EMT via induction of transcriptional repressors such as Slug and Snail [14, 15]. Thus, we

measured the expression of these transcriptional repressors in OSCC cells. As shown in **Figure 4A**, Hox9B knockdown significantly suppressed Slug and Snail expression levels. These results suggested that HoxB9 regulates the migration of OSCC cells by targeting EMT via the TGF-β1/Smad2/Slug signaling pathway.

Discussion

EMT is frequently observed at the invasive front of advanced tumors and is significantly correlated with metastasis in tumor progression [12, 13]. What's more, as a tumor-associated antigen, HoxB9 is also associated with cancer progression [16-18]. In this study, we used immunohistochemical staining to demonstrate that HoxB9 is over-expressed in OSCC and that high levels of HoxB9 are significantly associated with shorter overall survival in patients with OSCC. Furthermore, *in vitro* functional studies suggested that knockdown of HoxB9 in OSCC cells decreases cell migration and invasion. Additionally, HoxB9 may promote OSCC EMT by activating the TGF-β1/Smad2/Slug signaling pathway.

HoxB9 has been reported to be mediated via ERK5 signaling and BMI1 [19] and is an important prognostic factor for many cancers [9, 20, 21]. Silencing HoxB9 is associated with downregulation of CD56 and extrathyroidal extension of papillary thyroid carcinoma tumors [22] as well as the induction of tumor invasion and metastasis of breast and lung cancers [21]. Recently, a report showed that decreased expression of HoxB9 is related to poor overall survival in patients with gastric carcinoma, identifying an opposing role of HoxB9 in cancer [23], which suggested that HoxB9 may play diverse roles during cancer progression under various circumstances. In our study, we demonstrated that HoxB9 was over-expressed in OSCC and that high levels of HoxB9 were significantly associated with shorter overall survival in patients with OSCC.

The EMT is a key event for cancerous cells to acquire the capabilities of migration and invasion [24, 25]. These processes are temporally and spatially regulated in a strict manner by the expression and activation of many signaling molecules [26-28]. Recent reports have suggested that Slug is the key molecule regulating EMT in cancer [29, 30], and a number of studies reported that Slug-mediated epithelial-mesenchymal transition plays an important role in metastasis and apoptosis [31-33]. In addition, HoxB9 upregulation of cell-cell adhesion proteins including E-cadherin, Claudin-1, ZO-1 and Occludin has been identified in colon cancer [34]. Furthermore, HoxB9 was also found to induce EMT in breast cancer by activating the Wnt signaling pathway [7]. These results suggested that HoxB9 was associated with the EMT process. To better elucidate the invasive and metastatic mechanisms of HoxB9, the effect of HoxB9 knockdown on the EMT was investigated. The present study revealed that HoxB9 indeed plays a role in promoting EMT in OSCC, which may be related with the TGF-β1/Smad2/Slug signaling pathway.

Transforming growth factor-β1 (TGF-β1) is one of the critical growth factors that regulate tumor invasion and metastasis through the epithelial-mesenchymal transition (EMT) [35, 36]. Dysregulation of TGF-β1 had been known to contribute to the progression of pancreatic cancer [37]; however, the molecular mechanism is not fully elucidated. In the present study, we observed that TGF-β1 levels were decreased after HoxB9 knockdown. To investigate the involvement of TGF-β1-dependent signaling in HoxB9-induced EMT, we examined the expression of phospho-Smad2, Consistent with the changes in TGF-β1 activation, phospho-Smad2 expression was downregulated in Cal27 and SCC-25 cells with HoxB9 knockdown.

Of note, HoxB9 expression levels were not correlated with the OSCC sequence in the TNM classifications. The reason could be the inadequate number of patients included in the cohort. Therefore, the relationship between HoxB9 expression levels and the progression of OSCC remains to be elucidated using a larger patient cohort in future studies.

In conclusion, this study confirmed that HoxB9 is over-expressed in OSCC and that high levels of HoxB9 are significantly associated with shorter overall survival in patients with OSCC. Knockdown of HoxB9 inhibits the EMT process via the TGF-β1/Smad2/Slug signaling pathway, which plays an important role in this process. This finding supports the possibility of HoxB9 and/or its associated molecules as targets for anti-metastatic therapy against OSCC.

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Disclosure of conflict of interest

None.

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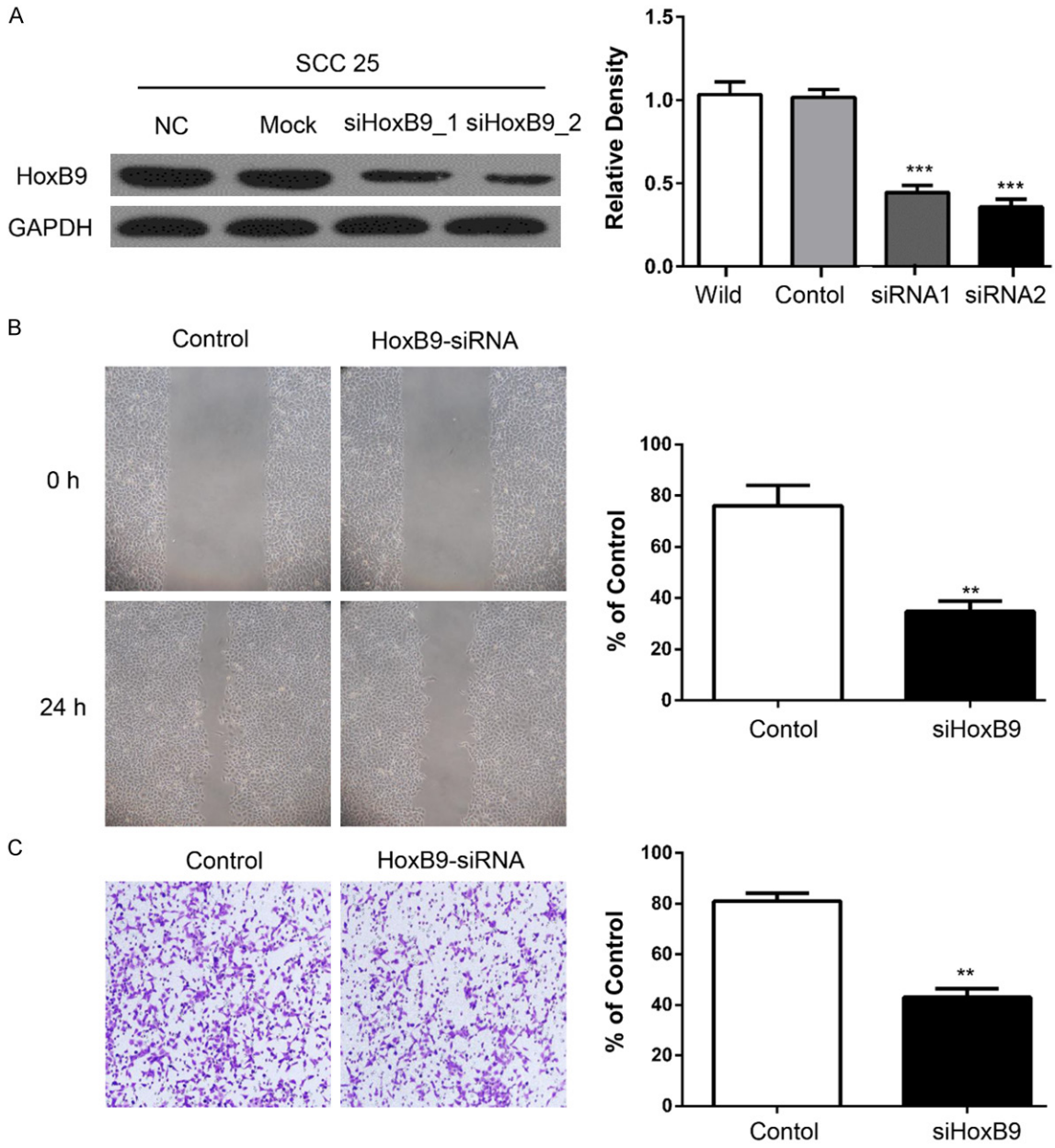
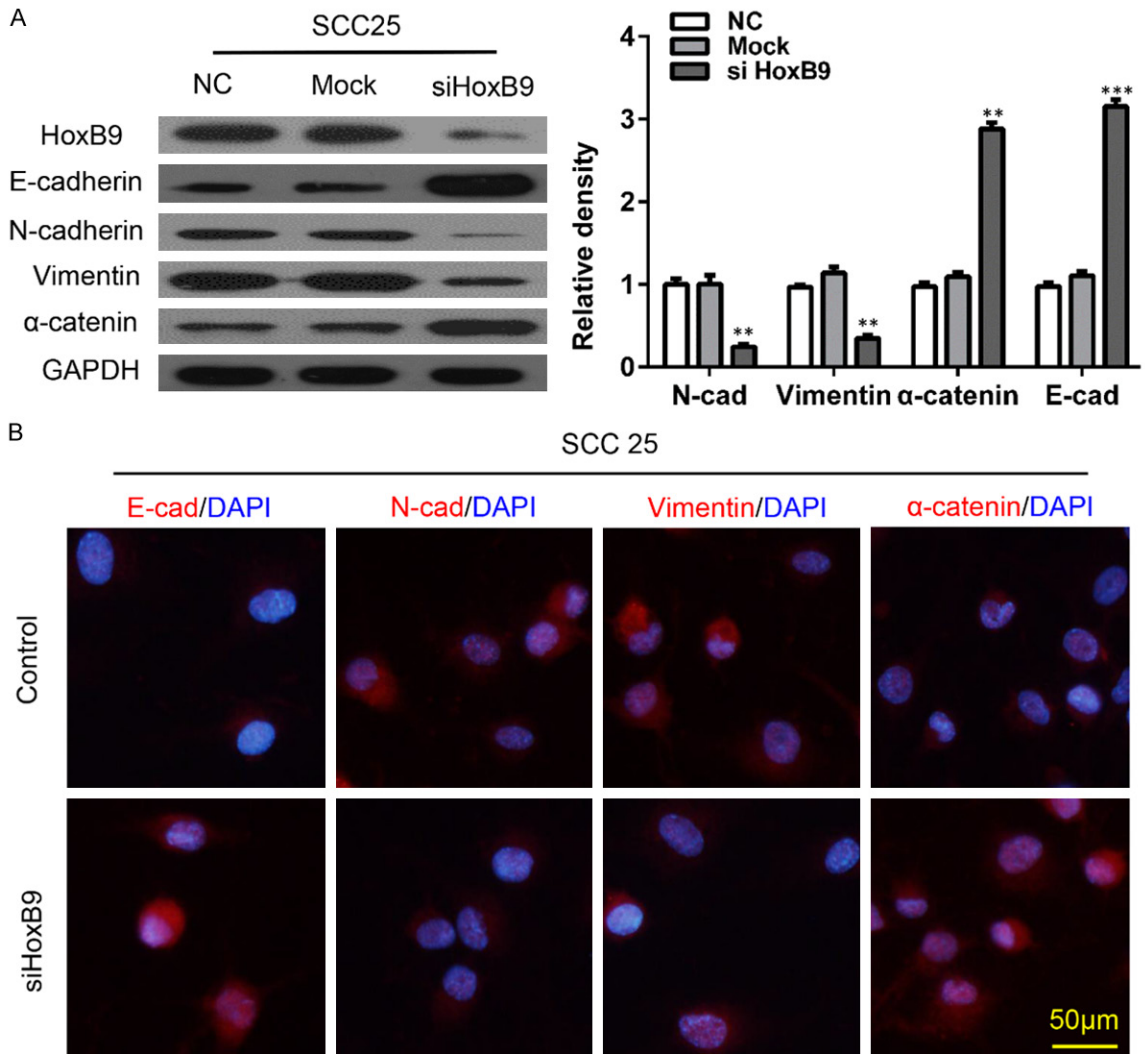
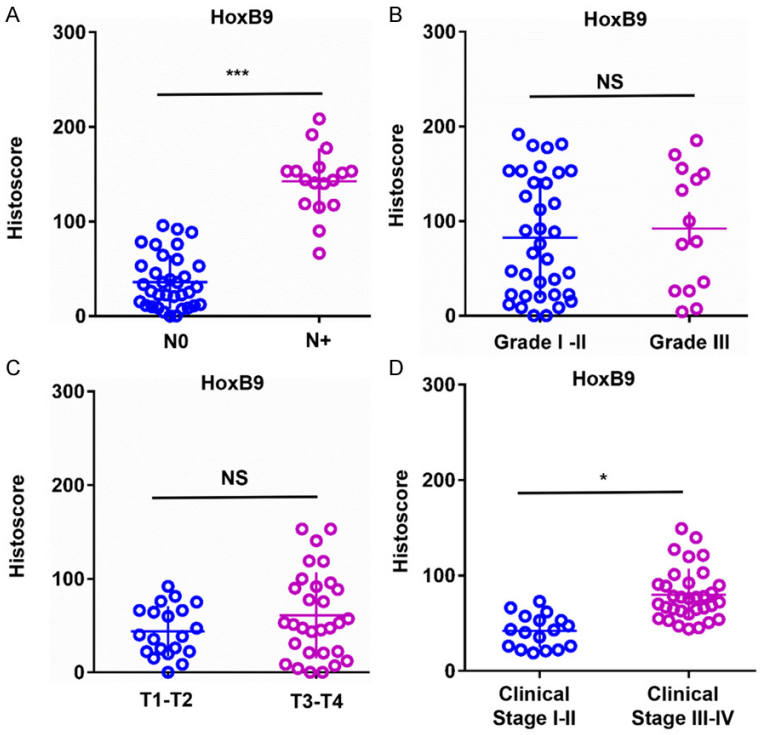


Supplementary Figure 1. Knockdown of HoxB9 decreases migration and invasion of SCC-25. A. Knockdown of HoxB9 by two different siRNA in SCC-25 cell line, GAPDH served as a loading control; Relative density data were calculated by Image J, and the data represented mean of three independent experiments. ***P < 0.001; B. Wound healing assay showed knockdown of HoxB9 suppressed the cell mobility of SCC-25 cell line, and quantification of wound closure shows the statistical significance of the difference (Mean ± SD; **P < 0.01, student t-test with GraphPad Prism 5.0); C. Transwell assay showed the migration abilities of SCC-25 were impaired after knocking down of HoxB9 compared with those of control group, and quantification of cell numbers with Image J "cell counter" module (Mean ± SEM; **P < 0.01, student t-test with GraphPad Prism 5.0 (n = 3).



Supplementary Figure 2. HoxB9 regulates the transition between epithelial and mesenchymal phenotypes in SCC-25 cells. A. SCC-25 cells were treated with siRNA for HoxB9, then the E-cadherin, N-cadherin, Vimentin and α-catenin levels were determined. GAPDH was the internal standard for protein loading. The values are presented as the means ± SEM. One-way ANOVA with post-Dunnett analysis was performed using GraphPad Prism 5.0. **P < 0.01, versus the control group. (n = 3); B. SCC-25 cells were treated with siRNA for HoxB9, the representative immunofluorescence of E-cadherin, N-cadherin, Vimentin and α-catenin were determined (Scale bars = 50 μm).



Supplementary Figure 3. HoxB9 expression in different Grades, T categories and N categories of OSCC. A. HoxB9 expression was correlated with lymph node metastasis of OSCC; B. HoxB9 expression was not correlated with different pathological grades (I-III) of OSCC; C. HoxB9 expression was not correlated with T category of OSCC; D. HoxB9 expression was correlated with clinical stages of OSCC (Mean ± SEM; *P < 0.05, ***P < 0.001, t test).