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Homeobox B9 is overexpressed in hepatocellular carcinomas and promotes tumor cell proliferation both in vitro and in vivo

Fangyi Li [a,1], Lei Dong [b,1,*], Rong Xing [c], Li Wang [a], Fengming Luan [a], Chenhui Yao [a], Xuening Ji [d], Lizhi Bai [e,*]

[a] Department of General Surgery, Dalian Municipal Friendship Hospital, No. 8 Sanba Square, Zhongshan District, Dalian 116001, China
[b] Department of Laparoscopic Surgery, First Affiliated Hospital of Dalian Medical University, No. 193 Lianhe Street, Shahekou District, Dalian 116001, China
[c] Department of Pathology and Pathophysiology, Dalian Medical University, No. 9 Lvshunnan Road, Lvshunkou District, Dalian 116044, China
[d] Department of Oncology, Zhongshan Hospital of Dalian University, No. 6 Jiefang Street, Zhongshan District, Dalian 116001, China
[e] Department of Emergency, Zhongshan Hospital of Dalian University, No. 6 Jiefang Street, Zhongshan District, Dalian 116001, China

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ABSTRACT

HomeoboxB9 (HOXB9), a nontransforming transcription factor that is overexpressed in multiple tumor types, alters tumor cell fate and promotes tumor progression. However, the role of HOXB9 in hepatocellular carcinoma (HCC) development has not been well studied. In this paper, we found that HOXB9 is overexpressed in human HCC samples. We investigated HOXB9 expression and its prognostic value for HCC. HCC surgical tissue samples were taken from 89 HCC patients. HOXB9 overexpression was observed in 65.2% of the cases, and the survival analysis showed that the HOXB9 overexpression group had significantly shorter overall survival time than the HOXB9 downexpression group. The ectopic expression of HOXB9 stimulated the proliferation of HCC cells; whereas the knockdown of HOXB9 produced an opposite effect. HOXB9 also modulated the tumorigenicity of HCC cells in vivo. Moreover, we found that the activation of TGF-β1 contributes to HOXB9-induced proliferation activities. The results provide the first evidence that HOXB9 is a critical regulator of tumor growth factor in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a highly lethal cancer whose prognosis is typically poor. HCC ranks third as the cause of cancer deaths in East Asia and sub-Saharan Africa [1,2] and second as the cause of male cancer deaths in China [3]. To date, the incidence of HCC is increasing in the United States and Europe [4,5]. HCC has poor prognosis, with its five-year survival rate as low as 25% to 39% after surgery [6]. The majority of HCC patients (>80%) present with an advanced stage for which chemotherapy and radiotherapy have limited efficacy [7]. Even for patients with their tumor resected, the recurrence rate can be as high as 50% at two years. Several clinicopathological parameters, including poorly differentiated phenotype, portal venous invasion, and intrahepatic metastasis, have been found to contribute to the poor prognosis of HCC. However, the mechanisms underlying the development of HCC remain unclear.

The gene amplification and consequent overexpression of many oncogenes have been frequently detectedin various types of solid tumors, including HCC [8–11]. These oncogenes may have an important role in tumor pathogenesis, probably because the overexpression of these oncogenes confers a growth advantage.HOXB9 is part of the HOX genes family cluster [12,13]. HOXB9 is involved in cell proliferation and differentiation and is also critical for embryonic segmentation [14]. Recent studies have demonstrated the implication of HOXB9 in tumorigenesis[15,16]. Several studies have suggested that HOXB9 protein is upregulated in breast tumors by altering the microenvironment, induces several tumorigenic phenotypes, and promotes disease progression [17–19]. HOXB9 expression has also been reported in lung cancer cell lines [13]. Changes in HOXB9 potentially exert a widely pleiotropic effect that alters cellular functions, including proliferation, differentiation, and apoptosis [20]. Thus, HOXB9 may act as an oncogene; however, whether HOXB9 has a role in HCC development and metastasis remains unknown.

Although the expression of HOXB9 has been studied in some cancers, little is known about its expression in HCC and its function in tumorigenesis. In this study, we investigated the roles of HOXB9 in HCC genesis. We showed that the HOXB9 expression was elevated in majority of the human HCC tissues examined. In human HCC cells, the overexpression of HOXB9 significantly enhanced cell proliferation in vitro. Consistently, HOXB9 overexpression also increased the tumorigenicity of HCC cells in nude

* Corresponding authors. Fax: +86 411 83635963 (L. Dong), +86 411 82108116 (L. Bai).
E-mail addresses: dlleidong@126.com (L. Dong), dllizhibai@126.com (L. Bai).
[1] These author contributed equally to this work.

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mouse xenograft. HOXB9alsoincreased the protein levels of TGF-β1 and its downstream target protein p-Smad2. HOXB9 knockdown by short hairpin RNA inhibited HCC cell proliferation both in vitro and in vivo. Collectively, these results indicate a pivotal role of HOXB9 in HCC cell proliferation.

2. Materials and methods

2.1. Samples, cells, and antibodies

Human normal liver tissue samples and HCC tissue samples were provided by the department of hepatobiliary surgery, the first affiliated hospital of Dalian medical University. All experiments were approved by the ethics committee of Dalian medical University and informed consent was obtained from all patients prior to specimen collection. Human HCC cell lines (BEL-7402, BEL-7404, BEL-7405, HepG2, Hep3B, and SNU475) and normal liver cell line THLE-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Minimum Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Mouse monoclonal HOXB9 (ab65063), p-Smad2 (ab53100), Smad2 (ab40855), and TGF-β1 (ab27969) antibodies were purchased from Abcam. Mouse monoclonal β-actin antibody (sc-47778) was product of Santa Cruz Biotech (Santa Cruz, CA).

2.2. qRT-PCR

Total RNA was extracted using Trizol reagent and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system. The primers used in the study were: HOXB9: sense 5′-CCGGCTACGGGGACAATAA-3″ andantisense 5′-GGTGTAGGGACAGCGCTTTTT-3′; GAPDH: sense5′-TGCCTC CTGCACCACCAACT-3′ and antisense 5′-CCCGTTCAGCTCAGGGATGA-3′.

2.3. Western blot

Samples and Cells were solubilized in radio immunoprecipitation assay lysis buffer [50 mmol/L Tris–HCl (pH 7.4), 1% NP40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonylfluoride, 1 mg/mL each of aprotinin, leupeptin, and pepstatin, 1 mmol/L Na_3VO_4, 1 mmol/L NaF]. The supernatants, which contained the whole-cell protein extracts, were obtained after centrifugation of the cell lysates at 10,000 g for 10 min at 4 °C. Twenty-microgramprotein samples were loaded on a sodium dodecylsulfate-PAGE gel (5% stacking gel and 12% separatinggel). The proteins were then transferred to polyvinylidenedifluoride membranes (Millipore, Bedford, MA). The membranes were first probed with a primary antibody and then with secondary antibody. The bound antibody was detected by enhanced chemiluminescence detection reagents (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions. The band intensity was quantitated with the use of Image Quant software (Molecular Dynamics, Sunnyvale, CA).

2.4. Plasmids construct

Human cDNA of HOXB9 was cloned as previously reported [17]. The full-length cDNAs were subcloned into the multiple cloning sites of the pBabe plasmid, forming the pBabeHOXB9 expression plasmids. Short hair-pinRNA (shRNA) targeting HOXB9 (sense: 5′-TCGACGCGAATCTCTCTTTGGCAAGTTCAAGAGACTTGCCAAAGAGAG ATTCGTTTTTTGGAAT-3′; antisense: 5′-CTAGATTCCAAAAAACGAAT CTCTCTTTGGCAAGTCTCTTGAACTTGCCAAAGAGAGATTCGCG-3′) was initially inserted into the Sal I and Xba I sites of pSuper plasmid, forming the pSuper shHOXB9 plasmids.

2.5. Generation of stable cell lines

BEL-4705 cell line was transfected with the pBabe orpBabe-HOXB9 plasmid using the Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). HepG2 cell line was transfected with the pSuper or pSuper-shHOXB9 plasmid using the Lipofectamine 2000. Stable transfectants were obtained after selectionby puromycin (Invitrogen; 10 μg/mL) for 2 weeks. Expression of HOXB9 mRNA and protein in stable cell lines were analyzed by qRT-PCR and Western blot, respectively.

2.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was carried out to determine the effect of overexpression or knockdown of HOXB9 in HCC cell proliferation. Five thousand cells were seeded into a 96-well plate. The cells were cultured in 100 μL growth medium. At various time points, 20 μL of sterile MTT dye (5 mg/mL; Sigma, St. Louis, MO) were added, followed by incubation at 37 °C for 4 h. The MTT solution was then replaced with DMSO (200 μL) and thoroughly mixed for 30 min. Spectrometric absorbance at 570 nm was measured by using a microplatereader (Spectra Max 340, Molecular Devices, Sunnyvale, CA) with a background subtraction at 660 nm.

2.7. Immunohistochemistry staining and semiquantitative analysis

Paraffin-embedded sections were deparaffinized, blocked, and incubated with 1:200 anti-HOXB9 antibody at 4 °C overnight. Horse-radish peroxidase-conjugated secondary antibody (1:500) was then added and further incubated for 1hat room temperature. The sections were developed using a 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (ThermoScien-tific, MA) at room temperature for 1–5 min and then counterstained with hematoxylin. Two independent, blinded investigators examined all tumor slides randomly. Five views were examined per slide, and 100 cells were observed per view at 400× magnification. Scores for HOXB9 cytoplasmic staining were calculated based on staining intensity (0, below the level of detection; 1, weak; 2, moderate; and 3, strong)and the percentage of cells staining at each intensity level (0–100%). The final score was calculated by multiplying the intensity score by the percentage, producing a scoring range of 0–300. The immunohistochemistry score cut-off point was established as 69 using X-tile software program (version 3.6.3, Yale University School of Medicine, CT USA).

2.8. Xenografted tumor model in vivo

Nude mice were purchased from the Shanghai Slac Laboratory Animal Co., Ltd. and maintained in microisolator cages. All animals were used in accordance with institutional guidelines and the current experiments were approved by the Use Committee for Animal Care. For subcutaneous inoculation, different numbers of tumor cells were resuspended in PBS medium with 50% Matrigel and inoculated subcutaneously into the 8-week-old nude mice. The tumors were measured weekly and the tumor volume was calculated according to the formula length × width^2/2. The mice were killed 42 days after the inoculation.

2.9. Statistical analysis

SPSS version 11.5 for Windows was used for all analyses. The χ^2 test was used to examine possible correlations between HOXB9

expression and clinicopathologic factors. The *t* test was used to compare data from the densitometry analysis of foci numbers. The Kaplan–Meier method was used to estimate the probability of patient survival. *P* value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. HOXB9 is overexpressed in human HCC samples

HOXB9 expression was determined by IHC in 89 surgical specimens of HCC and 32 normal liver tissues. A total of 58 HCC patients (65.2%) exhibited an overexpression of HOXB9 higher than that in normal liver tissue. The HOXB9 protein appeared to be expressed in both cytoplasmic and nuclear components of tumor cells with stronger signal seen in the cytoplasm (Fig. 1). Meanwhile, the normal liver tissues exhibited negative or low HOXB9 staining (Fig. 1). To evaluate the prognostic value of HOXB9 expression in HCC, we divided the HCC patients into HOXB9 high and low expression groups on the basis of a cutoff score of 69. Survival analysis reveals that HCC patients with high HOXB9 expression had poorer overall survival compared with patients with low HOXB9 expression ($P < 0.01$; Fig. 1J). We analyzed the relationship between HOXB9 staining and clinicopathological characteristics.

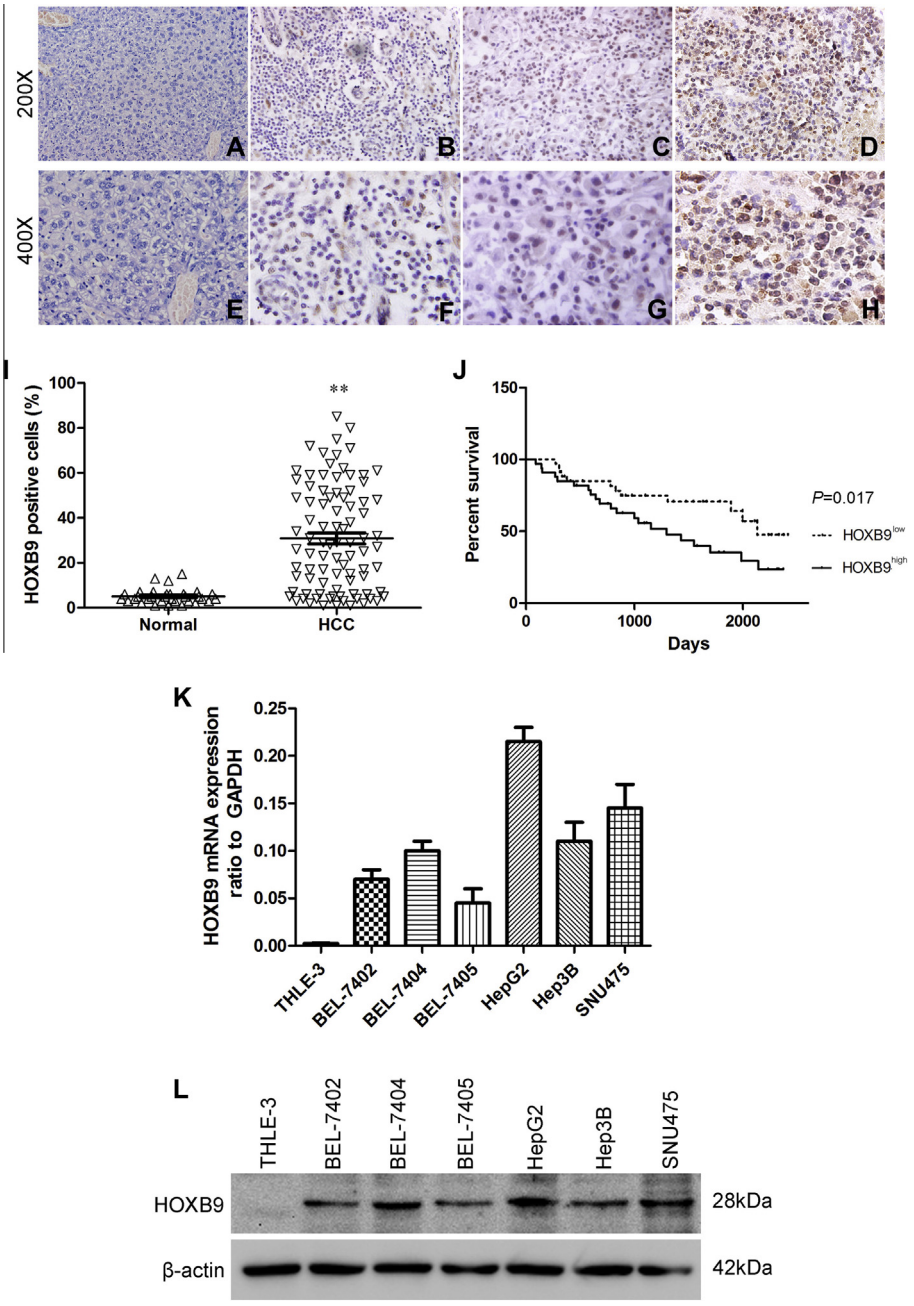

Fig. 1. Expressions of HOXB9 tissues and cell lines. (a × 200; e × 400) HOXB9 negative expression in normal liver tissue. (b × 200; f × 400) HOXB9 weak expression in HCC. (c × 200; g × 400) HOXB9 moderate expression in HCC. (d × 200; h × 400) HOXB9 strong expression in HCC. (i) Percentage of HOXB9 positive cells in normal liver tissues, and HCC tissues were assayed. (j) Kaplan–Meier survival analysis of primary HCC patients ($n = 89$) after surgical resection with high HOXB9 expression ($n = 58$) and low HOXB9 expression ($n = 31$). The survival rate for patients in the HOXB9-high group was significantly lower than that for patients in the HOXB9-low group (log rank, $P = 0.017$).Expression analysis of HOXB9 mRNA and protein in normal hepatocyte line (THLE-3) and HCC cell lines (BEL-7402, BEL-7404, BEL-7405, HepG2, Hep3B, and SNU475) by qRT-PCR (k) and Western blot (l).

HOXB9 expression was not correlated with sex, age, histological differentiation, metastasis, recurrence, serum AFP, or HBsAg status (Supplementarl Table 1). However, the results showed a statistical correlation of HOXB9 expression with HCC tumor size (Supplemental Table 1). These data showed that HOXB9 is the overexpression in HCC and indicated that HOXB9 expression is a prognostic predictor of poor clinical outcome in HCC patients.

We measured the HOXB9 mRNA and protein expression levels in six HCC cell lines (BEL-7402, BEL-7404, BEL-7405, HepG2, Hep3B, and SNU475) and one normal liver cell line, THLE-3, by qRT-PCR and Western blot, respectively (Fig. 1K and L). The results show that high levels of HOXB9 were expressed in nearly all tumor cell lines compared with the THLE-3 cell.

3.2. HOXB9 promotes HCC cell proliferation

To characterize the functional role of HOXB9 in HCC, we established HCC cell clone that stably expressed the overexpression of HOXB9 protein and then studied the effect of the overexpression on cell proliferation. To date, the HOXB9 expression plasmid pBabe-HOXB9 was first transfected into the BEL-7405 cell line. The BEL-7405 cell line was chosen for this study because it has the lowest expression of HOXB9 in the six HCC cell lines. After selection with puromycin, the expression of HOXB9 was assayed by qRT-PCR (Fig. 2A) and Western blot (Fig. 2B). A high level of HOXB9 was expressed in BEL-7405-pBabe-HOXB9 cell, whereas HOXB9 was less expressed in the control cells transfected with empty pBabe plasmid (BEL-7405-pBabe) cells. Compared with the vector-only control, the BEL-4705-pBabe-HOXB9 cell had significant increase in cell proliferation by MTT assay (Fig. 2C) and generated more numbers and larger colonies (Fig. 2D). We also retrovirally established the silencing of HOXB9 in the overexpression of HOXB9 cell line, HepG2 (designated as HepG2-shHOXB9) (Fig. 2E and F). By contrast, silencing of HOXB9 in HepG2 cell significantly reduces cell proliferation (Fig. 2G) and clonogenicity (Fig. 2H). These results suggest that HOXB9 is an important regulator of proliferation in HCC cells.

3.3. HOXB9 promotes tumorigenesis in vivo

To extend our in vitro observations, we investigated whether HOXB9 can regulate the tumorigenic capacity of HCC cells

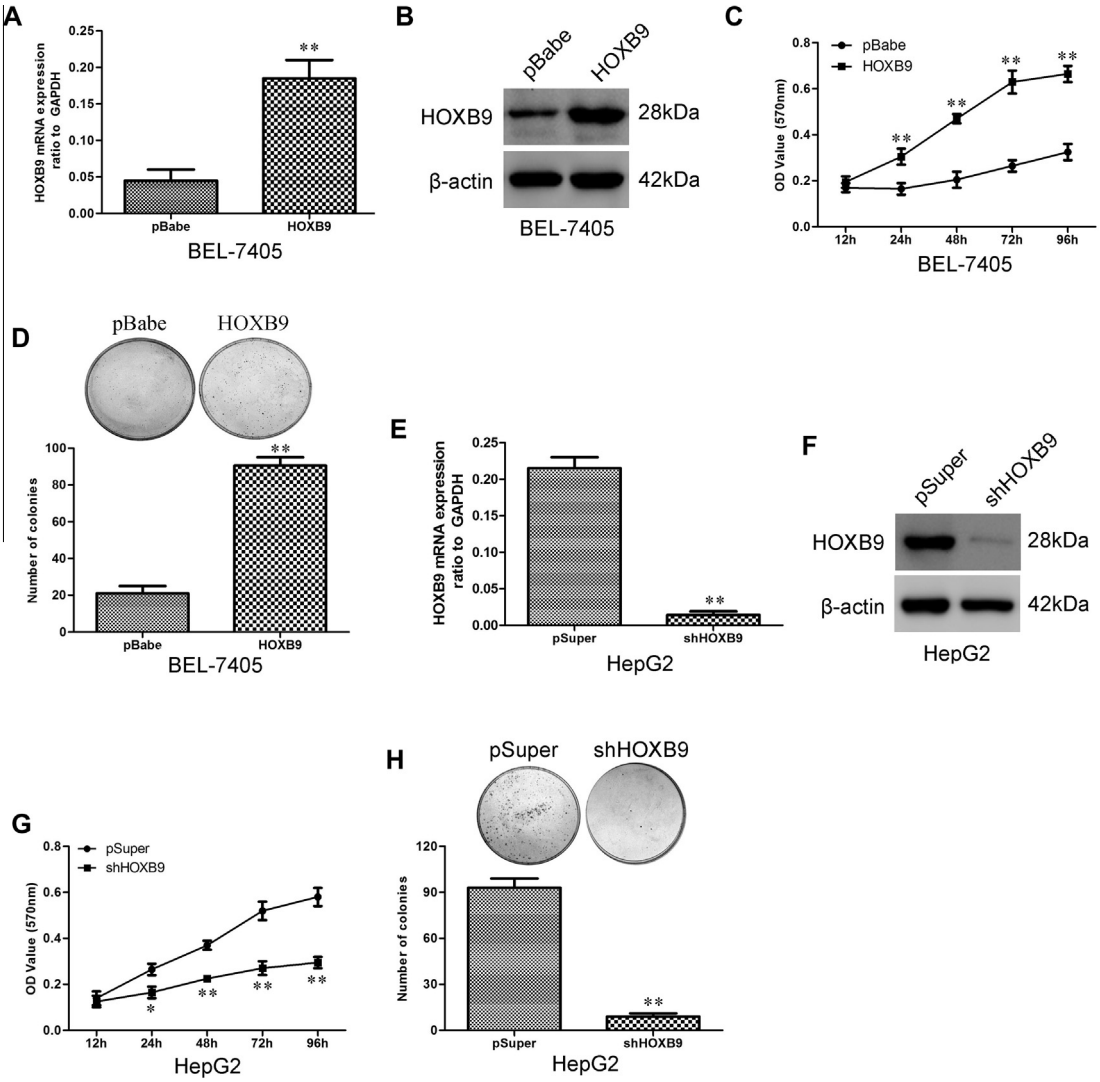

Fig. 2. Expression of HOXB9 modulates HCC cells proliferation. Ectopic expression of HOXB9 in BEL-4705 cell was established by retroviral transduction. The levels of HOXB9 in these resultant cell lines were verified by qRT-PCR (A) and Western blot (B). Cell in vitro proliferation was examined by MTT (C) and clone formation (D). The error bar indicates standard deviation. Silencing of HOXB9 expression by shRNA in HepG2 cell was established by retroviral transduction. The levels of HOXB9 in these resultant cell lines were verified by qRT-PCR (E) and Western blot (F). Cell in vitro proliferation was examined by MTT (G) and clone formation (H). $^{**}P < 0.01$ versus control cells. All results are from three independent experiments. The error bar indicates standard deviation.

in vivo.BEL-7405-pBabe-HOXB9, HepG2-shHOXB9, and their corresponding control cells were subcutaneously injected into nude mice. Tumor size was measured every week up to 42 days. As expected, the tumors from BEL-7405-pBabe-HOXB9 cells grew more rapidly at the implantation site than the control cells did (Fig. 3A–C). By contrast, silencing HOXB9 in the HepG2 cells led to a significant decrease in tumor volume and weight (Fig. 3A–C). The HOXB9 expression in xenograft was confirmed by immunohistochemical analysis (Fig. 3D). These results indicate that HOXB9 can regulate the tumorigenic capacity of HCC cells in vivo.

3.4. Activation of TGF-β1 contributes to HOXB9-induced oncogenic activities

Previous studies have shown that TGF-β1 is induced in HOXB9-expressing breast cancer cells [16]. Thus, we explored whether TGF-β1 is induced by HOXB9 in HCC cells. As shown in Fig. 4A, the HOXB9 overexpression in BEL-7405 cells significantly increased the expression of TGF-β1 and upregulated its downstream protein p-Smad2. On the contrary, the knockdown HOXB9 in HepG2 cells significantly decreased the expression of TGF-β1 and p-Smad2 (Fig. 4B). Treatment with LY364947, an inhibitor of TGF-β1 receptor signaling, significantly reduced the proliferation and clone formation property of BEL-7405-pBabe-HOXB9 cells (Fig. 4C and D). This finding indicates that the induction of TGF-β1 expression by HOXB9 activates a critical signaling pathways implicated in HCC formation.

4. Discussion

The role of HOXB9 in HCC was characterized in this study. Elevated levels of HOXB9 mRNA and protein were detected in majority of the HCC cell lines and HCC tissues examined compared with normal liver cells and normal liver tissues. HOXB9 overexpression was found to enhance cell proliferation and clone formation and accelerate tumor growth in nude mice in vivo. We also found that the knockdown of HOXB9 in HCC cells significantly inhibit proliferation, clone formation, and tumor growth in nude mice in vivo. These properties of HOXB9 were partly related to TGF-β1. Collectively, these findings suggest for the first time that HOXB9 is involved in HCC genesis.

HOXB9 is included in a cluster of Homeobox genes, and the encoded protein functions as a sequence-specific transcription factor [14]. Previous results have shown that HOXB9 is expressed differentially in normal and cancer tissues [15]. The overexpression of HOXB9 has been associated with progression and metastasis in leukemia, pediatric acute myeloid leukemia, lung cancer, breast cancer, and gastric cancer [13,15,21,22]. However, the role of altered HOXB9 expression in the progression of HCC remains elusive. The possible clinical significance of HOXB9 remains unknown in HCC patients. We examined the protein expression of HOXB9 in HCC cancer and then explored the relationships between HOXB9 expression and the clinicopathologic characteristics of patients with HCC. Our results show that HOXB9 was not detectable in normal human liver and was overexpressed in majority of the

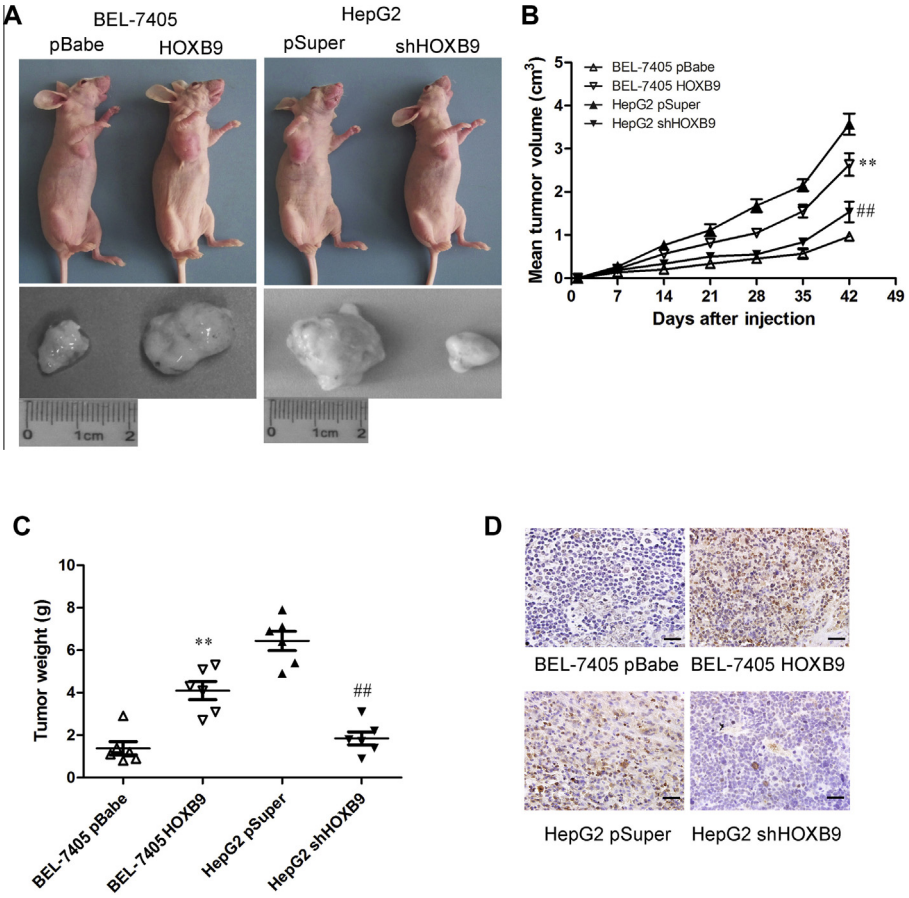

Fig. 3. HOXB9 modulates HCC cells tumorigenesis in vivo. (A) BEL-4705-pbabe, BEL-4705-HOXB9, HepG2-pSuper, and HepG2-shHOXB9 cells were inoculated subcutaneously into the right axilla of four eight-week-old female BALB/*c-nu/nu* mice. (B)Tumor volumes were measured every week after tumor cell injection, and the results are expressed as mean ± standard deviation. (C) Tumor weight was measured 42 days after tumor cell injection, and the results are expressed as mean ± standard deviation. (D) The HOXB9 expression in xenograft was confirmed by immunohistochemical analysis. $^{**}P < 0.01$ versus pBabe cells; $^{\#\#}P < 0.01$ versus pSuper cells. Scale bars = 50 μm.

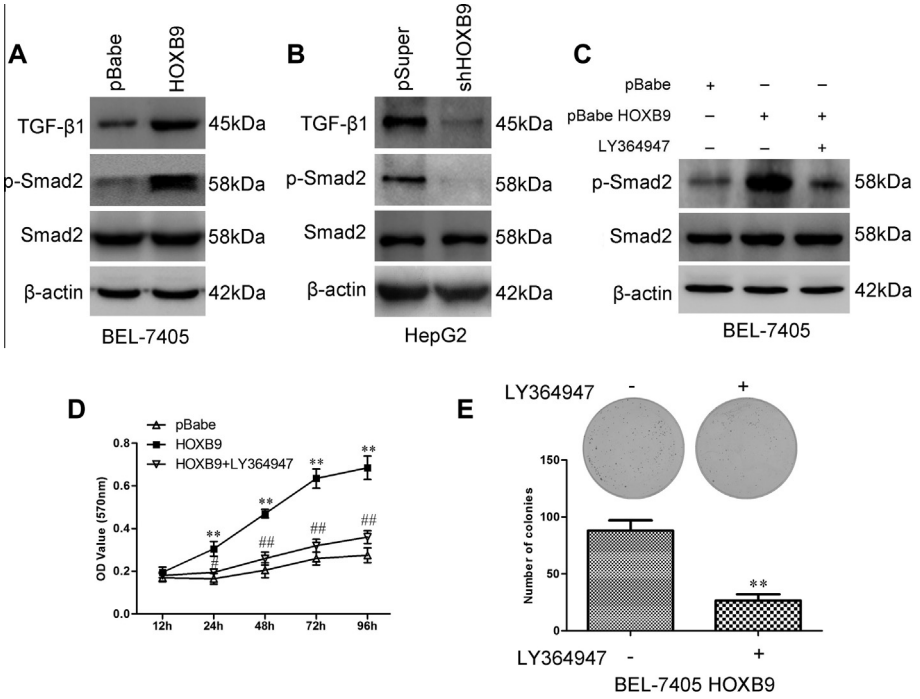

Fig. 4. Activation of TGF-β1 contributes to HOXB9-induced proliferation activities in HCC cells. (A) Western blot analysis of TGF-β1, phosphor-Smad2, and Smad2 protein levels in BEL-4705-pBabe and BEL-4705-HOXB9 cells. (B) Western blot analysis of TGF-β1, phosphor-Smad2, and Smad2 protein levels in HepG2-pSuper and HepG2-shHOXB9 cells. (C) Western blot analysis of phosphor-Smad2, and Smad2 protein levels in LY364947-treated HOXB9-BEL-4705 cells and its control. β-actin was used as the internal control for Western blot. (D) Growth rates were measured by MTT in LY364947-treated HOXB9-BEL-4705 cells and its control. Data are represented as mean ± standard deviation ($n = 6$; $^{**}P < 0.01$versus pBabe cells; $^{\#\#}P < 0.01$versus pBabe HOXB9 cells). (E) Clone formation of the LY364947-treated HOXB9-BEL-4705 cell and its control was evaluated by clone formation assay. The number of clones in each culture well was scored, and the results are expressed as mean ± standard deviation ($n = 6$; $^{**}P < 0.01$versus pBabe cells). All the results are from three or four independent experiments. The error bars indicate standard deviation.

high-grade HCC tissues examined. To clarify the prognostic significance, we analyzed the relationship of HOXB9 expression with the survival of 89 patients with HCC and revealed a link between overexpression and poor survival. The survival rate of patients with a high expression of HOXB9 was significantly less than that of patients with low expression.

We further showed that the overexpression of HOXB9 increased HCC cell proliferation and promoted tumorigenesis in vitro and in vivo, whereas the knockdown of HOXB9 expression by shRNA produced an inhibitory effect on tumorigenesis. These data are consistent to a recent study on the role of HOXB9 in gastric and breast carcinogenesis [17,23]. To the best of our knowledge, the current study is the first report demonstrating that HOXB9 can regulate the proliferation of HCC cells. Our findings are supported by a recent study showing that the overexpression of HOXB9 protein in breast cancer alters tumor cell fate and promotes tumor progression.

TGF-β1 signaling is involved in the regulation of proliferation, differentiation, and survival or apoptosis of many cells, including HCC cells [24]. TGF-β1 acts via specific receptors that activate multiple intracellular pathways, resulting in the phosphorylation of receptor-regulated Smad2/3 proteins that associate with the common mediator, Smad4. Such complex translocates to the nucleus, binds to DNA, and regulates the transcription of many genes [25]. The increased expression of TGF-β1 correlates with a degree of malignancy of human HCCs [26]. Previous studies have shown that HOXB9 expression led to the production of TGF-β1 in breast cancer cells and normal breast cells [16]. Therefore, we speculate that HOXB9 may also modulate TGF-β1 in HCC cells. In this study, we found that the overexpression of HOXB9 significantly increased the expression of TGF-β1 and its downstream protein p-Smad2, whereas the knockdown of HOXB9 produced an opposite effect. Inhibition of TGF-β1 signaling pathway by LY364947 reversed the induction of HOXB9 on HCC cells. Suppression of TGF-β1 signaling reduced the proliferation and clone formation property of T98G-pBabe-HOXB9 cells. Thus, aberrant HOXB9 in HCCs may enhance the oncogenic effects of activated TGF-β1 signaling pathway. However, further investigation on the mechanisms involved in the expression profile of the gene is needed.

We have demonstrated for the first time that HOXB9 is overexpressed in HCC tissues and that the overexpression of HOXB9 promoted HCC tumorigenesis in vitro and in vivo. Therefore, our data suggest that HOXB9 is potentially an important molecular target for the design of novel anti-HCC therapy.

Conflicts of interest statement

No conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.059.

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