ORIGINAL PAPER

Sox2 expression predicts poor survival of hepatocellular carcinoma patients and it promotes liver cancer cell invasion by activating Slug

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Abstract Sox2 is a major transcription factor essential to the stemness characteristics and is associated with various types of cancers. In this study, we investigated the expressions and functional roles of Sox2 in hepatocellular carcinoma (HCC). Our data show that high level of Sox2 expression correlates with metastasis and low survival rate in HCC. HCC cells overexpressing Sox2 are characterized by active epithelial-mesenchymal transition and exhibit increased ability of transwell invasion, soft agar colonization, and sphere formation. We also found Sox2 expression was correlated with the transcription activity of *SLUG* promoter region. These results present novel mechanistic insight into an important role of Sox2 in HCC and suggest a potential application of Sox2 in HCC prognosis and treatment.

Abbreviations
CSC Cancer stem-like cell
EMT Epithelial-mesenchymal transition
ESC Embryonic stem cell
GFP Green fluorescent protein
HCC Hepatocellular carcinoma
LV Lentivirus

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Introduction

Sox2, one of the key members of the Sox family gene, plays critical roles in embryonic pluripotent stem cells. Sox2 is also a key factor to reprogram differentiated cells into induced pluripotent stem cells [1]. Recent publications showed that Sox2 participated in oncogenesis and tumor progression of human colorectal [2], pancreatic [3], gastric [4] and breast [5] cancer, and osteosarcomas [6] and glioma [7, 8]. Sox2 is a novel predictor of poor prognosis for hepatocellular carcinoma (HCC) patients after hepatectomy [9]. Nevertheless, there is still no report describing the molecular role of Sox2 in HCC.

HCC is the third killing cancer in the world [10]. Although surgical resection improves survival of early-stage HCC patients, most HCC patients progress to late stages sooner or later and are to end up with intrahepatic spread and extrahepatic metastasis. Therefore, to investigate molecular mechanism of HCC invasion is crucial to improve the therapeutic effect of HCC treatment.

Epithelial-mesenchymal transition (EMT) was first recognized as a feature of morphogenesis during embryonic development. EMT is a process characterized by loss of epithelial traits, such as alteration of cell–cell adhesion mediated by E-Cadherin repression, accompanied by acquisition of mesenchymal characteristics, such as increased invasive mobility. These phenotypes of cells undergoing EMT are similar to those of invasive cancer cells and cancer stem-like cells (CSC). Accumulative researches revealed that the invasive cancer cells are critically involved with EMT

phenotypes and CSC characteristics [11–17]. Additionally, the subpopulation of cells with EMT phenotypes is a rich source of CSC and vice versa [14, 17, 18]. However, whether the EMT phenotypes can be regulated by stem cell transcription factors such as Sox2 is still poorly understood. In this study, we discovered Sox2 was highly expressed in metastatic HCC samples, and Sox2 expression in HCC cell lines was correlated with cell invasion ability, suggesting a positive involvement of Sox2 signaling in cell invasion. We further demonstrated that Sox2 activated EMT phenotypes in HCC cells and mediated transcription activity of *SLUG* gene. This report suggested that Sox2 had an important role in the metastasis of HCC and regulated EMT phenotypes.

Materials and methods

Cell lines and animals

Human HCC cell lines with stepwise metastatic potential (MHCC97L, MHCC97H, MHCCLM3) were established in Liver Cancer Institute, Zhongshan Hospital, Fudan University [19]. SMMC7721, a HCC cell line with low metastatic potential, was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Huh7 and PLC/PRC/5, another two HCC cell lines with low metastatic potential were obtained from American Type Culture Collection. All cell lines were cultured in DMEM supplemented with 10 % fetal bovine serum at 37 °C with a 5 % CO_2 in air atmosphere. These cell lines were routinely maintained in our institute.

Patients and follow-up

Seventy-five HCC tissue samples enrolled in this study for immunohistochemistry were collected from patients undergoing resection from 2005 to 2007 at the Liver Cancer Institute, Zhongshan Hospital, Fudan University. Fourteen cases of HCC and their paired adjacent non-tumor tissues randomly selected from this cohort using random numbers via SPSS software were used in western blot analysis. General clinical features of these 14 HCC patients were described in Supporting Table 1. Patients were monitored after surgery until September 2012. The procedure of postsurgical patient surveillance was described in previous publications [20, 21]. Overall survival was defined as the interval between surgery and death or between surgery and the time of last observation. The histological grade of tumor differentiation was determined according to the classification proposed by Edmondson and Steiner [22]. TNM stage was determined according to the 2010 International Union Against Cancer tumor-node-metastasis classification system. Another group of 48 frozen HCC tumor tissues for RNA extraction was collected from patients undergoing resection from 2008 to 2010 at the Liver Cancer Institute, Zhongshan Hospital, Fudan University. Ethical approval for this study was obtained from the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from each patient.

Quantitative RT-PCR

RNA was extracted from cultured cells or tissues using TRIzol (Invitrogen) according to the manufacturer's instruction. Total RNA was reversed transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kits (Fermentas). cDNA was prepared for subsequent PCR amplification with SYBR Premix Ex Taq (TAKARA) using IQ5 Realtime RT-PCR instrument (Bio-Rad). The used paired primers for each gene were listed in Supporting Table 2.

Western blot

Total cell or tissue lysates were generated, and proteins were separated on a 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes using conventional method. The membrane was blocked by 5 % milk or 2 % BSA at room temperature for 1 h. Then, the membrane was incubated with specific primary antibody with suitable dilution at 4 °C overnight. After 3 times washing by 0.1 % TBS-Tween20, the membrane was further incubated with horseradish-peroxidase-conjugated secondary antibody at room temperature for 1 h, and then washed again by 0.1 % TBS-Tween20 for 3 times. ECL prime western blotting detection reagents (GE) and ChemiDoc XRS + system (Bio-Rad) were used to visualize the bands on membrane. The used primary antibodies were listed in Supporting Table 3.

Immunohistochemistry

Tissue sections were formalin fixed, paraffin-embedded. Immunohistochemical staining was performed using the labeled streptavidin–biotin peroxidase complex method. The antigen was retrieved by heating in boiling temperature for 1 min using citrate antigen retrieval solution (Beyotime). The expression level of Sox2 in tissue microarray was judged according to the percentage of Sox2 positive cells in each tumor tissue. Specifically, a percentage of <10 % was judged negative and ≥10 % was positive. The stained tissue sections were analyzed by two pathologists without any knowledge about the patients' clinical information. The used primary antibody was listed in Supporting Table 3.

Immunofluorescence

Huh7, SMMC7721, or MHCCLM3 cells cultured on glass slides were fixed in 4 % paraformaldehyde for 15 min and permeabilized with 0.1 % Triton X-100 for 15 min at room temperature. Subsequently, cells were washed with phosphate-buffered saline (PBS) and blocked by 2 % BSA for 1 h at room temperature. Cells were incubated with primary antibody diluted in PBS at 4 °C overnight. A negative control (primary antibody omitted) was processed in every experiment. At the next day, cells were washed with PBS and incubated with working solution of Alexa Fluor 488-conjugated secondary antibody (Cell Signaling) at room temperature for 1 h. After rinsing in PBS, the slides were imaged immediately with a DM2500 fluorescence microscope (Leica). The used primary antibodies were listed in Supporting Table 3.

Sphere formation assay

Huh7, SMMC7721, or MHCCLM3 cells were plated in 6-well plates at a density of 400 cells per ml and cultured in a serum-free DMEM medium, supplemented with EGF (20 ng/ml), bFGF (20 ng/ml), and B27 supplement (Gibco). The number of spheres was counted 14 days later. All experiments were performed in triplicate.

Soft agar colonization

For clone formation assay, 1 ml of 0.6 % low melting point (LMP) agarose in complete medium was added to one well of 6-well plate first. After the medium became solid gel, 1 ml of 0.4 % LMP agarose in complete medium with 1000 Huh7, SMMC7721, or MHCCLM3 cells was added on top of the base gel. After culturing for 14 days, clone (>50 cells) numbers were assessed microscopically. All experiments were performed in triplicate.

Transwell invasion assay

Cell invasion assay was performed using 6-well transwell (8.0 μm pore size; Millipore) precoated with Matrigel (BD Biosciences). Huh7, SMMC7721, or MHCCLM3 cells (5×10^5) were suspended in 1.5 ml serum-free DMEM media and transferred into the inside chamber of 6-well cell culture insert with 8.0 μm pore size (Millipore). The total of 2.6 ml media with 20 % FBS was added into the outside well. After incubation of 36 h, the Matrigel and the cells remaining in the upper chamber were removed by cotton swabs. Cells on the lower surface of the membrane were fixed by cold methanol and subjected to Giemsa staining. Invasive cells were counted at five randomly selected views (at 200× magnification), and the average cell number per view was calculated. All experiments were performed in triplicate.

Lentiviral infection

The human Sox2 or *SLUG* CDS region was cloned from human genomic DNA and was confirmed by sequencing. Then, the PCR product was subcloned into pLV-EF1a-IRES-GFP. For shRNA knock-down systems, the pGCSIL-GFP cloning vector is a lentiviral vector expressing shRNA under the control of the U6 promoter. The Sox2- or *SLUG*-specific shRNA-targeting coding sequence (5′-GGACATGATCAG CATGTATCT-3′or 5′-GCATTTGCAGACAGGTCAAAT-3′) was cloned into the pFUGW. The pFUGW-scramble shRNA (5′-CCTGGAACAGTCCCTTCTATA-3′) was used as the control plasmid. The lentivirus was produced by transfecting the packaging plasmids as well as the transfer lentiviral plasmids into HEK-293T cells with Lipofectamine 2000 (Invitrogen). After 48 h of transfection, medium containing lentivirus was harvested and concentrated by ultracentrifugation. The concentrated lentivirus was stored in small aliquots at −80 °C. Huh7, SMMC7721, or MHCCLM3 cells were infected with lentivirus at a multiplicity of infection (MOI) of 10 for overnight in the presence of 8 μg/ml of polybrene (Sigma). After culturing 96 h, the efficiency of infection was more than 90 % according to FACS analysis of GFP fluorescence as internal control.

Statistics

The student's test was used to compare two groups of parametric variants. The chi-square test was used to analyze nonparametric variants. The Pearson's correlation coefficient was used to analyze the correlation between parametric variants. The Kaplan–Meier method was used for survival analysis. SPSS 13.0 was used to process the statistical analysis, and GraphPad Prism 5 was used to draw the graphs. In this manuscript, bar graphs show mean ± standard error of the mean (SEM) performed in at least 3 times compared with mock cells. $P < 0.05$ was considered statistically significant and marked with *.

Results

Elevated Sox2 expression is correlated with poor survival and metastasis of HCC patients

First, Sox2 expression in 14 pairs of HCC and adjacent tissues was measured using western blot. The result showed that Sox2 expression was higher in HCC tissues than their paired adjacent non-HCC tissues (Fig. 1a). The general information of these 14 patients was described in

Supporting Table 1. Furthermore, HCC tissues from 75 HCC patients with survival information from a 60-month follow-up period were collected for production of tissue microarray and immunohistochemistry analysis. The result indicated that the localization of Sox2 was both in the cytoplasm and the nucleus of HCC cells and found that about 61 % of patients (46/75) stained positively with the Sox2 antibody. The stained tissue sections were judged to be positive or negative as described in materials and methods. The typical images of negative and positive staining of Sox2 were showed in Fig. 1b. In these Sox2 positive HCCs, metastatic tumors were detected in 50 % of them (23/46). However, in the Sox2 negative HCC, only 17 % of them (5/29) had detectable metastatic tumors ($P < 0.05$) (Table 1). Kaplan–Meier survival analysis showed the survival of patients with positive Sox2 expression was significantly poorer than those with negative Sox2 expression (Fig. 1c). The Sox2 expression in several HCC cell lines with varying metastatic potential was also measured by western blot analysis. The staining using anti-Sox2 antibody was strong in highly metastatic MHCC97H and MHCCLM3 cells, while moderate or faint in low metastatic MHCC97L or Huh7, PLC/PRC/5, and SMMC7721 cells, respectively (Fig. 1d).

Sox2 activates epithelial-mesenchymal transition (EMT) in HCC cells

To investigate the effect of Sox2 expression in EMT process, we overexpressed Sox2 in a Sox2 negative Huh7 and SMMC7721 cell line and inhibited Sox2 in a Sox2 positive MHCCLM3 cell line using lentiviral transfection. Increased expression of Sox2 led to dramatically morphological changes of Huh7 and SMMC7721 cells from an epithelial-like phenotype to a mesenchymal-like phenotype (Fig. 2a), while the corresponding morphological changes in MHCCLM3 cells treated with LV-shSox2 were not observed (data not shown) probably because the inhibition of Sox2 by shRNA was not enough to produce the phenotype at the cellular level.

The expression of classical factors involved in the EMT process including E-Cadherin, Vimentin, and Slug were
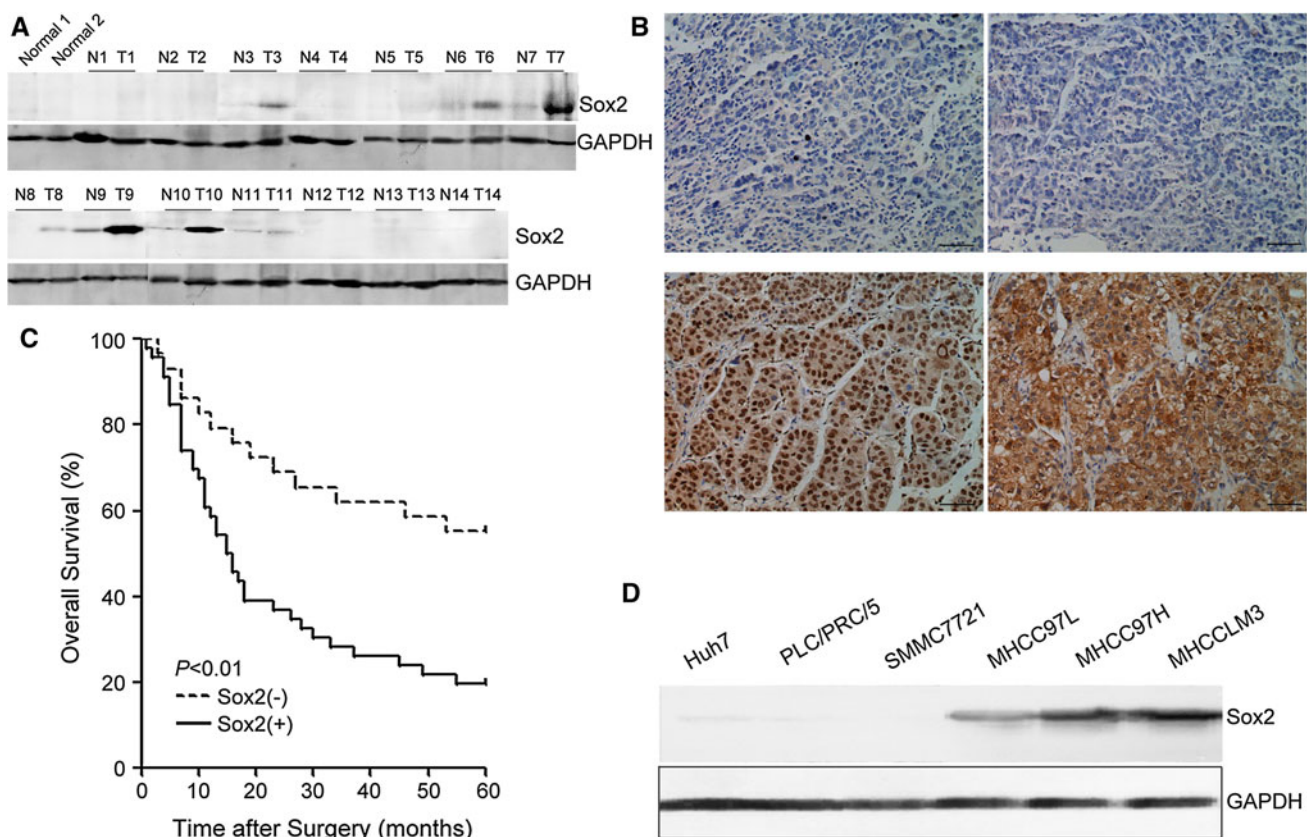
Fig. 1 Expression of Sox2 in HCC tissues and cell lines. a Sox2 expression in 14 pairs of HCC tissues (T) and adjacent non-HCC tissues (N). b Representative negative and positive Sox2 expression in immunohistochemistry (scale bars, 50 μm). c Kaplan–Meier analysis of overall survival in HCC patients with negative Sox2 expression ($n = 29$) or positive Sox2 expression ($n = 46$) using SPSS 13.0. d Sox2 expression in the indicated HCC cell lines

Table 1 Correlation between the Sox2 expression and clinicopathologic characteristics in HCC ($n = 75$)

Clinicopathological indexes	Sox2		
	Negative	Positive	P
Age(year)			
≤50	19	27	0.555
>50	10	19	
Sex			
Female	4	6	0.926
Male	25	40	
HbsAg			
Negative	6	13	0.463
Positive	23	33	
AFP (ng/ml)			
≤20	11	18	0.917
>20	18	28	
GGT (U/L)			
≤50	9	21	0.208
>50	20	25	
Liver cirrhosis			
No	5	8	0.987
Yes	24	38	
Tumor size (cm)			
≤5	14	20	0.684
>5	15	26	
Tumor number			
Single	16	39	0.954
Multiple	3	7	
Microvascular invasion			
Absence	21	25	0.118
Present	8	21	
Metastasis			
Absence	24	23	0.004
Present	5	23	
Tumor differentiation			
I + II	21	22	0.036
III + IV	8	24	
TNM stage			
I	4	4	0.486
II + III + IV	25	42	

Chi-sqaure tests

AFP alpha-fetoprotein, *GGT* gamma-glutamyl transferase, *TNM* tumor-node-metastasis

assessed by realtime PCR, western blot and immunofluorescence. In Huh7 and SMMC7721 cells, we found a significant inhibition of E-Cadherin and remarkable elevation of Vimentin and Slug when Sox2 was overexpressed. In MHCCLM3 cells, the expression of E-Cadherin was increased while Vimentin and Slug were inhibited when Sox2 was knocked down (Fig. 2b, c, d).

Sox2 enhances invasive potential and CSC property of HCC cells in vitro

To investigate the effect of Sox2 on cell colonization and invasion, sphere formation, soft agar colonization, and transwell invasion assays were carried out using stable Huh7, SMMC7721, and MHCCLM3 cells transfected by lentivirus with plasmid vectors encoding Sox2 cDNA or shRNA against Sox2. We found Huh7 and SMMC7721 cells with Sox2 overexpression acquired stronger ability in sphere formation, soft agar colonization, and transwell invasion than mock cells, while inhibiting Sox2 expression in MHCCLM3 weakened the cell ability of sphere formation, soft agar colonization, and transwell invasion (Fig. 3). This evidence implied that Sox2 might have driven HCC cells toward a state closer to CSC with higher invasion ability.

Transcription of *SLUG* gene is regulated by Sox2 and may be involved in Sox2-induced acquisition of invasive ability and EMT phenotypes of HCC cells

Since Sox2 was a transcription factor regulating multiple target genes and could stimulated Slug expression, we asked whether Sox2 enhanced promoter activity of *SLUG* gene. First, a putative Sox2-binding motif (TACAAAG) was found close to the translational initiation site (ATG) of *SLUG* gene. Then, a DNA sequence covering 1 kb upstream of *SLUG* gene was inserted into the pGL3 luciferase reporter vector. The construct was transiently transfected into Huh7, SMMC7721, and MHCCLM3 cells, and the transcription activities derived from this construct was measured using a dual-luciferase detection system. Overexpression of Sox2 in Huh7 and SMMC7721 cells led to upregulation of the luciferase reporter containing the putative Sox2-binding motif, whereas inhibiting Sox2 expression in MHCCLM3 cells resulted in downregulation of the luciferase reporter (Fig. 4a). These results showed Sox2 could regulate the transcription activity of *SLUG* promoter.

We further asked whether Slug was involved in Sox2-induced promotion of cell invasion. When Slug was overexpressed in Huh7 and SMMC7721 cells, E-Cadherin expression was almost abolished and Vimentin expression was remarkably increased. In MHCCLM3, when Slug was knocked down, E-Cadherin expression was inhibited and Vimentin expression was remarkably decreased. Nevertheless, the forced expression or inhibition of Slug did not influence the Sox2 expression (Fig. 4b), which indicated that Slug might be a downstream target of Sox2 signaling. Meanwhile, overexpression of Slug in Huh7 and SMMC7721 cells promoted cell invasion and inhibition of Slug in MHCCLM3 cells decreased cell invasion (Fig. 4c). These results suggested that Slug might mediate Sox2-induced E-Cadherin downregulation, Vimentin upregulation, and
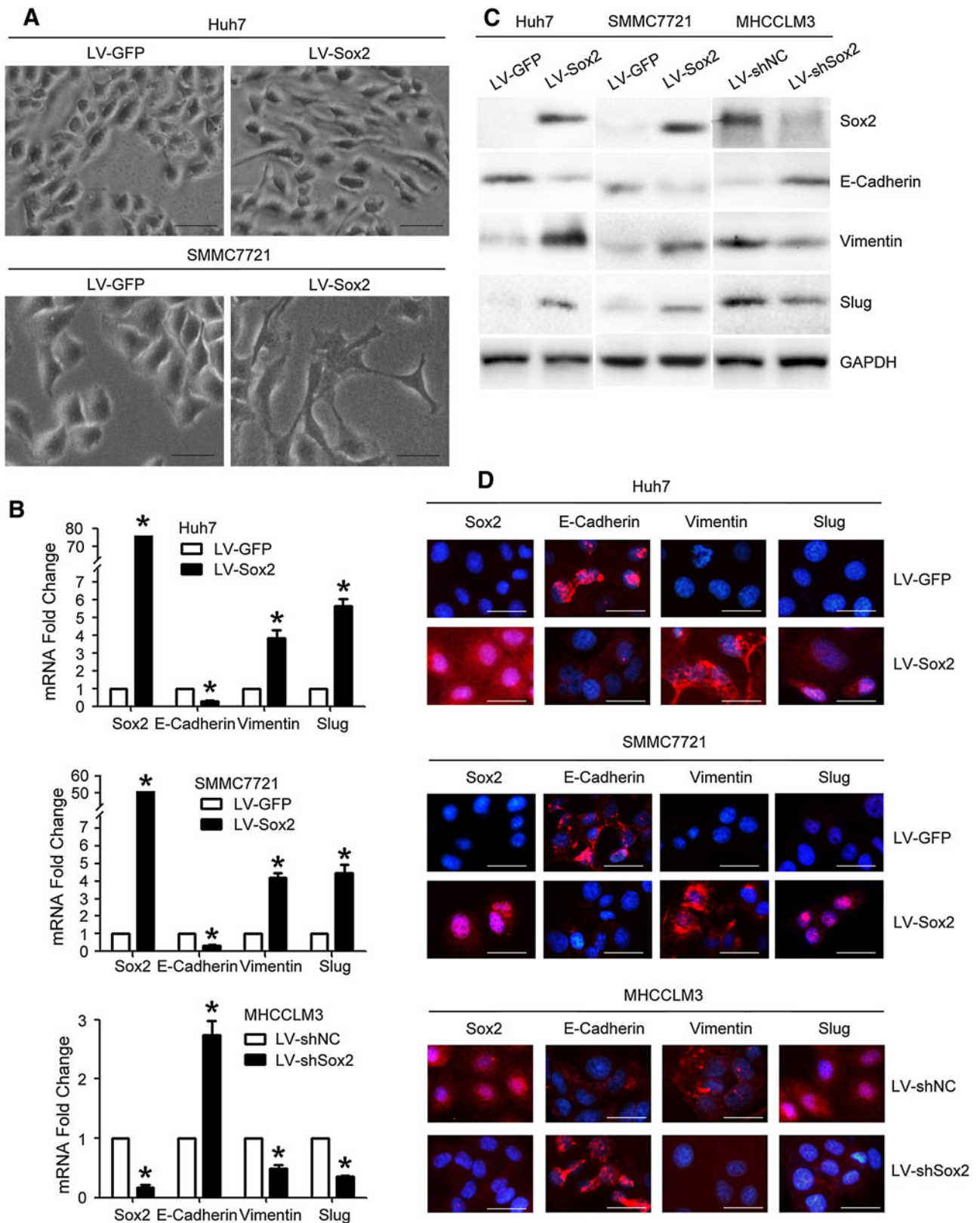
Fig. 2 Sox2 increased epithelial marker expression and decreased mesenchymal marker expression in HCC cells. **a** The morphological changes of Huh7 and SMMC7721 cells after Sox2 overexpression (*Scale bars*, 25 μm). **b** Realtime analysis of EMT markers in indicated cell lines. **c** Western blot analysis of EMT markers in indicated cell lines. **d** Immunofluorescence analysis of EMT markers in indicated cell lines (*Scale bars*, 25 μm)
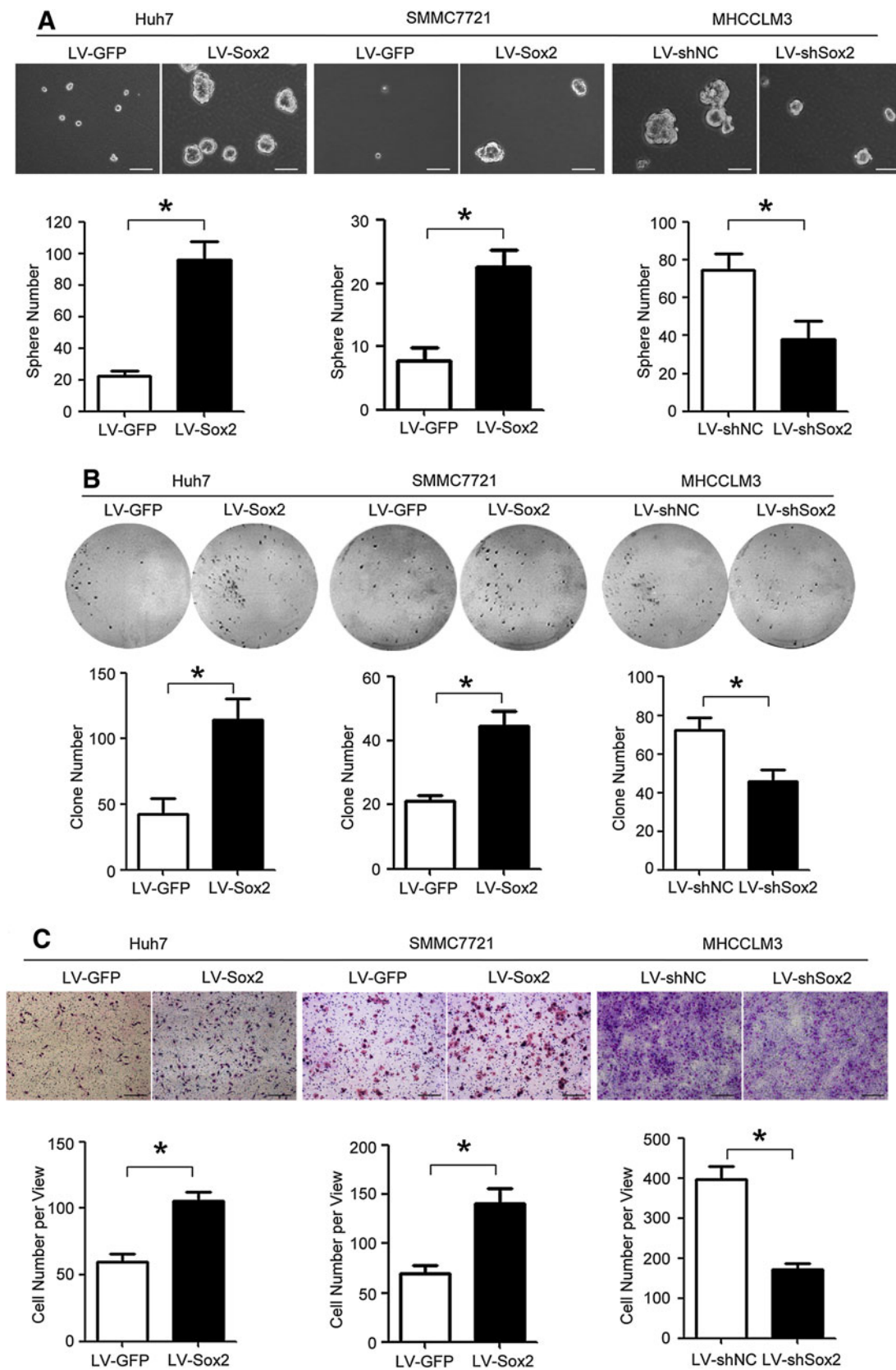
Fig. 3 Sox2 enhanced the abilities of *sphere* formation, soft agar colonization, and Matrigel invasion in HCC cells. **a** *Sphere* formation assay of indicated cell lines (*Scale bars*, 50 μm). **b** Soft agar colonization of indicated cell lines. **c** Transwell invasion assay of indicated cell lines, followed by giemsa staining (*Scale bars*, 100 μm)
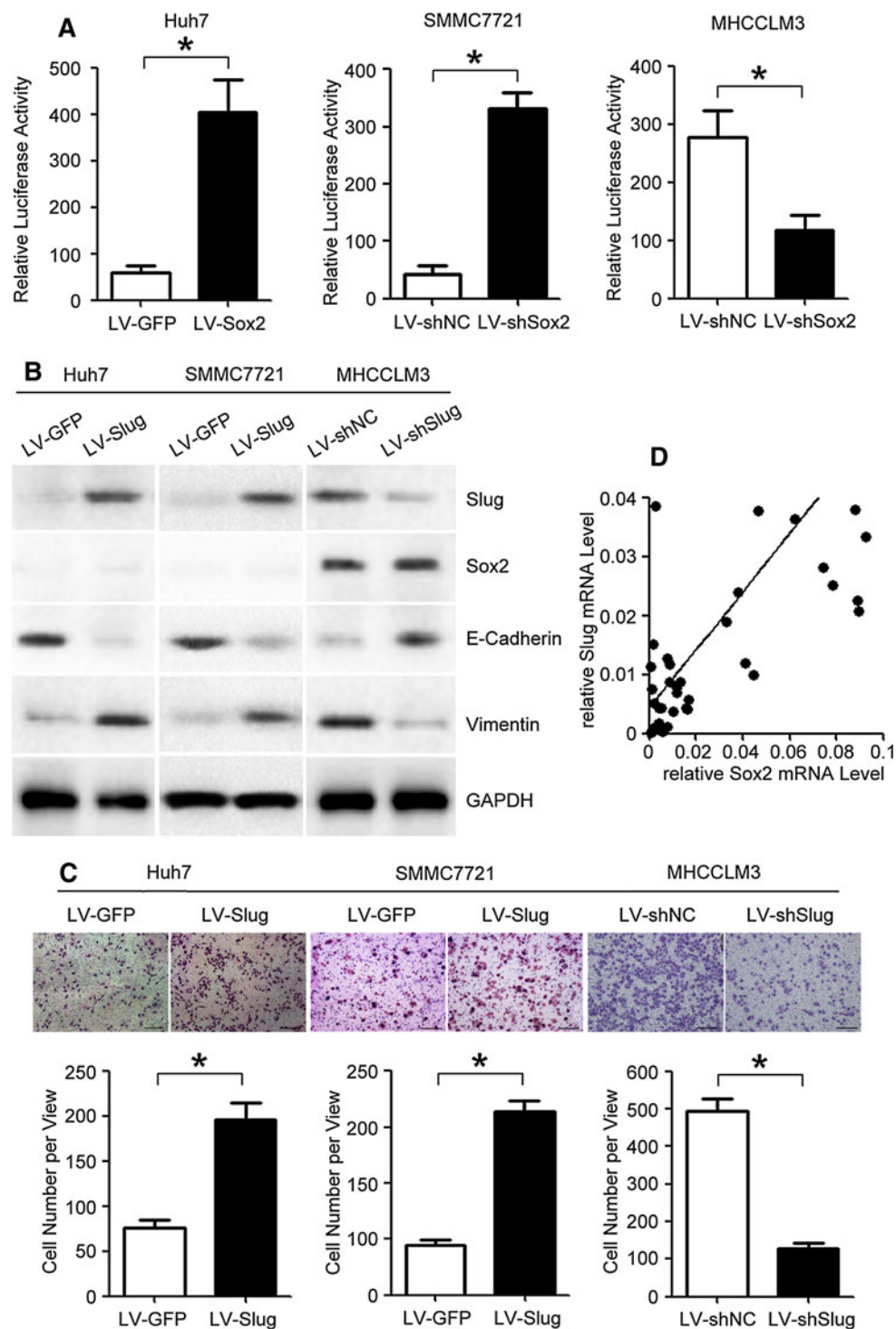
Fig. 4 Slug was regulated by Sox2 and promoted invasive ability of HCC cells. **a** Luciferase activity assay of indicated cell lines. Firefly luciferase activity was normalized by Renilla luciferase activity. **b** Western blot analysis of Sox2 expression and EMT markers in indicated cell lines. **c** Transwell invasion assay of indicated cell lines, followed by giemsa staining (*Scale bars*, 100 μm). **d** Relative mRNA levels (normalized by GAPDH) of Sox2 and Slug were examined in 48 HCC tissues and analyzed for the correlation between Sox2 and Slug mRNA levels using SPSS 13.0 by Pearson's correlation coefficient

acquisition of invasive ability in HCC cells. We further examined the correlation between the expression of Sox2 and Slug in another 48 HCC tumor samples using realtime PCR.

As shown in Fig. 4d, the level of Sox2 was positively correlated with the level of Slug in HCC ($r^2 = 0.7290$, $P < 0.0001$).

Discussion

In this study, we found the overexpression of Sox2 to be closely correlated with the poor survival and metastasis of HCC patients. Functional analysis of Sox2 in invasive property of Huh7, SMMC7721, and MHCCLM3 cell lines revealed that the acquisition of EMT phenotypes through activating *SLUG* transcription activity was a possible underlying mechanism of Sox2-induced cell invasion.

One of the observations in this study is that Sox2 expression was low in non-tumor liver tissues, increased in noninvasive HCC, and reached the highest level in invasive HCC (Fig. 1a; Table 1). This expression profile suggests a role of Sox2 in the progression of HCC. Moreover, Sox2 might be a potential biomarker for HCC prognosis, since strong expression of Sox2 in tumor tissues was in correlation with metastasis and poor survival. However, the sample size of this study is small ($n = 75$), and all the patients were from one hospital in Shanghai, China. Although these patients lived in different areas of China, the majority of them were from East China or South China. Therefore, in the future study, it is necessary to use a larger sample size to evaluate the power of Sox2 as a predictive biomarker for HCC metastasis and poor survival, and the new sample pool should be constructed using a stratified sampling strategy.

Another interesting finding was that Sox2 located not only in nuclei but also in the cytosol of HCC cancer tissues and cell lines (Figs. 1b, 2d). This pattern of Sox2 location in HCC corresponds with that in colorectal [2] and prostate [23] cancer cells. The nuclei localized Sox2 may function as a transcriptional factor, while the function of the cytosol localized Sox2 needs to be further investigated.

We also found overexpression of Sox2–activated cell EMT process, and a reciprocal process was carried out when Sox2 was knocked down, based on the cell morphological alteration and expression change of epithelial and mesenchymal markers (Fig. 2). Increasing evidence suggests that in HCC cells, the acquisition of an EMT phenotype is correlated with increasing capacity of invading surrounding tissues [24–28]. Therefore, not surprisingly, we found Sox2 enhanced cell invasion as well as CSC properties in HCC cells (Fig. 3). Cumulatively, we proposed that EMT was involved in Sox2-induced HCC invasion. The EMT is also marked by activation of some transcriptional factors including Slug, Snail, or Twist. We also found Sox2 overexpression led to Slug activation in both mRNA and protein levels. A Sox2-binding motif was found at the -847 to -841 base pair upstream from the ATG translational initiation site of *SLUG* gene, and Sox2 could enhance transcription activity of *SLUG* promoter (Fig. 4a), which indicated Sox2 might regulate *SLUG* transcription directly.

Elevated expression of Slug is associated with advanced tumor grade, metastasis, and poor prognosis in various types of cancers [29–32]. In this study, we found overexpression of Slug enhanced cell invasion, and knockdown of Slug inhibited cell invasion (Fig. 4c). These results, combined with the evidences that Sox2 activated Slug expression, demonstrated that Sox2 promoted cell invasion through Slug-dependent signaling pathways, including inhibition of E-Cadherin and elevation of Vimentin. Supporting the in vitro results, we found that Sox2 expression in HCC tissues was positively correlated with the expression of Slug (Fig. 4d).

It is not surprising to find a link between Sox2 and EMT, since Sox2 is a key transcriptional factor regulating self-renew of stem cell and EMT is also a critical program in the early embryonic development. Furthermore, recent reports showed Sox2 and other stemness-related transcriptional factors including Nanog and Oct4 were overexpressed in various types of cancers [33–37], and the EMT process is often activated in the acquisition of cancer cell invasion. In this study, we suggested Slug, a key transcriptional factor of EMT, might be a downstream target of Sox2 in HCC. However, the regulation mechanism between EMT and stemness-related transcriptional factors and their roles in cancer metastasis are worthy of intensive investigation.

In summary, this study demonstrated Sox2 expression was correlated with HCC metastasis and prognosis, and Sox2–activated cell invasion and EMT through regulating Slug expression in HCC cells.

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