

Mesoderm/mesenchyme homeobox 1 may promote tumor progression in human hepatocellular carcinoma

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Conflict of interest

None declared

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Abstract

Background. The clinical response rate for molecularly targeted medications is limited despite significant advancements in molecularly targeted therapy for hepatocellular carcinoma (HCC). Therefore, it is necessary to find new and robust therapeutic targets for the treatment of HCC. Recent research has shown that mesoderm/mesenchyme homeobox gene 1 (*Meox1*) is closely associated with cancer progression.

Objectives. The aim of this study was to evaluate the clinical relevance as well as biological function of *Meox1* in HCC.

Materials and methods. *Meox1* protein expression level was identified through immunohistochemistry (IHC) examination of pathological tissues from 25 HCC patients. The aim of the analysis was to investigate the relationship between clinicopathological traits and *Meox1* expression. Biological function assays of *Meox1* in HCC, including proliferation, colony formation, migration, and invasion, were performed with Huh7 and Hep3B cells.

Results. In this study, *Meox1* expression in HCC tissues was significantly higher ($p < 0.05$) compared to paracancerous tissues. Especially in HCC tissues of patients with cirrhosis, the level of *Meox1* expression was significantly elevated when compared to HCC tissues of patients without cirrhosis ($p < 0.05$). High *Meox1* expression was significantly associated with tumor-node-metastasis (TNM) stage ($p < 0.05$) and the Barcelona Clinic Liver Cancer (BCLC) stage ($p < 0.05$). Moreover, *Meox1* silencing suppressed the proliferation, colony formation, migration, and invasion of Huh7 and Hep3B cells.

Conclusions. Our data reveal that *Meox1* may play a crucial role in the development of HCC, and given the function of *Meox1* in proliferation and metastasis, targeting *Meox1* may offer a promising approach for combined and adjuvant therapeutics of HCC.

Key words: progression, proliferation, hepatocellular carcinoma, metastasis, mesoderm/mesenchyme homeobox gene 1

Background

Hepatocellular carcinoma (HCC), which accounts for about 75–85% of primary liver cancer cases in China, is the leading cause of cancer-related deaths.^{1,2} The main therapeutic strategies for HCC patients with early-stage disease are surgery and radiofrequency ablation.^{3–5} However, most HCC patients are at an advanced stage at the first diagnosis and are treated with transcatheter arterial chemoembolization (TACE).⁵ Molecularly targeted therapy includes various kinase inhibitors,⁶ such as sorafenib,⁷ lenvatinib⁸ and apatinib,⁹ and immune checkpoint inhibitors, such as nivolumab¹⁰ and pembrolizumab.¹¹ Despite significant advancements in molecularly targeted therapy for HCC, these medications have a limited clinical response rate,¹² which emphasizes the need to explore novel and effective therapeutic targets for HCC treatment.

A key transcription factor called mesoderm/mesenchyme homeobox I (*Meox1*) is necessary for cell proliferation and differentiation, and organ formation throughout embryonic development.^{13,14} To date, many studies linked aberrant expression of *Meox1* to the development of cancer. The *Meox1* protein was markedly upregulated in human non-small cell lung cancer (NSCLC) tissues and linked to unfavorable prognosis. The inhibition of *Meox1* expression suppressed lung cancer cell proliferation and mammosphere formation in vitro.¹⁵ The *Meox1* protein was also abnormally expressed in ovarian cancer, which promotes cell growth through interaction with *PBX1*.¹⁶ Moreover, *Meox1* was crucial in breast cancer stem cell (CSC) maintenance and epithelial–mesenchymal transition, and it was linked to unfavorable survival outcomes, breast cancer stage and lymph node metastasis in trastuzumab-resistant *PTEN*-deficient breast cancer.¹⁷ In addition, *Meox1* knockdown inhibited the proliferation of triple-negative breast cancer cells in vitro and tumor growth in vivo.¹⁸ However, the clinical significance and biological role of *Meox1* in HCC have not been investigated.

During our research, we not only investigated the expression level of *Meox1* in human HCC tissues and evaluated the relationship between *Meox1* expression and poor progression in patients with HCC, but also elucidated the role of *Meox1* in HCC cell malignancy in vitro for the first time.

Objectives

The aim of this study was to assess the clinical significance and biological role of *Meox1* in HCC.

Materials and methods

Collection of clinical samples

Pathological specimens were collected from 25 patients with HCC who underwent surgical operations

at the 980th Hospital of People's Liberation Army (PLA) Joint Logistics Support Force (Shijiazhuang, China) between January 2018 and November 2022. The clinicopathological parameters, including age, gender, pathogeny, cirrhosis, tumor size, tumor number, vascular invasion, lymph node metastasis, tumor-node-metastasis (TNM) stage,¹⁹ the Barcelona Clinic Liver Cancer (BCLC) stage system,²⁰ and results of biochemical tests, including alpha-fetoprotein (AFP), carcinoma embryonic antigen (CEA), prothrombin time (PT), albumin, as well as total bilirubin, were collected. Radiotherapy, chemotherapy or other biological therapies were not administered to any of the patients. Furthermore, no occurrences of malignant tumors, cardiovascular or cerebrovascular ailments, diabetes, pulmonary fibrosis, or kidney disease were observed among the patients. The Ethics Committee of the 980th Hospital of the PLA Joint Logistics Support Force gave its approval to this study (approval No. 2022-KY-127). All patients signed written informed consent.

Immunohistochemistry analysis

The protein level of *Meox1* was analyzed using immunohistochemistry (IHC) staining. Briefly, after deparaffinization in xylene and in various concentrations of alcohol, antigen retrieval was conducted using ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0), followed by treatment with 3% hydrogen peroxide for 25 min in the dark at room temperature. After that, the specimens were incubated with primary antibodies against *Meox1* (1:500; cat. No. ab105349; Abcam, Cambridge, UK) overnight at 4°C after being blocked with 3% bovine serum albumin (BSA) for 30 min.

A semiquantitative scoring system was used to assess *Meox1* expression in accordance with the percentage of positive cells and staining intensity. Pathologists scored the expression in a blinded manner.^{15,21} Four categories were established for the percentage of positive cells: 0 (0~5%), 1 (6~25%), 2 (26~50%) and 3 (51~100%). The staining intensity was divided into 4 levels: 0 (no staining), 1 (faint staining), 2 (moderate staining), and 3 (strong staining). The multiplication of both factors determined a positive grade that could be negative (0), weakly positive (from 1 to 4), moderately positive (from 5 to 8), or strongly positive (from 9 to 12). *Meox1* expression was classified into 2 categories: low expression (negative and weakly positive) or high expression (moderately and strongly positive).

Cell lines and culture

The Huh7 and Hep3B cell lines were stored at the Hebei Key Lab of Laboratory Animal Science of Hebei Medical University (Shijiazhuang, China) and cultured as previously described.^{21,22}

RNA interference and stable-knockdown cell screening

Recombinant lentivirus carrying specific short hairpin RNA (shRNA) for *Meox1* or a negative control was packaged by GenePharma Co., Ltd. (Shanghai, China). Two different *Meox1* shRNAs were used to perform the experiment. The sequence for *Meox1* shRNA 1 was 5'-GAA ATC ATC CAG GCG GAG AAA-3'; *Meox1* shRNA 2 was as follows: 5'-CTG CCA ATG AGA CAG AGA A-3';²³ The negative control shRNA sequence was as follows: 5'-TTC TCC GAA CGT GTC ACG T-3'.

To conduct *Meox1* inhibition experiments, we infected HCC cells (Huh7 and Hep3B) with recombinant lentivirus carrying its specific shRNA (sh-*Meox1*) and negative control shRNA (sh-NC). Stable cell clones with *Meox1* knockdown were established via 0.6 µg/mL puromycin selection for Huh7 cells and 0.3 µg/mL puromycin for Hep3B cells.

Western blotting

Radioimmunoprecipitation (RIPA) lysis buffer (Solarbio, Beijing, China) containing freshly added phenylmethyl sulphonyl fluoride (PMSF; Solarbio) was used to extract total protein from cells based on the manufacturer's instructions. A bicinchoninic acid assay (BCA) protein assay kit (Solarbio) was used to test the concentrations of protein according to the manufacturer's guidelines. The protein samples underwent separation utilizing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then moved onto a nitrocellulose membrane (MilliporeSigma, St. Louis, USA), followed by blocking with 5% non-fat dry milk. Primary antibodies, including anti-*Meox1* (1:1,000; cat. No. ab105349; Abcam) and anti-*GAPDH* (1:10,000; cat. No. ab181602; Abcam), were used to conduct the experiments. The secondary antibody used was horseradish peroxidase (HRP)-conjugated Affinipure goat anti-rabbit immunoglobulin G (IgG) (H+L) (1:5,000; cat. No. SA00001-2; Proteintech, San Diego, USA). The immunoblots were detected as previously described.²²

Cell proliferation assay

Cell Counting Kit-8 (CKK-8; Dojindo Lab, Kumamoto, Japan) was employed to assess cell proliferation. The analysis was conducted according to the previously described methods.²²

Colony formation assay

Six-well plates containing 500 cells/well were used to seed cell lines, which were then routinely cultivated for 2 weeks. The plates were discarded after a visible cell colony formed, rinsed with phosphate-buffered saline (PBS) and treated with methyl alcohol for 30 min, followed by staining with 0.5% crystal violet for 20 min to count

the cell number. The colony formation efficiency was calculated using the following formula: colony number divided by seeded cell number multiplied by 100%.

Cell migration and invasion assay

Eight-micrometer Transwell® chambers containing polycarbonate filters (Corning Company; Corning, USA) were used to conduct the cell invasion and migration assays. For the invasion assay, the chambers were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, USA), and the migration assay was carried out without Matrigel. The analysis was conducted as previously described.²²

Statistical analyses

Statistical analysis was performed using IBM SPSS 26.0 software (IBM Corp., Armonk, USA). Pathological specimens were collected from 25 patients with HCC undergoing surgical operations at the 980th Hospital of PLA Joint Logistics Support Force. Three separate independent runs of each cell function experiment (cell proliferation, colony formation, cell invasion and migration assay) were conducted. The frequency or medians (interquartile range (IQR)) was used to represent the values. The Fisher's exact test or non-parametric Mann-Whitney U test or Kruskal-Wallis H test were utilized for examining the data, as appropriate. The Spearman's correlation analysis was utilized for examining the relationship between the 2 variables. Survival curves for HCC were produced using the Kaplan-Meier method and the log-rank test. All comparisons were 2-tailed, and a $p < 0.05$ denoted statistical significance.

Results

High expression of *Meox1* in HCC tissues

To evaluate the expression level of *Meox1* in human HCC tissues, we conducted an IHC analysis. The results revealed that *Meox1* was primarily localized in the nucleus (Fig. 1A). In the 25 HCC tissues, the *Meox1* showed negative expression in 2, while weak, moderate and strong positive expression in 6, 14 and 3, respectively (Table 1). Among the 25 paracancerous tissues, 4 were negative and 13 and 8 were weakly and moderately positive, respectively, and there was no strongly positive expression (Table 1). *Meox1* expression was significantly higher in HCC tissues compared to paracancerous tissues ($p < 0.05$, Mann-Whitney U test). Furthermore, we compared the percentage of positive cells expressing *Meox1* in hepatocellular carcinoma (HCC) and paracancerous tissues (Fig. 1B). The median (IQR) of positive cells percentage of *Meox1* in HCC was 0.7318 (0.2904–0.8486), while in paracancerous tissue it was 0.3607 (0.0932–0.663); a statistically significant distinction existed ($p = 0.048$, Mann-Whitney U test). The result indicate that *Meox1* is highly expressed in HCC tissues.

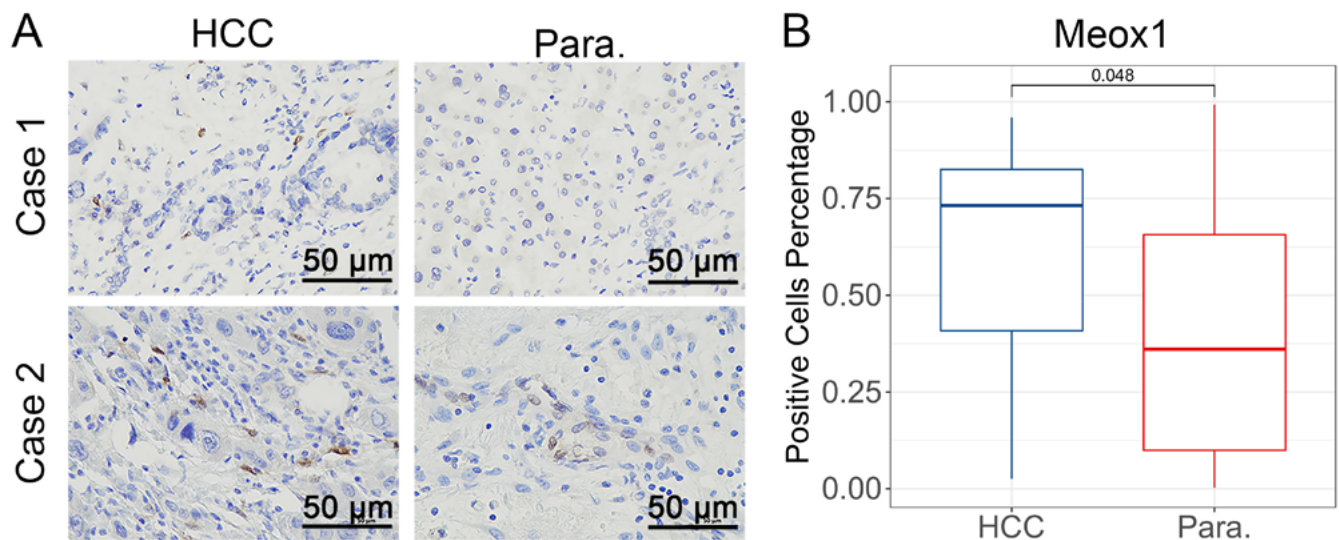


Fig. 1. The expression of *Meox1* in HCC and paracancerous tissues was determined using immunohistochemistry (IHC). A. Representative staining in HCC tissues and paracancerous tissues at $\times 400$. *Meox1* protein was mainly localized in the nucleus. The brown signal is the *Meox1* protein, and the blue signal is the nucleus; B. The median (interquartile range (IQR)) of *Meox1*-positive cells percentage in HCC was 0.7318 (0.2904–0.8486), while in paracancerous tissue it was 0.3607 (0.0932–0.663). Mann–Whitney U test was used to examine the difference of *Meox1*-positive cells percentage between HCC and paracancerous tissues ($p = 0.048$, Mann–Whitney U test)

HCC – hepatocellular carcinoma; Para. – paracancerous tissue.

Table 1. The expression of *Meox1* in HCC and paracancerous tissues

Immunohistochemical grade	HCC tissues (n = 25, 100%)	Paracancerous tissues (n = 25, 100%)	p-value*
Negative (–)	2 (8%)	4 (16%)	0.011
Weakly positive (+)	6 (24%)	13 (52%)	
Moderately positive (++)	14 (56%)	8 (32%)	
Strongly positive (+++)	3 (12%)	0 (0%)	

*The non-parametric Mann–Whitney U test was used to examine the difference of *Meox1* expression between HCC and paracancerous tissues.

Table 2. The expression of *Meox1* in hepatocellular carcinoma (HCC) tissues with or without cirrhosis

Cirrhosis	Number of cases (%)	Low expression	High expression	p-value*
No	7 (28%)	5	2	0.017
Yes	18 (72%)	3	15	

* The Fisher's exact test was used to examine the data.

High expression of *Meox1* in HCC patients with cirrhosis

We further observed the expression level of *Meox1* in HCC tissues of patients with and without cirrhosis. The results indicated that among 18 HCC patients with cirrhosis, *Meox1* expression was low in 3 HCC tissues and high in 15. In the following 7 patients without cirrhosis, *Meox1* expression was low in 5 HCC tissues and high in 2 (Table 2). The expression of *Meox1* was markedly elevated in HCC tissues from patients with cirrhosis compared to patients without cirrhosis ($p < 0.05$, Fisher's exact test).

We also observed the expression level of *Meox1* in paracancerous tissues of patients with and without cirrhosis (Table 3). In 18 HCC patients with cirrhosis, 10 patients had

low *Meox1* expression while 8 had high expression, respectively. However, in 7 HCC without cirrhosis, *Meox1* was expressed at low levels in 7 patients, and there was no high expression. The results show that there is no significant difference in *Meox1* expression between paracancerous tissues of HCC with cirrhosis and HCC without cirrhosis ($p = 0.057$, Fisher's exact test).

Meox1 expression level linked to poor progression in HCC

Statistical analysis revealed a significant relationship between *Meox1* expression and BCLC stage ($p < 0.05$, Fisher's exact test) as well as TNM stage ($p < 0.05$, Fisher's exact test). However, *Meox1* expression did not show

Table 3. The expression of *Meox1* in paracancerous tissues with or without cirrhosis

Cirrhosis	Number of cases (%)	Low expression	High expression	p-value*
No	7 (28%)	7	0	0.057
Yes	18 (72%)	10	8	

* The Fisher's exact test was used to examine the data.

Table 4. The relationship between the expression of *Meox1* and clinical characteristics in hepatocellular carcinoma (HCC) patients

Variables	Number of cases (%)	Low expression	High expression	p-value*
Gender	25	8	17	0.231
Male	22 (88%)	6	16	
Female	3 (12%)	2	1	
Age [years]	25	8	17	0.411
≤60	12 (48%)	5	7	
>60	13 (52%)	3	10	
Pathogeny	25	8	17	0.828
HBV	17 (68%)	6	11	
HCV	3 (12%)	1	2	
unknown	5 (20%)	1	4	
Tumor size [cm]	25	8	17	0.695
≤5	14 (56%)	5	9	
>5	11 (44%)	3	8	
Tumor number	25	8	17	0.140
Single	20 (80%)	8	12	
Multiple	5 (20%)	0	5	
Vascular invasion	25	8	17	0.057
No	18 (72%)	8	10	
Yes	7 (28%)	0	7	
Lymph node metastasis	25	8	17	0.527
No	22 (88%)	8	14	
Yes	3 (12%)	0	3	
TNM stage	25	8	17	0.008
I–II	15 (60%)	8	7	
III–IV	10 (40%)	0	10	
BCLC stage	25	8	17	0.022
0+A	16 (64%)	8	8	
B+C	9 (36%)	0	9	

* The Fisher exact test was used to examine the data; HBV – hepatitis B Virus; HCV – hepatitis C virus; TNM – tumor-node-metastasis; BCLC – Barcelona Clinic Liver Cancer staging.

any statistically significant relationships with other clinico-pathological traits such as age, sex, tumor size, tumor number, vascular invasion, and lymph node metastasis (Table 4). In addition, *Meox1* expression and clinical parameters (Table 5), such as alpha-fetoprotein (AFP), carcinoma embryonic antigen (CEA), CA125, CA199, prothrombin time (PT), albumin, as well as total bilirubin, did not significantly correlate, according to Spearman's analysis. These results showed that abnormal expression of *Meox1* may be correlated with the progression of HCC.

Gene expression of *Meox1* in HCC

We further investigated the gene expression of *Meox1* in HCC using the Gene Expression Profiling Interactive Analysis (GEPIA) database. The analysis revealed that the expression of the *Meox1* gene was notably increased in 369 HCC tissues than in 160 normal tissues (Fig. 2A).

Table 5. Spearman's analysis of the correlation between the expression of *Meox1* and clinical parameters

Clinical parameter	<i>Meox1</i> expression	
	Spearman's correlation	p-value*
AFP	0.188	0.369
CEA	0.23	0.303
CA125	–0.272	0.246
CA199	0.097	0.683
PT	–0.327	0.171
Albumin	0.206	0.384
Total bilirubin	0.114	0.633

* The correlation between *Meox1* expression and clinical parameters were examined using Spearman's analysis; AFP – alpha-fetoprotein; CEA – carcinoma embryonic antigen; CA125 – cancer antigen 125; CA199 – cancer antigen 199; PT – prothrombin time.

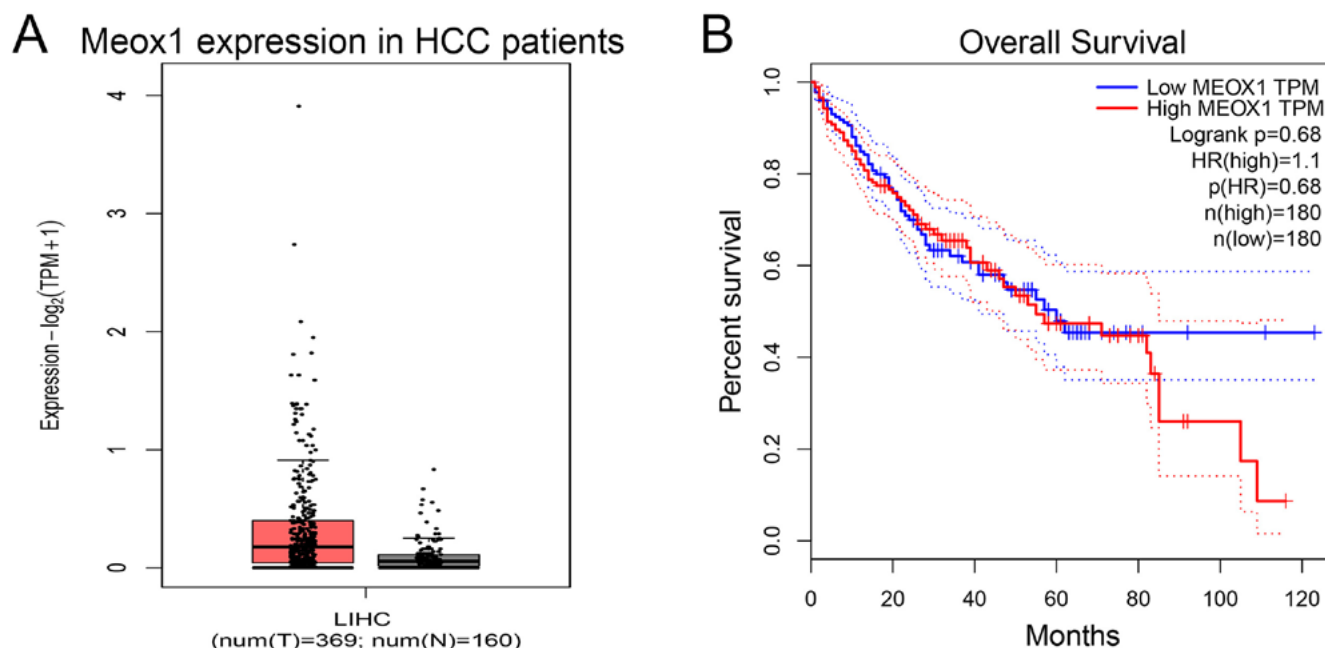


Fig. 2. The expression of *Meox1* in patients with liver hepatocellular carcinoma (LIHC) and overall survival (OS) curves. A. Data from the Gene Expression Profiling Interactive Analysis (GEPIA) database showed that the expression of *Meox1* was higher in hepatocellular carcinoma (HCC) tissues than in normal tissues; B. Survival curves for LIHC patients from the GEPIA database [$p = 0.68$, Kaplan–Meier method, log-rank test].

However, the survival analysis suggested that high *Meox1* expression may not be significantly correlated with overall survival (OS) in HCC (Fig. 2B).

Meox1 silencing suppressed HCC cell malignancy in vitro

To explore the role of *Meox1* in HCC cell malignancy, *Meox1* expression was reduced by infecting HCC cells (Huh7 and Hep3B cells) with recombinant lentiviruses containing its particular shRNA (sh-*Meox1*). As shown in Fig. 3A and 3B, HCC cell lines with stable *Meox1* knockdown were successfully constructed, and the knockdown efficiency was confirmed. Initially, we observed the function of *Meox1* in cell proliferation using CCK-8 assay. The results indicated that the growth rate of Huh7 cells with knockdown using 2 sh-*Meox1* was significantly decreased compared to those in sh-NC and the blank control at 72 h, 96 h and 120 h (Fig. 3C, $p < 0.05$, respectively, Kruskal–Wallis H test). The same phenomenon was observed in Hep3B cells (Fig. 3D, $p < 0.05$, Kruskal–Wallis H test), indicating that *Meox1* silencing suppressed the proliferation of HCC cells. In addition, colony formation assays revealed that the size and the number of colonies were markedly decreased in both Huh7 and Hep3B cells with *Meox1* knockdown compared to those in the sh-NC and blank control groups (Fig. 4, $p < 0.05$, Kruskal–Wallis H test). The data suggested that *Meox1* silencing inhibited colony formation of HCC cells and may exert a negative effect on cell self-renewal function.

Cell migration and invasion are important characteristics of malignancy.^{24,25} Therefore, we assessed

the contribution of *Meox1* in HCC cell migration and invasion using a transwell (An experimental method used for the study of biological processes such as cell migration and invasion) assay (Fig. 5). The findings demonstrate that the migration of cells significantly decreased when *Meox1* was knocked down in both Huh7 and Hep3B cells, compared to the sh-NC and blank control groups. In Huh7 and Hep3B cells with *Meox1* knockdown, the number of invading cells notably decreased, indicating that *Meox1* regulates both migration and invasion properties in HCC cells. Based on the data, silencing *Meox1* reduced the malignancy of HCC cells and may be critical for HCC development.

Discussion

The discovery of novel and effective therapeutic targets for HCC treatment is crucial for clinical application. Accumulated evidence has shown that *Meox1* is closely related to the progression of several cancers, including lung,¹⁵ breast^{17,18} and ovarian cancer.¹⁶ Studies have confirmed that *Meox1* expression in tumors was increased. This study aimed to evaluate *Meox1* expression in HCC and investigate how it affected the biological functions of HCC cells.

We first assessed the expression level of *Meox1* in HCC through IHC and bioinformatics analysis. The findings demonstrated a notable upregulation of *Meox1* in HCC tissues, aligning with previous studies conducted on lung, breast and ovarian cancer. Importantly, our observations indicated a notable increase in *Meox1* expression levels in HCC tissues from patients with cirrhosis compared

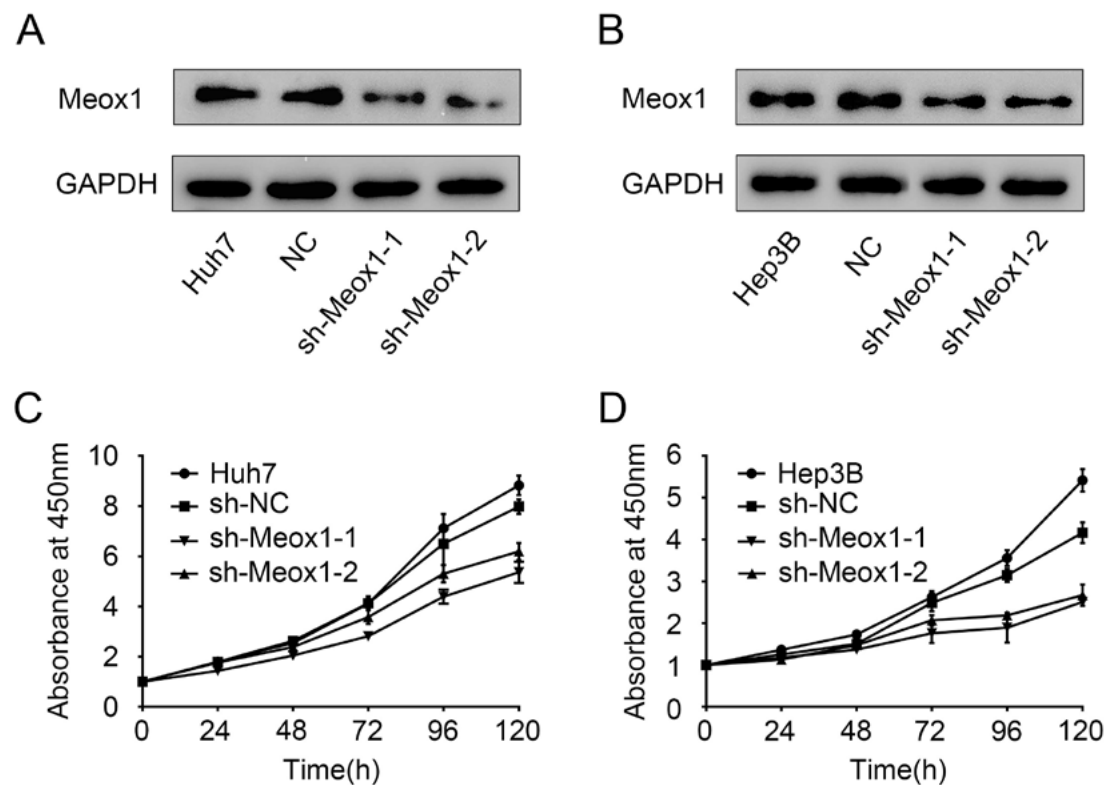


Fig. 3. *Meox1* gene silencing inhibits the proliferation of hepatocellular carcinoma (HCC) cells. Stable cell clones with *Meox1* knockdown were established by infecting HCC cells (Huh7 and Hep3B) with recombinant lentivirus carrying its specific shRNA (sh-*Meox1*) and negative control shRNA (sh-NC) with puromycin selection. A. The expression of *Meox1* in *Meox1* knockdown Huh7 cell lines was detected using western blot analysis ($n = 3$); B. The expression of *Meox1* in *Meox1* knockdown Hep3B cell lines was detected with western blot analysis ($n = 3$). Cell lines in the logarithmic growth phase were resuspended and seeded into 96-well plates at a density of 1×10^3 cells/well. Ten microliters of Cell Counting Kit-8 (CCK-8) were added to each well at the indicated times (0, 24, 48, 72, 96, and 120 h) and then incubated for 2 h at 37°C in a 5% humidified CO_2 incubator. The optical density (OD) value was measured at a wavelength of 450 nm every 24 h using a microplate reader; C. Proliferation effect of *Meox1* in Huh7 cells. The growth rate of Huh7 cells with knockdown using 2 sh-*Meox1* was significantly decreased compared to that with sh-NC and the blank control at 72 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test); the same phenomenon was also found at 96 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test) and 120 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test); D. Proliferation effect of *Meox1* in Hep3B cells. The growth rate of Hep3B cells with knockdown using 2 sh-*Meox1* was significantly decreased compared to that with sh-NC and the blank control at 72 h ($n = 3$, $p < 0.05$, the Kruskal–Wallis H test); the same phenomenon was also found at 96 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test) and 120 h ($n = 3$, $p < 0.05$, Kruskal–Wallis H test).

to those without cirrhosis. Advanced liver fibrosis and cirrhosis are principle risk factors for HCC, and up to 90% of cases are based on this background.^{26,27} The tumor microenvironment (TME) has a vital function in progression of HCC. Liver fibrosis can promote HCC progression by regulating the TME, including cytokine secretion, immune surveillance, tumor angiogenesis, and extracellular matrix synthesis.^{28,29} According to numerous research, *Meox1* is crucial in organ fibrosis.^{23,30–33} More recently, 1 study found that *Meox1* is a central regulator in the transformation of fibroblasts to profibrotic myofibroblasts and is necessary for tumor growth factor beta (*TGF β*)-induced fibroblast activation.³³ In addition, *Meox1* may promote hepatic stellate cell (HSC) activation, which is a crucial event during liver fibrosis.³³ We speculated that abnormal *Meox1* expression might be closely linked to liver fibrosis as well as HCC and involved in the TME, but this hypothesis needs to be further explored.

Previous data from breast cancer have shown that elevated *Meox1* is linked to an advanced tumor stage and poor OS.¹⁷ Another study on lung cancer also indicated

that increased *Meox1* promoted tumor progression and contributed to shorter OS, increased lymph node metastasis, and advanced stage of HCC, and was an independent poor prognosis predictive factor identified with Cox multivariate regression analysis.¹⁵ We further evaluated the potential implication of *Meox1* in HCC, and clinical relevance analysis showed that high *Meox1* expression was positively correlated with advanced tumor stage. However, the survival analysis from GEPIA databases indicated that high *Meox1* expression may not be significantly correlated with OS in HCC. We speculate that this variation may be brought about by *Meox1*'s various tumor-specific actions.

Meox1 is considered an important transcription factor that promotes tumor cell growth in ovarian cancer.¹⁶ Inhibition of *Meox1* expression also effectively suppressed the proliferation of lung cancer¹⁵ and breast cancer cells.^{17,18} We constructed *Meox1* stable knockdown HCC cell lines to examine *Meox1*'s role in HCC. It was found that inhibiting *Meox1* expression significantly suppressed the proliferation and colony formation of 2 different HCC cell lines. These results imply that *Meox1* may be crucial

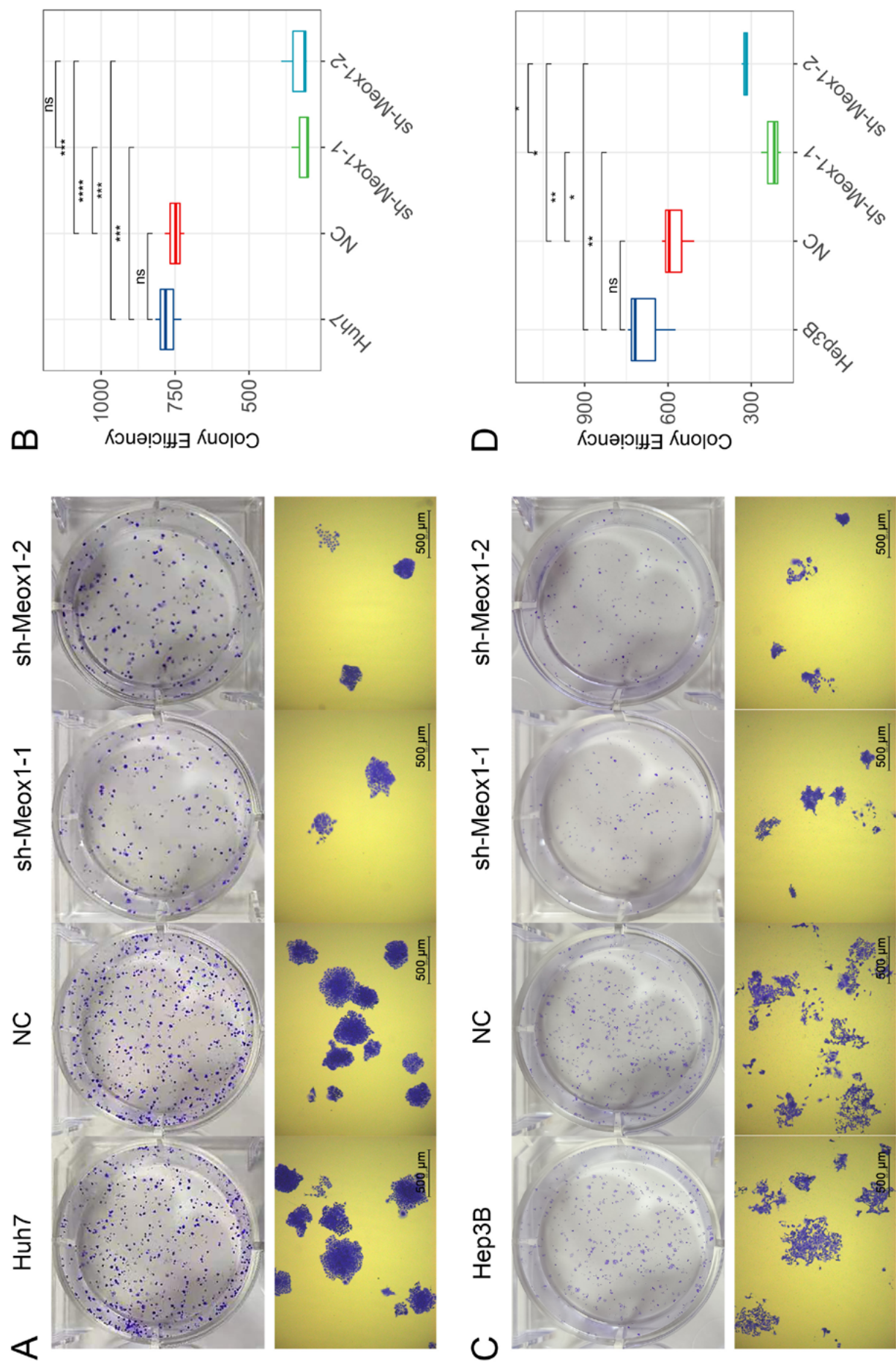


Fig. 4. *Meox1* gene silencing inhibits the colony formation of hepatocellular carcinoma (HCC) cells. **A.** *Meox1* gene silencing inhibits colony formation of Huh7 cells; **B.** Quantification of results from colony formation in **A**; the number of colonies were markedly decreased in Huh7 cells with *Meox1* knockdown compared to those in the sh-NC and blank control groups ($n = 3$, $p < 0.05$, Kruskal–Wallis H test); **C.** *Meox1* gene silencing inhibits colony formation of Hep3B cells; **D.** The quantification of results from colony formation in **C**; the number of colonies were markedly decreased in Hep3B cells with *Meox1* knockdown compared to those in the sh-NC and blank control groups ($n = 3$, $p < 0.05$, Kruskal–Wallis H test)

ns – no significant difference; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

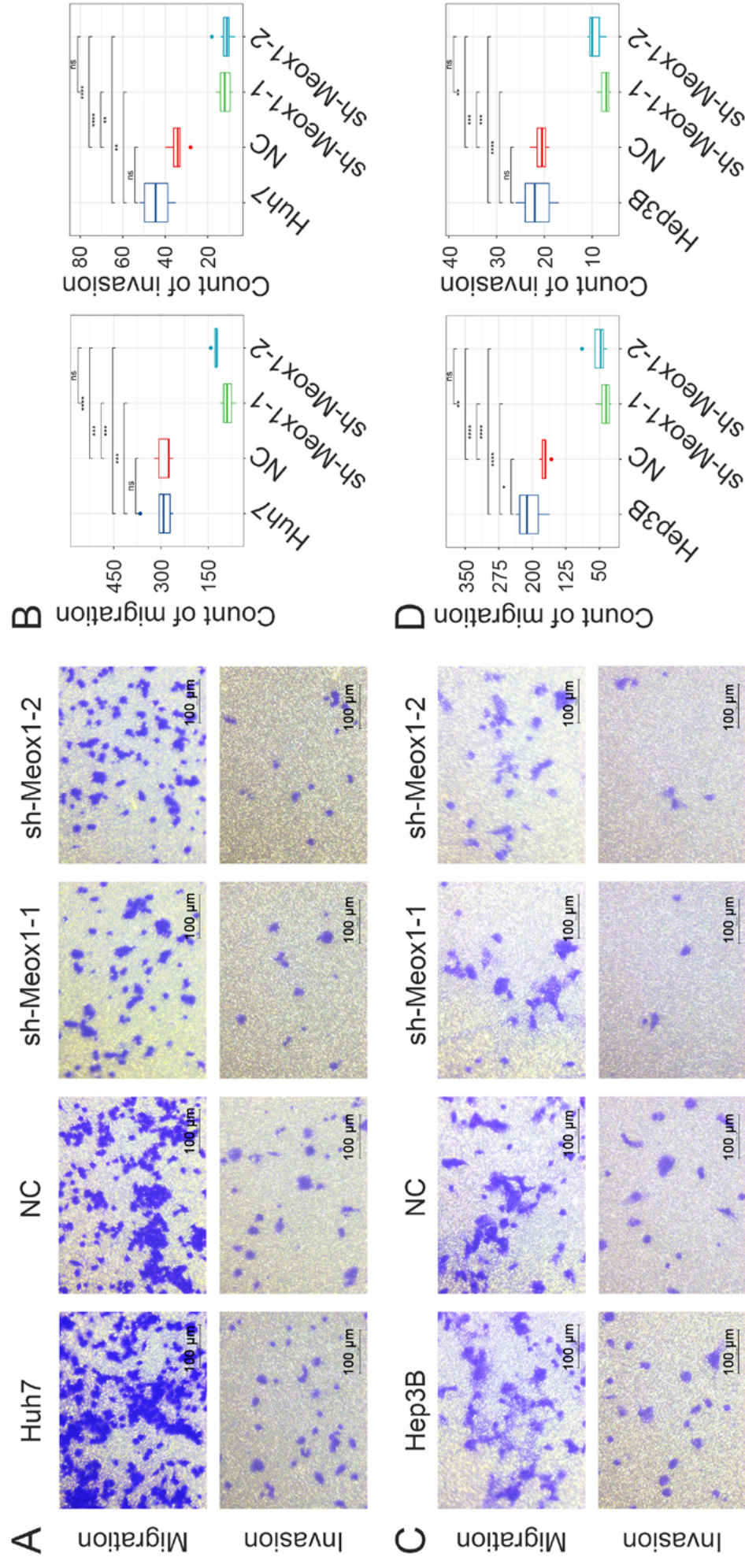


Fig. 5. *Meox1* gene silencing inhibits the migration and invasion of hepatocellular carcinoma (HCC) cells. The cell lines were seeded into the Transwell (an experimental method used for the study of biological processes such as cell migration and invasion) chamber at a density of 1×10^4 /well and incubated for 24 h for the migration assay and at a density of 3×10^4 /well, and then incubated for 48 h for the invasion assay. After the indicated time, the cells were fixed with methyl alcohol for 30 min, followed by staining with 0.5% crystal violet for 20 min. The stained cells were counted under an inverted microscope in 5 randomly selected fields at a magnification of $\times 100$. **A:** Effect of *Meox1* knockdown on the migration and invasion of Huh7 cells ($n = 3$). **B:** Quantification of results from migration in **A**; the migration of cells was significantly decreased when compared to the negative control shRNA (sh-NC) and blank control groups when *Meox1* was knocked down in Huh7 cells (left, $p < 0.05$, Kruskal–Wallis H test). The quantification of results from invasion in **A**; the invasion of cells was significantly decreased when compared to the sh-NC and blank control groups when *Meox1* was knocked down in Huh7 cells (right, $p < 0.05$, Kruskal–Wallis H test). **C:** Effect of *Meox1* knockdown on the migration as well as invasion of Hep3B cells ($n = 3$). **D:** The quantification of results from migration in **C**; the migration of cells was significantly decreased when compared to the sh-NC and blank control groups when *Meox1* was knocked down in Hep3B cells (left, $p < 0.05$, Kruskal–Wallis H test). The quantification of results from invasion in **C**; the invasion of cells was significantly decreased when compared to the sh-NC and blank control groups when *Meox1* was knocked down in Hep3B cells (right, $p < 0.05$, Kruskal–Wallis H test)

ns – no significant difference; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

in regulating cell proliferation. Moreover, experiments with shRNA knockdown showed that downregulation of *Meox1* expression reduced migration and invasion in 2 different HCC cell lines, indicating that *Meox1* could regulate HCC cell metastasis. These results suggest that targeting *Meox1* not only decreases the rapid proliferative behavior, but also suppresses the aggressive metastatic potential to reduce the malignancy of HCC cells, and in consideration of the significant correlation between the *Meox1* and vascular invasion and advanced tumor stage in HCC, it is plausible that abnormal expression of *Meox1* may accelerate the progression of HCC. Future investigations should involve HCC clinical research with large samples, and HCC animal experiments are warranted to verify the role of *Meox1* in HCC. The detailed molecular mechanism of the role of *Meox1* in HCC progression deserves further research.

Limitations

The study may have been conducted in a specific HCC model or population, which means that the results may not be applicable to other types of cancers or populations.

Conclusions

Our studies found that *Meox1* was highly expressed in HCC tissues, especially in HCC with cirrhosis, and was closely correlated with advanced stage of HCC. Moreover, *Meox1* silencing suppressed the proliferation, colony formation, migration, and invasion of HCC cells. Given the physiological significance of *Meox1* in proliferation and metastatic features and the implications of these findings for the progression of HCC, targeting *Meox1* may offer a possible strategy for adjuvant and combination therapies of HCC.

Supplementary data

The Supplementary materials are available at <https://doi.org/10.5281/zenodo.10090681>. The package includes the following files:

Supplementary Fig. 1 Kruskal-Wallis H test was utilized for examining the data from colony formation of Huh7 cells.

Supplementary Fig. 2 Kruskal-Wallis H test was utilized for examining the data from colony formation of Hep3B cells.

Supplementary Fig. 3. Kruskal-Wallis H test was utilized for examining the data from migration of Huh7 cells.

Supplementary Fig. 4. Kruskal-Wallis H test was utilized for examining the data from invasion of Huh7 cells.

Supplementary Fig. 5. Kruskal-Wallis H test was utilized for examining the data from migration of Hep3B cells.

Supplementary Fig. 6. Kruskal-Wallis H test was utilized for examining the data from invasion of Hep3B cells.

Data availability


The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.


Consent for publication

Not applicable.


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