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ORIGINAL RESEARCH

TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 Signaling Axis Promotes Viability, Migration, Invasion and EMT of Hepatocellular Carcinoma Cells

Kangjun Zhang[1]
Taishi Fang[1]
Yajie Shao[2]
Yanhui Wu[3]

[1]Hepatic Surgery Department, The Third People's Hospital of Shenzhen, Shenzhen City, Guangdong Province, People's Republic of China; [2]Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan City, Hubei Province, People's Republic of China; [3]Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan City, Hubei Province, People's Republic of China

Correspondence: Yanhui Wu
Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095 Jiefang Avenue, Qiaokou District, Wuhan City, Hubei Province, 430030, People's Republic of China
Tel +86-027-83665313
Email Wuyanhai_yhiw@163.com

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Introduction: Enhancer of zeste homolog 2 (EZH2) is implicated in hepatocellular carcinoma (HCC), but whether transforming growth factor-β (TGF-β)-metastasis associated 1 (MTA1)-SMAD7-SMAD3-SRY-Box Transcription Factor 4 (SOX4)-EZH2 signaling axis, in which EZH2 participates, is also involved in HCC remained unknown.

Methods: Data on EZH2 expression in liver hepatocellular carcinoma (LIHC) and its relation with prognosis of HCC patients were predicted and analyzed using online databases. Following transfection with or without TGF-β1, HCC cell viability, migration and invasion were determined with MTT, Scratch and Transwell assays. Relative expressions of epithelial-to-mesenchymal transition (EMT)-related factors (N-Cadherin, Vimentin, and E-Cadherin) and TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis factors (TGF-β, MTA1, SMAD7, phosphorylated-SMAD3, SOX4 and EZH2) were calculated via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot.

Results: EZH2 was upregulated in HCC, which was related to poor prognosis. Silencing EZH2 suppressed EZH2 expression and HCC cell viability, migration, and invasion, and increased E-Cadherin expression yet decreased N-Cadherin and Vimentin expression, whereas EZH2 overexpression did conversely. Also, silencing EZH2 reversed the effects of TGF-β1 on promoting viability, migration, and invasion, as well as N-Cadherin and Vimentin expressions, yet suppressing E-Cadherin expression in HCC cells. In addition, TGF-β1 promoted TGF-β, MTA1, SOX4 and EZH2 expressions and p-SMAD3/SMAD3 ratio yet suppressed SMAD7, whereas silencing EZH2 solely reversed the effects of TGF-β1 on EZH2 expression in HCC cells.

Conclusion: The present study provides a theoretical basis for TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling cascade in viability, migration, invasion, and EMT of HCC cells. Inhibiting these signals may represent a therapeutic pathway for the treatment of metastatic HCC.

Keywords: hepatocellular carcinoma, enhancer of zeste homolog 2, transforming growth factor-β, metastasis, metastasis associated 1-SMAD7-SMAD3-SRY-box transcription factor 4 axis, MTA1-SMAD7-SMAD3-SOX4 axis

Introduction

As the most prevalent form of liver cancer, hepatocellular carcinoma (HCC), at present, is not only a leading cause of cancer-associated death globally but also a highly aggressive disease with a poor prognosis.[1,2] HCC is also a complicated

disease with a variety of pathogenic mechanisms caused by several risk factors, of which chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and chronic alcohol consumption have been underlined as those predominant, making it difficult to characterize within a single biomarker.[3,4] From approximately 1995 until perhaps 5 years ago, radiofrequency ablation and subsequent microwave ablation have been the dominant percutaneous ablative technologies for the management of HCC. However, the location and number of tumors are critical and can be a limitation for the use of these technologies.[5] Surgical operation has been suggested and considered as the only potentially curative strategy for patients with HCC nowadays; however, chances that patients will be eventually "cured" after resection remain poor and ill-defined.[6] Although a number of consistent alterations to metabolic genes have been identified in HCC, their prognostic values are inadequately discussed and need to be further elucidated in detail.[7] Therefore, it has become urgently needed to further work out a viable therapeutic method and target for HCC.

As the main enzymatic catalytic polycomb repressive complex 2 (PRC2) subunit and one of the most important histone methyltransferases, enhancer of zeste homolog 2 (EZH2) has the capacity on alteration on downstream target gene expressions via Lys-27 trimethylation in histone 3 (H3K27me3) which is a transcriptionally repressive epigenetic mark regulating gene expression, differentiation and development, thus establishing and maintaining H3K27 trimethylation repressive marks.[8,9] A previous study has identified the role of EZH2 in cell proliferation, apoptosis and senescence and the application of targeting EZH2 for cancer therapy.[10] It has been uncovered that EZH2 is overexpressed and can be used as a therapeutic target for ovarian cancer.[11] Also, EZH2 can act as one of the key driving oncogenes and be adopted as the biomarker for aggressive prostate cancer.[12] In addition, EZH2 oncogenic deregulation has been indicated as a possible opportunity for targeted therapy for lung cancer.[13] Furthermore, the implication and the promotive effects of EZH2 in HCC development and progression have been additionally discovered.[14] Previous studies have also shown that EZH2 could modulate transforming growth factor-β (TGF-β) pathway in HCC, and the implication of TGF-β-Metastasis Associated 1 (MTA1)-SRY-Box Transcription Factor 4 (SOX4)-EZH2 signaling axis in tumor metastasis and epithelial-to-mesenchymal transition (EMT).[15,16] Nevertheless, whether TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis was involved in HCC awaited to be further elucidated. Therefore, we aimed to discover and discuss the detailed role of EZH2 and the possible participation of TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in HCC cells, hoping to work out a viable therapy for HCC in the future.

Materials and Methods

Ethical Statement

The current study has been approved by the ethics committee of The Third People's Hospital of Shenzhen (serial number: NK2017090101), and also obtained the written informed consents from recruited patients who agreed the usage of their tissue in our study. This study was conducted in accordance with the Declaration of Helsinki.[17]

Bioinformatic and Survival Analysis

To determine the role of EZH2 played in HCC, data on EZH2 expression in liver hepatocellular carcinoma (LIHC) was collected from gene expression profiling interactive analysis 2 (GEPIA 2, http://gepia2.cancer-pku.cn/#analysis).

In order to assess and evaluate EZH2 expression in HCC patients, patients were assigned to high or low EZH2 groups, and their survival rates were predicted using Kaplan–Meier Plotter (http://kmplot.com/analysis/index.php), and monitored and determined every 10 months. Survival analysis was plotted via Kaplan–Meier Curve.

Clinical Samples

Clinical samples used for our current study were collected from tissues of patients with HCC and normal healthy patients in The Third People's Hospital of Shenzhen from 2017 September to 2019 August (n=70 for each). All patients enrolled were conformed to the criteria: (a) no pre-surgery chemotherapy or radiotherapy; (b) no other cancers, autoimmune diseases, or infectious diseases, and so on. All tissue samples were carefully collected during surgery and were washed with phosphate buffered saline (PBS, P1022, Solarbio, Beijing, China) followed by being stored in −80°C refrigerator for subsequent analysis. Meanwhile, we also collected and summarized the basic characteristics of patients, including sex, age, tumor size, hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol consumption, serum alpha-fetoprotein (AFP) level, Number (No.) of tumor nodules, Cirrhosis; Venus

infiltration, Edmondson-Steiner grading, and tumor-node-metastasis (TNM) stage, which are detailed in Table 1. The patients were assigned to high- or low-EZH2 expression groups in accordance with the median of the RNA expression level of EZH2.

Cell Culture and Treatment

Human liver epithelial cell-line THLE-2 (BNCC322618), and HCC cell line (SNU-182, BNCC353594; SUN-387, BNCC253492; SNU-423, BNCC234890; and PLC/PRF/5, BNCC293647) were ordered from BeiNa Bio (Beijing, China). THLE-2 cells were grown in RPMI-1640 medium (90022, Solarbio, China) with 10% fetal bovine serum (FBS, F2442, Sigma-Aldrich, St. Louis, MO, USA), and SNU-182, SUN-387, and SNU-423 cells were maintained in RPMI-1640 medium with 10% FBS, 20 mmol/L 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, H4034, Sigma-Aldrich, USA), 2 g/mL sodium bicarbonate (S5761, Sigma-Aldrich, USA) and 100 U/mL-100 mg/mL penicillin-streptomycin (P4333, Sigma-Aldrich, USA). In addition, PLC/PRF/5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 90113, Solarbio, China) with 10% FBS and 1% penicillin-streptomycin. All the cells were cultured at 37°C with 5% CO_2. As EZH2 expression was highest in PLC/PRF/5 cells, these two cells were used for our studies. Also, TGF-β1 (T1654) was obtained from Sigma-Aldrich and was added into HCC PLC/PRF/5 cells at a concentration of 5 ng/mL for 24 hours as confirmed in a previous study.[18]

Transfection

Short hairpin RNA targeting EZH2 (shEZH2) was purchased from Gene Pharma (Shanghai, China). EZH2 overexpression was made with pcDNA 3.1 plasmid (VT1001, Youbio, Changsha, China), and the controls for both shEZH2 and EZH2 (Vector) were additionally obtained from Gene Pharma. For transfection, 1×10^6 cells/well HCC cells were cultured in 6-well plates and when they became 90% confluence, transfection was performed at room temperature (RT) using Lipofectamine 2000 reagent (11668–030, Invitrogen, Carlsbad, CA, USA). Sequence for transfection is provided in Table 2.

MTT Assay

5×10^3 cells/well treated or transfected HCC cells were cultured in 96-well plates at 37°C, 5% CO_2 for 0, 24 and 48 hours. 10 μL MTT reagent (C0009S, Beyotime, Shanghai, China) was added into each well at 37°C for another 4 hours. OD value was recorded using iMark microplate reader (Bio-Rad, Hercules, CA, USA) at an absorbance of 570 nm.

Table 1 Basic Characteristics of Patients

Characteristics	EZH2 Expression		*P* value
	Low (n = 35)	High (n = 35)	
Sex			0.607
male	23	25	
Female	12	10	
Age (y)			0.322
< 50	15	11	
≥50	20	24	
HCV			0.614
No	32	34	
Yes	3	1	
Alcohol consumption			0.629
No	16	14	
Yes	19	21	
Tumor size (cm)			0.007
≤ 4	19	8	
> 4	16	27	
HBV			0.467
Absent	16	13	
Present	19	22	
Serum AFP level (ng/mL)			0.470
< 20	17	14	
≥20	18	21	
No. of tumor nodules			0.303
1	22	26	
≥2	13	9	
Cirrhosis			0.337
Absent	18	14	
Present	17	21	
Venous infiltration			0.212
Absent	25	20	
Present	10	15	
Edmondson-Steiner grading			0.007
I-II	27	16	
III-IV	8	19	
TNM stage			0.008
I-II	24	13	
III-IV	11	22	

Abbreviations: HBV, hepatitis B virus; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis; HCV, hepatitis C virus.

Table 2 Sequences for Transfection

Gene	Sequence (5′->3′)
shEZH2 sense obligo	CCGGGTTGGAGACTGCTGGTTTAAACTCG AGTTTAAACCAGCAGTCTCCAACTTTTTG
shEZH2 antisense obligo	AATTCAAAAAGTTGGAGACTGCTGGTTTA AACTCGAGTTTAAACCAGCAGTCTCCAAC

Scratch Assay

1×10^5 cells/well transfected or treated HCC cells were grown in a 6-well plate, and after cells reached approximately 80% confluence, an artificial scratch was made using a sterile pipette tip. HCC cells were further cultured in a serum-free medium at 37°C, 5% CO_2. Cell images were observed, and photos were taken in an inverted optical microscope (GX71, Olympus, Tokyo, Japan) at 0 and 24 hours under × 100 magnification.

Transwell Assay

1×10^5 cells/well treated or transfected HCC cells with 200 μL non-serum medium were transferred to Transwell chambers of 8-μm pore (CLS-3464, Sigma-Aldrich, USA) which have been pre-coated with Matrigel (356237, Corning, Inc., Corning, NY, USA) at 37°C with 5% CO_2, and 600 μL complete medium was added into corresponding lower Transwell chamber. 24 hours later, all invaded cells were fixed in Methanol (322415, Sigma-Aldrich, USA) and stained using 0.1% Giemsa (C0133, Beyotime, China). All cells were calculated in five randomly picked fields, and photos were taken in an inverted optical microscope under × 250 magnification.

RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Trizol (T9424, Sigma-Aldrich, USA) was used for total RNA extraction of treated or transfected HCC cells and tissues. All RNA extracted was subsequently preserved in −80°C. A NanoDrop Lite spectrophotometer (ND-LITE, Thermo Fisher Scientific, Waltham, MA, USA) was used for concentration determination. CDNA was then synthesized via a first strand cDNA synthesis kit (D7178S, Beyotime, China), and RT-qPCR was conducted by RT-qPCR kit (D7277S, Beyotime, China) and operated in Touch real-time PCR system (CFX86, Bio-Rad, USA) under the conditions: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 62°C. Relative expressions were calculated using the $2^{-\Delta\Delta CT}$ method, in which GAPDH was used as an internal reference.[19] Primer sequences are referred to in Table 3.

Western Blot

Relative protein expressions of migration and invasion-related (N-Cadherin, Vimentin, and E-Cadherin) and TGF-β1-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis-related factors (TGF-β; MTA1; SMAD7; phosphorylated-SMAD3, p-SMAD3; SMAD3; SOX4; and EZH2) were measured via Western blot as previously described.[16] Total protein was lysed and extracted with RIPA buffer (P0013C, Beyotime, China) after treated or transfected HCC cell collection, and the concentration of protein was measured with bicinchoninic acid (BCA) protein reagent (P0012S, Beyotime, China). 30 μg sample protein lysates were electrophoresed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (P0012A, Beyotime, China), and transferred into polyvinylidene fluoride (PVDF) membrane (FFP33, Beyotime, China) blocked with fat-free milk (5%) for 2 hours and incubated in indicated primary antibodies, including those against N-Cadherin (ab18203, 1:2000, 130 kDa), Vimentin (ab92547, 1:5000, 54 kDa), E-Cadherin (ab231303, 1:2000, 97

Table 3 Primers for RT-qPCR

Gene	Primers (5′->3′)
EZH2	
Forward Reverse	GAAGTTTTAGATCAGGATGG TGTCTAGAGCTGTTTCTGTG
N-Cadherin	
Forward Reverse	GCTGGTATCTATGAAGTTCC TAGGTGTAAGCTCTCTATGG
Vimentin	
Forward Reverse	CTTAAAGGAACCAATGAGTC GAGAAGTTTCGTTGATAACC
E-Cadherin	
Forward Reverse	GATGATGTGAACACCTACAA GTAGCTATGATTAGGGCTGT
GAPDH	
Forward Reverse	TTTTTGGTTTTAGGGTTAGTTAGTA AAAACCTCCTATAATATCCCTCCTC

kDa), TGF-β (ab215715, 1:1000, 44 kDa), MTA1 (ab71153, 1:2000, 81 kDa), SMAD7 (ab90086, 1:500, 46 kDa), p-SMAD3 (ab52903, 1:2000, 48 kDa), SMAD3 (ab40854, 1:2000, 55 kDa), SOX4 (ab70598, 1:2000, 48 kDa) and EZH2 (ab191080, 1:500, 93 kDa) at 4°C overnight, and the membrane was also incubated with primary antibodies against internal control GAPDH (ab8245, 1:2000, 36 kDa) at 4°C overnight. The membrane was incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit IgG H&L (ab205718, 1:2000) and goat anti-mouse IgG H&L (ab205719, 1:2000) for an hour at RT and washed using tris-buffer saline tween (TBST, T1085, Solarbio, China) for three times. All primary and secondary antibodies were ordered from Abcam (Cambridge, UK).

Enhanced chemiluminescence (ECL) reagent (P0018S, Beyotime, China) was finally used for visualization after protein band collection. Data were analyzed with iBright CL750 Imaging System (A44116, Thermo Fisher Scientific, USA) and grey values were calculated via ImageJ (ver. 5.0, Bio-Rad, USA).

Statistical Analysis

Each experiment was repeated over three times in an independent manner. Data were expressed as mean ± standard deviation (SD). Survival rates of HCC patients were analyzed using Kaplan–Meier Curve. GraphPad 8.0 (GraphPad, Inc., La Jolla, CA, USA) and SPSS 20.0 (SPSS, Chicago, IL, USA) were both used for statistical analysis. Statistical significance was determined by one-way ANOVA followed by Dunnett or Tukey's post hoc test and paired *t* test. Statistical significance was determined when $P<0.05$.

Results

EZH2 Was Upregulated in HCC, Which Was Related to Poor Prognosis of Patients

To determine the role of EZH2 played in HCC, data on EZH2 expression in LIHC was firstly collected from GEPIA 2, which showed an upregulated EZH2 expression in LIHC (Figure 1A, $P<0.05$). Also, results from Kaplan–Meier Plotter showed that high EZH2 expression was related to poor survival rate of HCC patients (Figure 1B). In addition, our data on patients with HCC presented that high EZH2 expression was significantly associated with tumor size, Edmondson-Steiner grading and TNM tumor stage (Table 1). Furthermore, we discovered that EZH2 expression was increased in both HCC tissue and cells, with highest expression in PLC/PRF/5 cells (Figure 1C and D, $P<0.001$). Therefore, PLC/PRF/5 cells were used for subsequent studies.

EZH2 Regulated EZH2 Expression and Cell Viability, Migration and Invasion and Epithelial-to-Mesenchymal Transition (EMT)-Related Markers in HCC Cells

Then, we transfected shEZH2 and EZH2 overexpression plasmid into HCC cells to further determine the role and effect of EZH2 played in HCC, and silencing EZH2 suppressed EZH2 expression and cell viability at 24 and 48 hours in HCC cells (Figure 2A–D, $P<0.05$). Also, results from Scratch assay showed that silencing EZH2 inhibited HCC cell migration (Figure 2E and F, $P<0.001$). However, overexpressed EZH2 promoted EZH2 expression and cell viability and migration in HCC cells (Figure 2A–F, $P<0.001$). Furthermore, according to the results of Transwell assay, silencing EZH2 suppressed, whereas EZH2 overexpression promoted HCC cell invasion (Figure 3A and B, $P<0.001$). As decreased E-cadherin and increased Vimentin/N-cadherin expression are conventional EMT markers, we subsequently measured their expressions in transfected HCC cells and silencing EZH2 in HCC cells suppressed N-Cadherin and Vimentin yet promoted E-Cadherin, whereas EZH2 overexpression led to dropped E-Cadherin yet raised N-Cadherin and Vimentin in HCC cells (Figure 3C–E, $P<0.05$).

Silencing EZH2 Reversed the Effects of TGF-β1 on Viability, Migration and Invasion in HCC Cells

We also transfected shEZH2 and treated the cells with TGF-β1 to detect the effects of EZH2 and TGF-β1 on HCC cells. It was discovered from the results of MTT assay and Scratch assay that TGF-β1 had a promotive effect on cell viability at 24 and 48 hours and cell migration, whereas silencing EZH2 reversed the promotive effects of TGF-β1 (Figure 4A–C, $P<0.05$). Meanwhile, in the results of Transwell assay, TGF-β1 promoted cell invasion, while silencing EZH2 reversed the effects of TGF-β1 (Figure 5A and B, $P<0.05$).

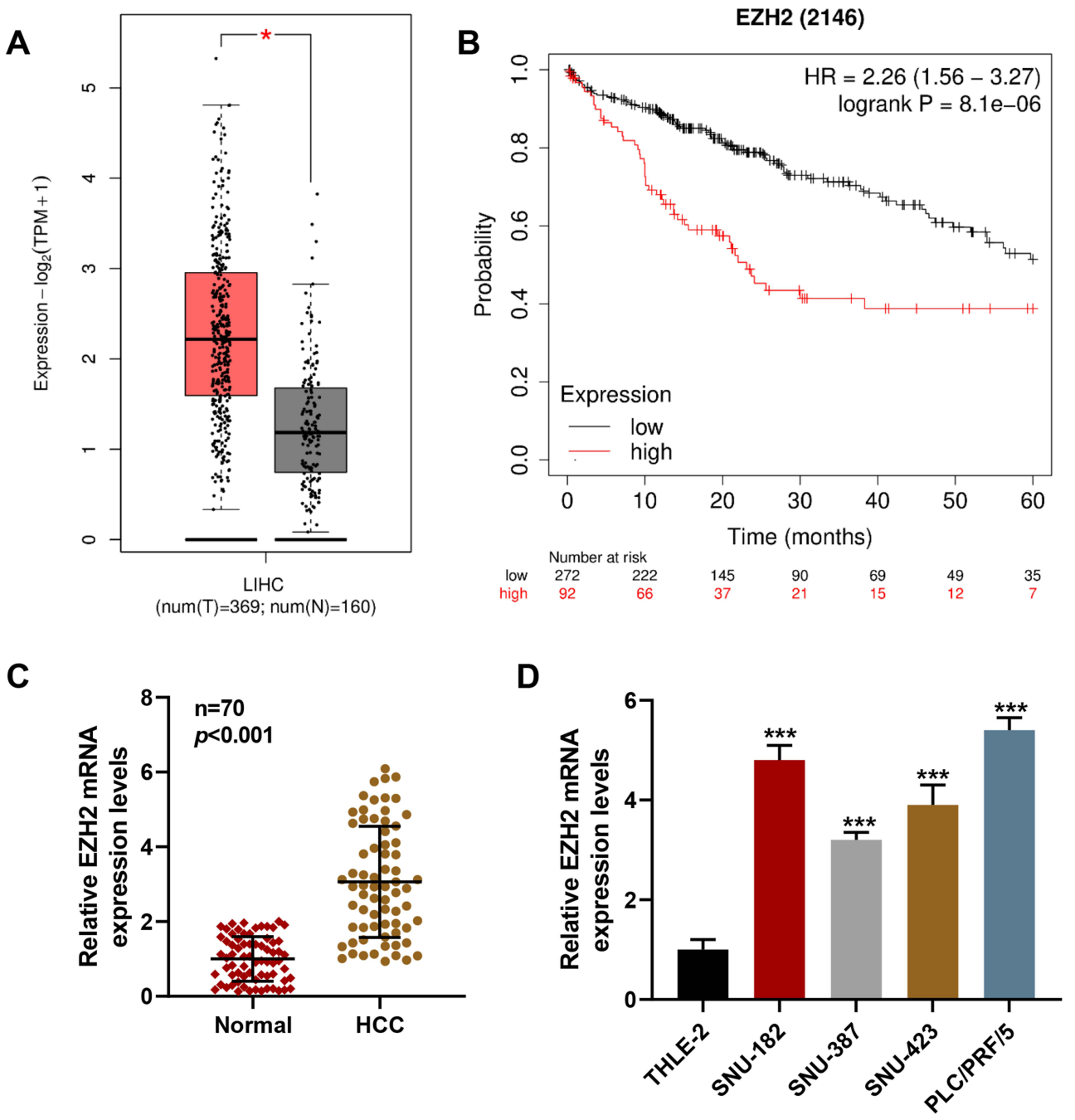

Figure 1 EZH2 was upregulated in HCC, which was associated with poor prognosis of patients. (**A**) Data on EZH2 expression in LIHC were collected from GEPIA 2 (http://gepia2.cancer-pku.cn/#analysis). (**B**) Survival probability of patients with HCC was predicted using Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php). (**C**) Relative EZH2 expression in HCC and Normal tissue was measured via RT-qPCR. GAPDH was used as internal control. (**D**) Relative EZH2 expression in HCC cells (SNU-182, SNU-387, SNU-423, and PLC/PRF/5) and liver epithelial cell line THLE-2 was quantified via RT-qPCR. GAPDH was used as internal control. All experiments have been performed in triplicate and data were expressed as mean ± standard deviation (SD). (**A**) *P<0.001, vs Non-LIHC (N); (**D**) ***P<0.001, vs.

Abbreviations: THLE-2. EZH2, enhancer of zeste homolog 2; LIHC, liver hepatocellular carcinoma; GEPIA 2, gene expression profiling interactive analysis 2; HCC, hepatocellular carcinoma; RT-qPCR, quantitative real-time polymerase chain reaction.

Furthermore, based on results, TGF-β1 resulted in increased N-Cadherin and Vimentin yet decreased E-Cadherin (Figure 5C–E, P<0.05). However, silencing EZH2 reversed the effects of TGF-β1 on promoting N-Cadherin and Vimentin yet suppressing E-Cadherin in HCC cells (Figure 5C–E, P<0.01).

Silencing EZH2 Solely Reversed Effects of TGF-β1 on EZH2 Expression in TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 Signaling Axis in HCC Cells

To further detect the mechanism, we measured expressions of proteins related to TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in HCC cells following TGF-β1 and silencing EZH2. It was found that TGF-β1 promoted TGF-β, MTA1, SOX4 and EZH2 expression and p-SMAD3/SMAD3 ratio yet suppressed SMAD7, however, silencing EZH2 solely reversed the effects of TGF-β1 on EZH2 expression in HCC cells, while it had no effects on TGF-β, MTA1, SMAD7 and SOX4 expressions,

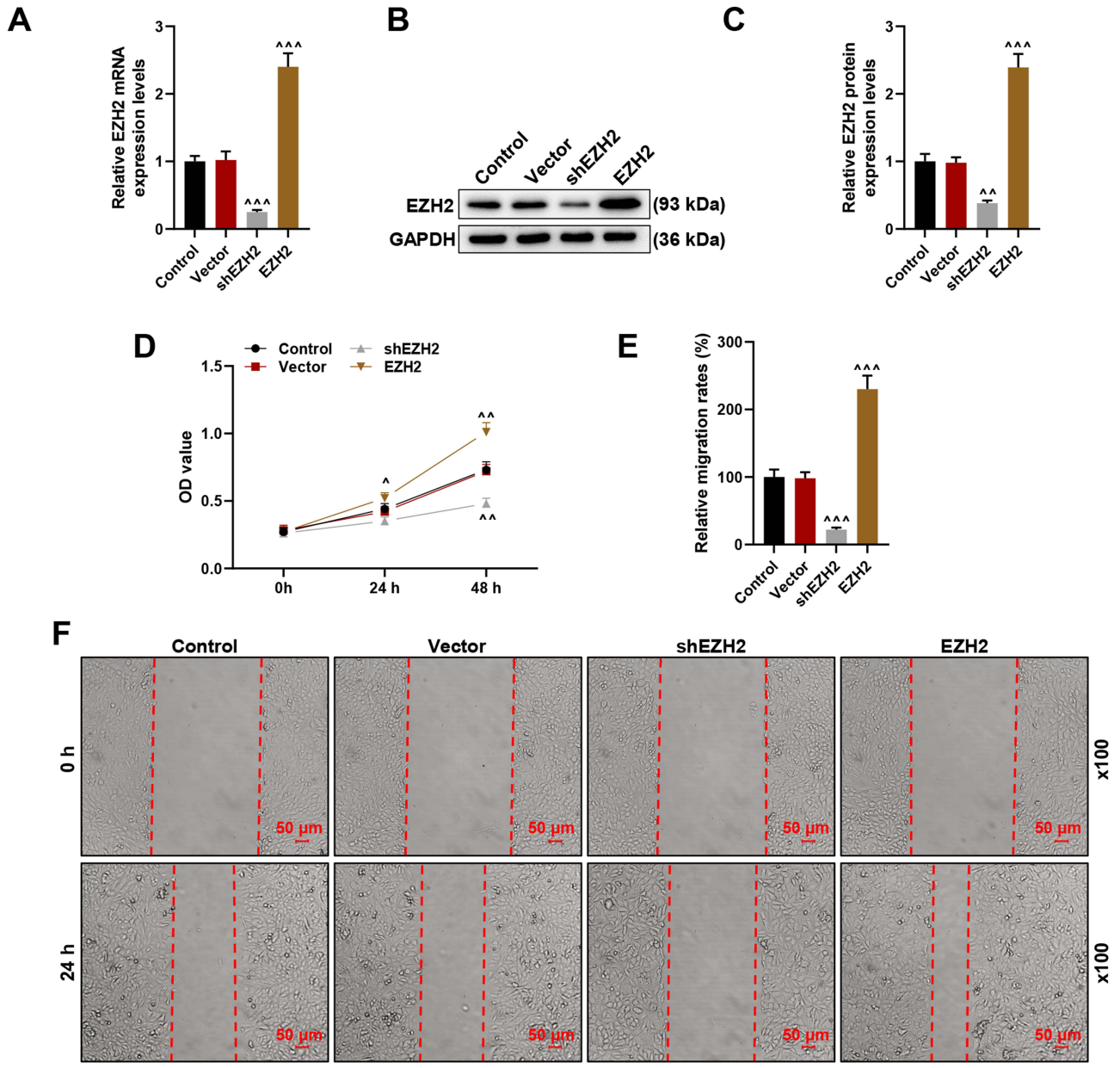

Figure 2 Silencing EZH2 suppressed EZH2 expression and HCC cell viability and migration, whereas EZH2 overexpression did conversely. (**A**) Relative EZH2 mRNA expression following shEZH2 or EZH2 overexpression plasmid transfection in HCC cell was calculated via RT-qPCR. GAPDH was chosen as internal reference. (**B** and **C**) Relative EZH2 protein expression following shEZH2 or EZH2 overexpression plasmid transfection in HCC cell was calculated via Western blot. GAPDH was chosen as internal reference. (**D**) MTT assay was used to determine the viability of HCC cells following shEZH2 or EZH2 overexpression plasmid transfection at 0, 24 and 48 hours. (**E** and **F**) Scratch assay were used to detect the migration of HCC cells at 0 and 24 hours following shEZH2 or EZH2 overexpression plasmid transfection. Magnification: × 100. All experiments have been performed in independent triplicate and data were expressed as mean ± standard deviation (SD). ^P<0.05, ^^P<0.01, ^^^P<0.001, vs Vector. **Abbreviation:** shRNA, short hairpin RNA.

as well as p-SMAD3/SMAD3 ratios (Figure 6A–C, $P<0.01$). All the results further verified the TGF-β-MTA1- SMAD7-SMAD3-SOX4-EZH2 signaling cascade in HCC cells (Figure 7).

Discussion

For those large and diverse families, which have epigenetically repressed effects on transcription of key developmental genes, the polycomb group (PcG) proteins are those which help maintenance on gene-expression pattern of cells set in early development via regulation of chromatin structure, of which PRC2 has been identified as one of the main PcG complexes.[20,21] EZH2, additionally, has been recognized as those genes encoding proteins, which comprise the core components of PRC2.[22] Increasing evidence has addressed the role of EZH2 in different human malignancies, including ovarian cancer, prostate cancer, lung cancer and even HCC.[11–14] in our study, we provided another evidence of EZH2 on HCC and its interaction with TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in HCC cells. To begin with, we discovered that EZH2 was upregulated in HCC, which was related to poor prognosis. We also found that EZH2 overexpression promoted HCC cell viability, migration and invasion, with upregulated N-Cadherin and Vimentin yet downregulated E-Cadherin, while silencing EZH2 did conversely. In

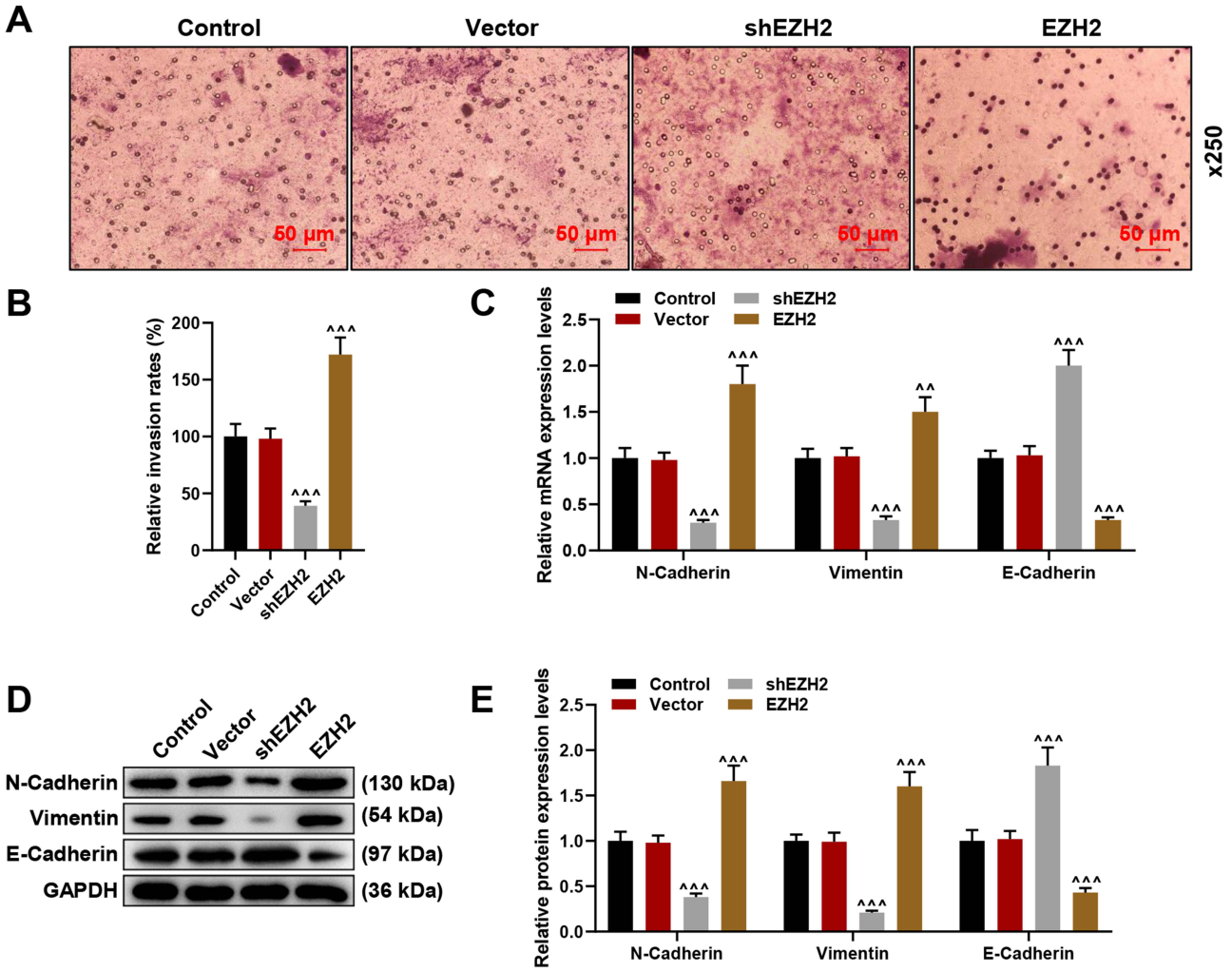

Figure 3 Silencing EZH2 suppressed HCC cell invasion and regulated and epithelial-to-mesenchymal transition (EMT)-related markers' expression, whereas EZH2 overexpression did oppositely. (**A** and **B**) Transwell assay were used to detect the invasion of HCC cells at 24 hours following shEZH2 or EZH2 overexpression plasmid transfection. Magnification: × 250. (**C**) Relative mRNA expression of EMT-related factors (N-Cadherin, Vimentin and E-Cadherin) in HCC cells following shEZH2 or EZH2 overexpression plasmid transfection was quantified using RT-qPCR. GAPDH was used as internal reference. (**D** and **E**) Relative mRNA expression of EMT-related factors (N-Cadherin, Vimentin and E-Cadherin) in HCC cells following shEZH2 or EZH2 overexpression plasmid transfection was quantified using RT-qPCR. GAPDH was used as internal reference. All experiments have been performed in independent triplicate and data were expressed as mean ± standard deviation (SD). ^^$P<0.01$, ^^^$P<0.001$, vs Vector.

addition, it was discovered that silencing EZH2 reversed the effects of TGF-β1 in HCC cells. Furthermore, to the best of our knowledge, we firstly put forward that TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis was implicated in HCC in vitro via promoting HCC cell viability, migration, invasion and EMT process.

Cancer cell proliferation is essential in cancer development, as manifested and demonstrated by altered expressions and/or activities of cell cycle-associated proteins.[23] Metastasis of cancer, as defined, is the dissemination of cancer cell from tumor sites to the succeeding new colonies seeding in distant tissues, and a multistep process, known as the invasion-metastasis cascade, has also been found to have an implication.[24] As a developmental program, EMT has a critical function in metastasis, and in the process of metastatic progression, some modifications both genetic and epigenetic can endow malignant cells with the properties that have effects on modulation of metastatic capacity.[25,26] E-Cadherin, N-Cadherin, and Vimentin are those EMT-related factors.[27] As two members of the Cadherin family, E-Cadherin and N-Cadherin are the most prominent cell adhesion molecules, and E-Cadherin is a glycoprotein with transmembrane ability, which is pivotal to cell-to-cell adhesion and acts as tumor-suppressor, while N-Cadherin, on the opposite confers malignant cells upon metastasis acquisition or promotion.[28,29] In addition, Vimentin, an intermediate filament protein that is upregulated characteristically in those cells with EMT, has been regarded as an EMT marker as well.[30] It has

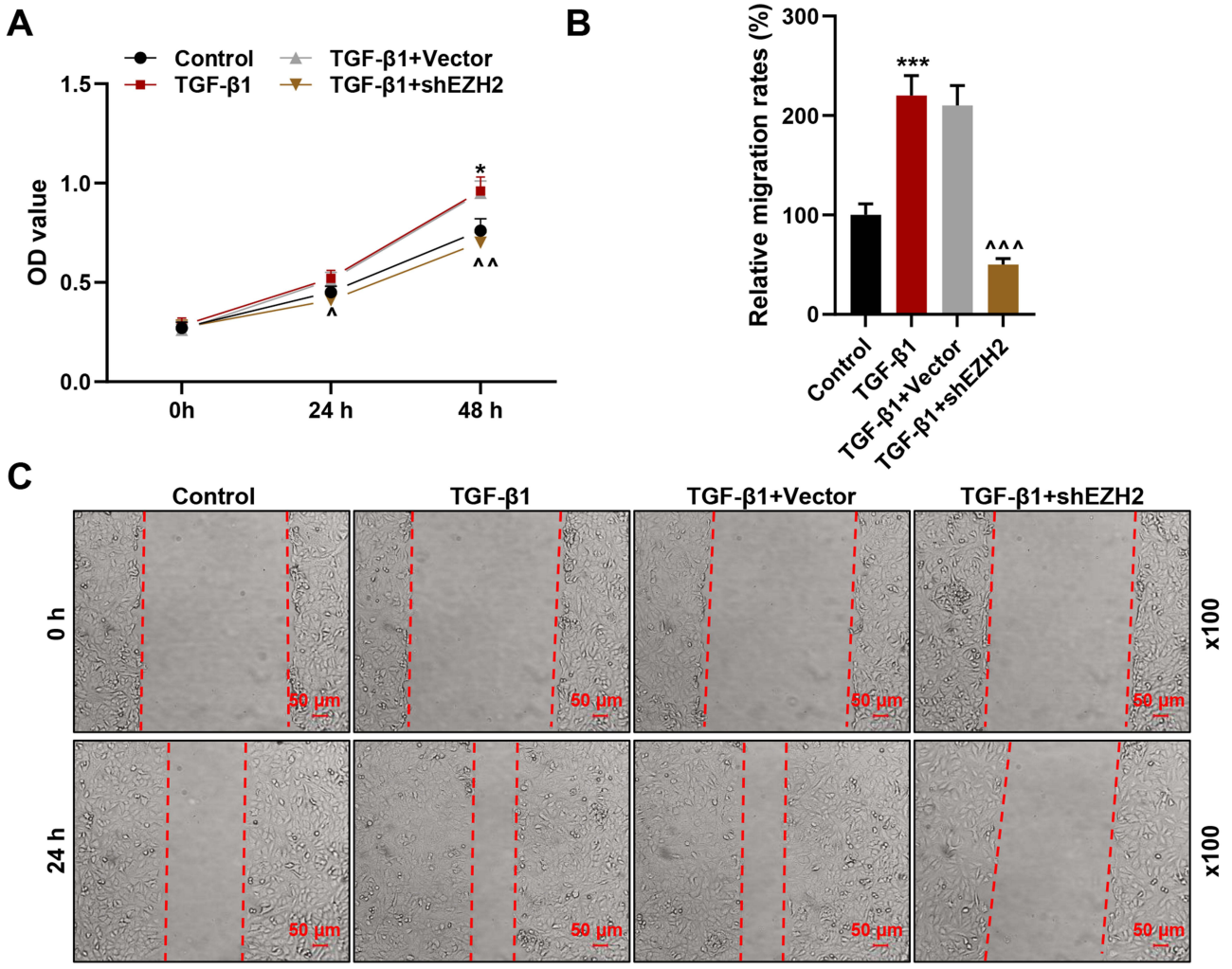

Figure 4 Silencing EZH2 reversed the effects of TGF-β1 on HCC cell viability and migration. (**A**) MTT assay was used to determine the viability of HCC cells following shEZH2 transfection and TGF-β1 at 0, 24 and 48 hours. (**B** and **C**) Scratch assay were used to detect the migration of HCC cells at 0 and 24 hours following shEZH2 or EZH2 overexpression plasmid transfection. Magnification: × 100. All experiments have been performed in independent triplicate and data were expressed as mean ± standard deviation (SD). **P*<0.05, ****P*<0.001, vs Control; ^*P*<0.05, ^^*P*<0.01, ^^^*P*<0.001, vs TGF-β1+Vector.
Abbreviation: TGF-β1, transforming growth factor-β1.

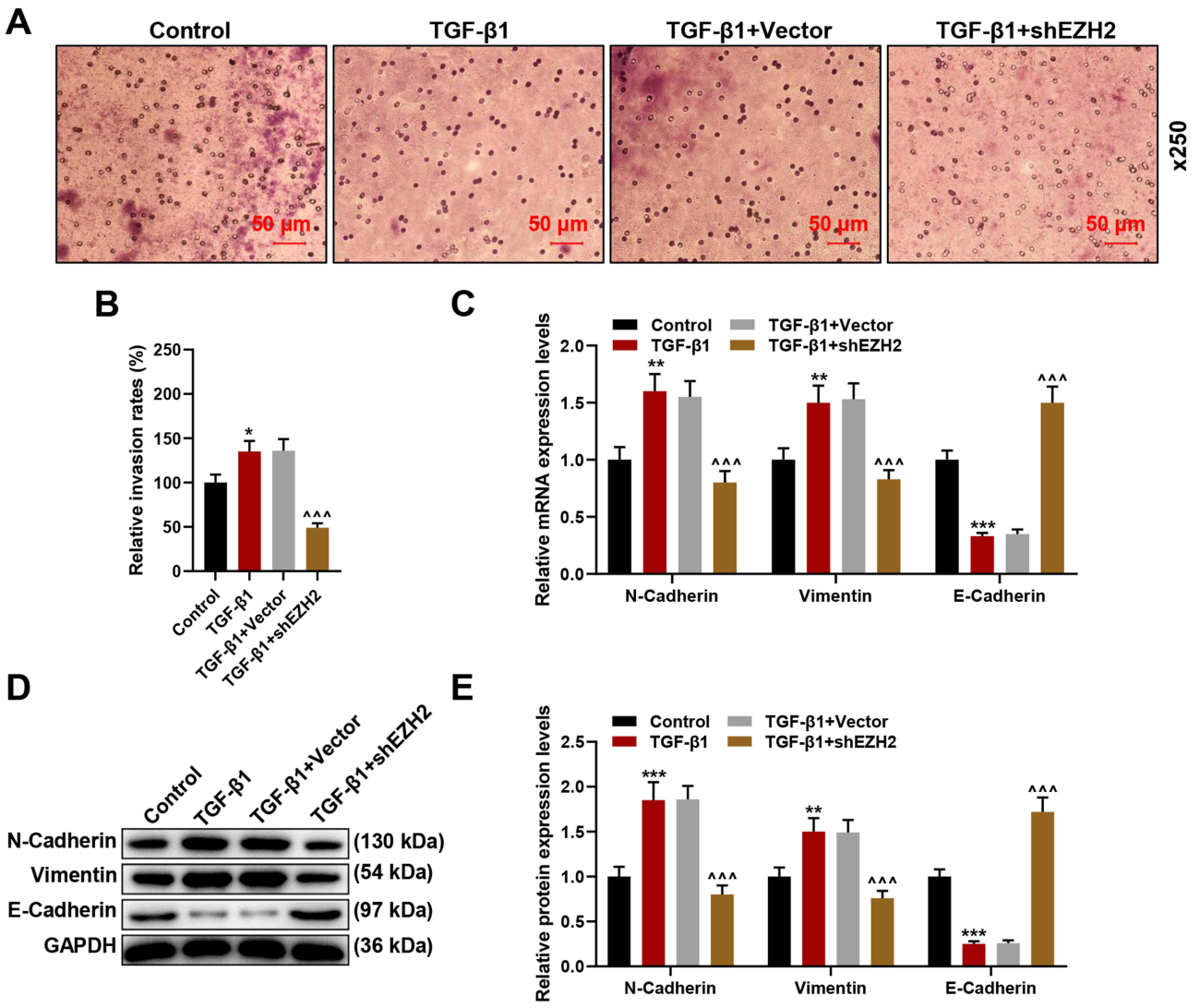

Figure 5 Silencing EZH2 reversed the effects of TGF-β1 on HCC cell invasion and epithelial-to-mesenchymal transition (EMT)-related markers' expression. (**A** and **B**) Transwell assay was used to detect invasion at 24 hours of HCC cells following shEZH2 transfection and TGF-β1. Magnification: × 250. (**C**) Relative mRNA expression of migration- and invasion-related factors (N-Cadherin, Vimentin and E-Cadherin) in HCC cells following shEZH2 transfection and TGF-β1 was quantified using RT-qPCR. GAPDH was used as internal reference. (**D** and **E**) Relative mRNA expression of EMT-related factors (N-Cadherin, Vimentin and E-Cadherin) in HCC cells following shEZH2 transfection and TGF-β1 transfection was quantified using RT-qPCR. GAPDH was used as internal reference. All experiments have been performed in independent triplicate and data were expressed as mean ± standard deviation (SD). **P*<0.05, ***P*<0.01, ****P*<0.001, vs Control; ^^^*P*<0.001, vs TGF-β1+Vector.

been discovered and discussed that silencing EZH2 could decrease cell migration and invasion, with increased E-Cadherin yet decreased N-Cadherin and Vimentin in head and neck squamous cell carcinoma (HNSCC).[31] In our study, overexpressed EZH2 promoted HCC cell viability, migration and invasion, with increased N-Cadherin and Vimentin yet decreased E-Cadherin; silencing EZH2, however, exerted contrary effects, which provided another evidence on the role of EZH2 played in HCC.

TGF-β-MTA1-SOX4-EZH2 signaling axis may be implicated in the mechanism to promote EMT in tumor metastasis,[16] which made us wonder whether TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis would have the same function. As a member of the TGF-β family of pleiotropic cytokines, TGF-β is a possible inducer of developmental and fibrogenic EMTs, and its aberrance and EMT are also involved in the pathogenesis of cancers.[32,33] It has been additionally found that TGF-β could induce and stimulate MTA1, a gene that plays a significant role in cancer metastasis and repressed SMAD7, which subsequently led to activation of TGF-β pathway.[34,35] SMAD7 has been shown to be an inhibitor of SMAD3, whose activation was also controlled by MTA1.[36,37] SOX4 is a member of the SOX

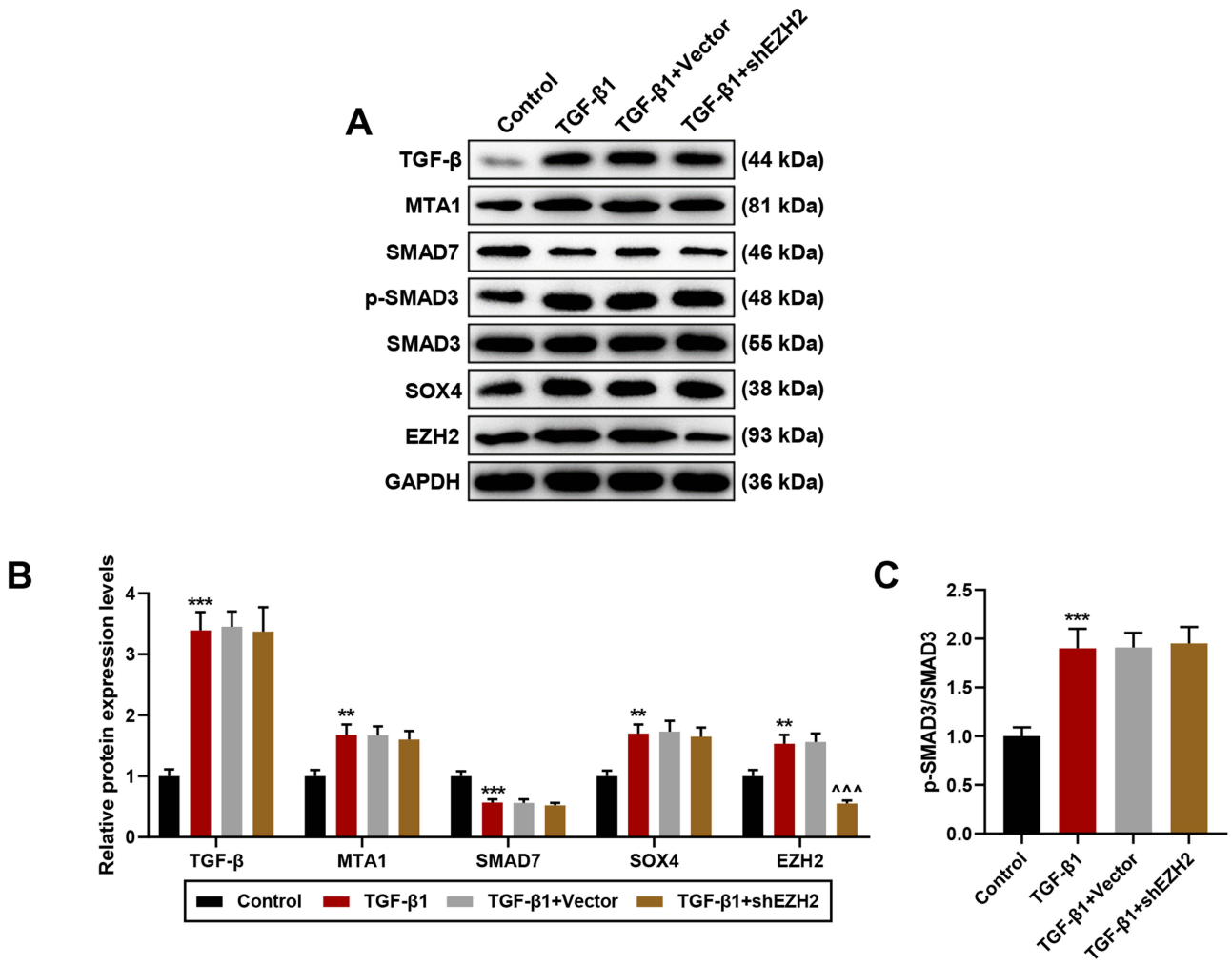

Figure 6 Silencing EZH2 solely reversed effects of TGF-β1 on EZH2 expression in TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in HCC cells. (**A**–**C**) Relative expressions of TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis-related proteins were measured via Western blot, and ratios of p-SMAD3/SMAD3 were determined as well. GAPDH was used as internal control. All experiments have been performed in independent triplicate and data were expressed as mean ± standard deviation (SD). ***P*<0.01, ****P*<0.001, vs Control; ^^^*P*<0.001, vs TGF-β1+Vector.

Abbreviations: MTA1, Metastasis Associated 1; SOX4, SRY-Box Transcription Factor 4; p-SMAD3, phosphorylated-SMAD3.

family which, in stem cells, is highly expressed and could interact with and be regulated by TGF-β and its downstream molecule SMAD3.[38] Moreover, SOX4 has the capability to induce EZH2 transcription, and thus reprograms the cancer epigenome so as to promote EMT and metastasis.[39] In our study, we found that following TGF-β treatment, TGF-β, MTA1, SOX4 and EZH2 expressions and p-SMAD3/SMAD3 ratios were increased, whereas SMAD7 was down-regulated. A reversed effect on EZH2 expression solely, however, was found following silencing EZH2 in HCC cells, which further pointed out the role and effect of TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in HCC.

The present study provides a crucial part for EZH2 in prognosis of HCC and theoretical basis for TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling cascade in EMT of HCC cells. Inhibiting these signals may represent a therapeutic pathway for the treatment of metastatic HCC. Nevertheless, there are some limitations we wanted to address. In our study, we solely confirmed that TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis may be involved in HCC cells in vitro, but whether this novel-discovered signaling axis was implicated in vivo needs to be further proved and validated. Another limitation of the study of how the signaling axis has been defined was not studied. The TGFβ-MTA1-SMAD7-SMAD3-SMAD3-SOX4-EZH2 axis is described, but the intermediary mediators are varied, such as other SMAD members and other SOX. In addition, transcription factors that trigger EMT and are

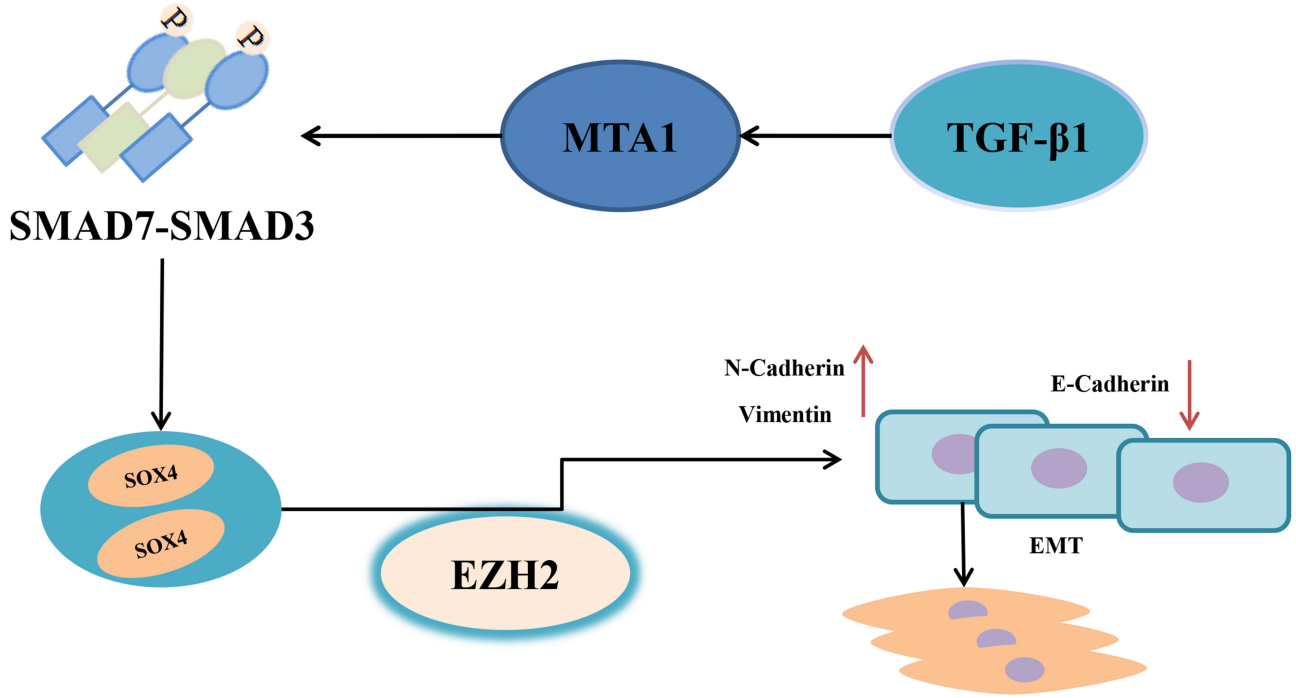

Figure 7 Schematic diagram showing the TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in promoting EMT in HCC cells.
Abbreviations: TGF-β, transforming growth factor-β; MTA1, Metastasis Associated 1; SOX4, SRY-Box Transcription Factor 4; HCC, hepatocellular carcinoma; EMT, epithelial-to-mesenchymal transition.

downstream of the pathway are multiple. Therefore, future studies are needed to complete our results.

Conclusion

In our current study, we unveiled another evidence with regard to the role of EZH2 on HCC, and its interaction with TGF-β1, a member of TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis. To be specific, EZH2 was upregulated in HCC, and EZH2 overexpression promoted cell viability, migration and invasion, while silencing EZH2 did contrarily. Furthermore, EZH2 silence reversed the effects of TGF-β1. These results in our study not only provide evidence on EZH2 but also propose a brand-new discovery of TGF-β-MTA1-SMAD7-SMAD3-SOX4-EZH2 signaling axis in HCC, and we hope that results from our study can help gain better insights on HCC and possible strategy for HCC in the near future.

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Disclosure

The authors declare no conflicts of interest.

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