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Original Article

ZEB1 promotes colorectal cancer cell invasion and disease progression by enhanced LOXL2 transcription

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Abstract: Disease progression after curative surgery is still the main challenge for colorectal cancer (CRC). Identifying biomarkers and precise mechanisms in CRC disease progression is necessary for therapeutic improvement. As a transcription factor, ZEB1 promotes malignancy, but the precise mechanism by which ZEB1-dependent transcriptional regulation remains largely undefined. In this study, the transcriptional regulation of lysyl oxidase-like 2 (LOXL2) by ZEB1 in CRC was investigated. Our data show that ZEB1 enhanced LOXL2 transcription through direct binding to its promoter. The gain of function assays of ZEB1 showed increased cell proliferation, migration, and invasion. The inhibition of LOXL2 impaired the invasion and migratory ability of CRC cells, but had no effect on cell proliferation *in vitro* and *in vivo*. Immunohistochemical staining of tumor tissues indicated that elevated ZEB1/LOXL2 expression was significantly associated with lymph node metastasis and TNM stage. More importantly, elevated ZEB1/LOXL2 expression was an independent prognostic factor in CRC patients. These findings provide a molecular basis for the promotion of an invasive cancer phenotype by ZEB1-LOXL2 overexpression. Our results identify ZEB1/LOXL2 as a prognostic biomarker and potential therapeutic target against progression of CRC.

Keywords: Colorectal cancer, disease progression, ZEB1, LOXL2, transcription, invasion

Introduction

Colorectal cancer (CRC) is one of the leading causes of morbidity and mortality [1]. About half of the CRC patients suffer disease progression within five years [2]. Local recurrence or distant metastasis after curative surgery leads to shorter survival, which is still the main challenge for therapeutic efficiency in CRC patients [3]. Therefore, the identification of molecular markers that participate in tumor progression and metastasis, is necessary for personalized therapy and life expectancy improvement of CRC patients.

As a transcription factor, Zinc finger E-box binding homeobox 1 (ZEB1) has emerged as a key player in cancer progression [4-6]. Aberrant expression of ZEB1 transcriptionally regulates gene expression status to enhance aggression and malignant disease progression in various cancers [7-9]. Previous studies indicated an increased ZEB1 expression in CRC cell lines and tumor tissues [10]. ZEB1 expression showed different clinical correlation and functional roles in the different studies, such as epithelial-mesenchymal transition and crosstalk with extracellular matrix [11-13]. However, the precise molecular mechanisms behind the downstream modification of ZEB1 in CRC are still unclear. Further studies of clinical significance are still needed on the precise mechanisms of ZEB1-dependent transcriptional regulation in CRC.

Lysyl oxidase-like 2 (LOXL2) is a member of the lysyl oxidase (LOX) family, which is an extracellular copper-dependent amine oxidases cluster [14]. LOXL2 participates in the formation of collagen-elastin crosslinks in extracellular matrix [15, 16]. Elevated LOXL2 expression is observed in many types of tumors to predict poor outcome, and it may function in malignant biologic behavior [17, 18]. LOXL2 is also a candidate downstream molecular of ZEB1 transcriptional regulation in lung cancer [19]. The underlying gene regulation and clinical significance of LOXL2 in CRC is worth exploring. In

the present study, the modification mechanism of LOXL2 by ZEB1 in CRC cell lines was evaluated. The functional roles of ZEB1/LOXL2 were analyzed in CRC cell lines. Furthermore, ZEB1 and LOXL2 expression in CRC tissues were also examined for clinical significance.

Materials and methods

Cell culture

CRC cell lines (HCT116 and SW480) and 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), which were cultured in Dulbecco's Modified Eagle Medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, NY).

Cell transfection

For the ectopic expression of ZEB1, cells were transfected with pCI-neo-RL-ZEB1 plasmid, which was a gift from Greg Goodall (#35535, Addgene) with Lipofectamine 2000 (Invitrogen, Green Island, CA) according to the manufacturer's instructions. For LOXL2 knockdown, cells were transfected with shRNA targeting LOXL2 with a sequence of 5'-GCCACAUAGGUGGUUCCUUCAUU-3') or scramble shRNA with Lipofectamine 2000 (Invitrogen).

Western blotting

Western blotting was performed as described elsewhere [20]. The antibodies against LOXL2 (ab96233, Abcam, Cambridge, MA), ZEB1 (ab180905, Abcam), and GAPDH (#5174, Cell Signaling Technology, Danvers, MA) were used in the immunoblotting assays.

Quantitative RT-PCR

Quantitative real-time PCR was performed as previously described [20]. Primer sequences used in each PCR set are: LOXL2: Forward 5'-AGGACATTCGGATTCGAGCC-3', Reverse 5'-CTTCCTCCGTGAGGCAAAC-3'; GAPDH: Forward 5'-TGTGGGCATCAATGGATTTGG-3', Reverse 5'-ACACCATGTATTCCGGGTCAAT-3'.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed with ChIP Assay Kit (P2078, Beyotime Inst Biotech, Shanghai, China) as described elsewhere [21]. Immunoprecipitation was carried out using an anti-ZEB1 antibody or mock IgG control (Abcam). Promoter segment enrichment was analyzed by qPCR to investigate ZEB1 binding sites in the LOXL2 promoter as previously reported [19].

Luciferase reporter assays

Luciferase reporter assays were carried out by transfection of 500 ng of the reporter constructs in specified cell lines with ZEB1 overexpression. Assays used Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Cell proliferation assay

For the proliferation analysis, Cell Counting Kit-8 (Beyotime Inst Biotech, China) was used, according to the manufacturer's instructions. Tumor cells were seeded in a 96-well flat-bottomed plate (2000 cells/well), and were cultured with indicated treatment. Relative proliferation rates were calculated with Multiskan Spectrum 1500 (Thermo Scientific, PA) for light absorbance at 450 nm at the indicated time.

Cell invasion assays

Cell invasion assays were performed as previously reported [20]. Transwell inserts (8 μm) were pre-coated with Matrigel or 100 μl of 0.2 mg/ml collagen type I (BD Biosciences, San Jose, CA). β-aminopropionitrile (BAPN, Sigma, St. Louis, MO) was added in the outer compartment as indicated. Invaded cells were imaged by bright field microscopy and counted using ImageJ software.

Wound-healing assay

Indicated cells (10^6) were cultured in 6-well plates for 8 hours. Then the medium was changed to a low-serum DMEM medium (1% fetal bovine serum). Wound scratching was produced. The cells were cultured for another 48 hours, and captured images were collected during the 48 hours. Cell migration was determined by the percentage of the remaining cell-free distance compared with that of the initial wound.

Tumorigenesis assays

Subcutaneous xenografts were planted as previous reported [20]. Cells were subcutaneously injected for a xenograft in the right flanks of

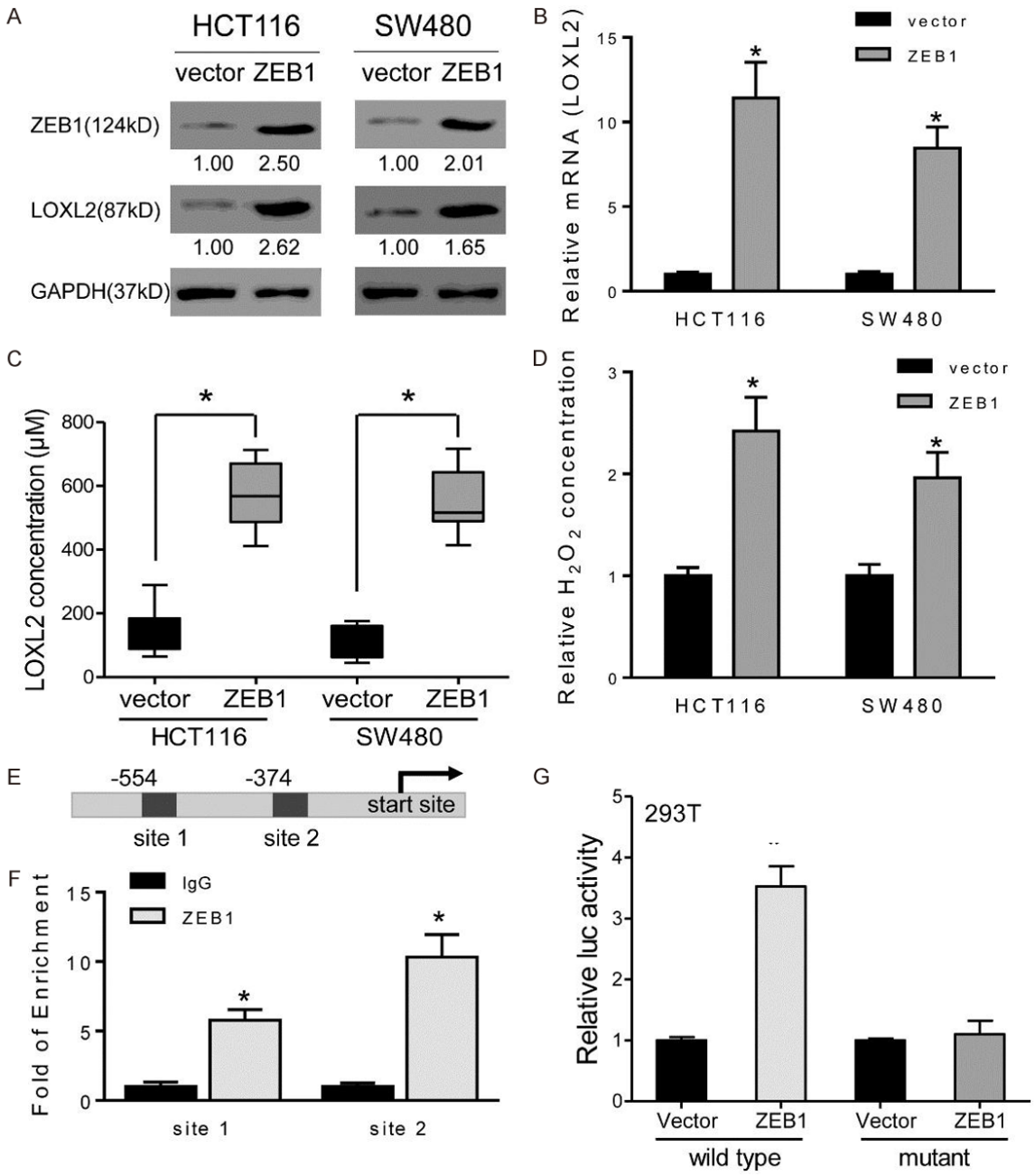


Figure 1. ZEB1 transcriptionally elevates LOXL2 expression in colorectal cancer cells. A. ZEB1-expressing plasmids were transfected into HCT116 and SW480 cells. The expression levels of ZEB1 and LOXL2 were evaluated with western blotting. B. Box plot depicting LOXL2 mRNA levels in transfected cells as assessed by qRT-PCR. GAPDH was used as control. C. ELISA assays of secreted LOXL2 in conditioned media of transfected CRC cell lines. D. Amplex Red assay to determine LOXL2 enzymatic activity in conditioned media of transfected cells. E. The schematic showed two predicted ZEB1 binding sites in LOXL2 promoter region, which was analyzed with the JASPER transcription factor binding database (http://jaspar.genereg.net/). F. Fold enrichment was measured by qPCR analysis of LOXL2 promoter segments, which was analyzed by chromatin immunoprecipitation in HCT116-ZEB1 and control cells. ZEB1 antibody or control IgG was used in ChIP assays. G. LOXL2 reporter was constructed and transfected into 293T cells, as well as ZEB1 expressing plasmid or vector control and relative luciferase activity was determined. Wild type and mutant potential ZEB1 binding sites were constructed as indicated in the schematic. Three experimental replicates were performed for each experiment. *, $P < 0.05$.

nude mice (6 weeks old with an average weight of 20 g). All the tumors were allowed to grow for 37 days with the volume not exceeding 600 mm^3. Tumors volumes were measured with

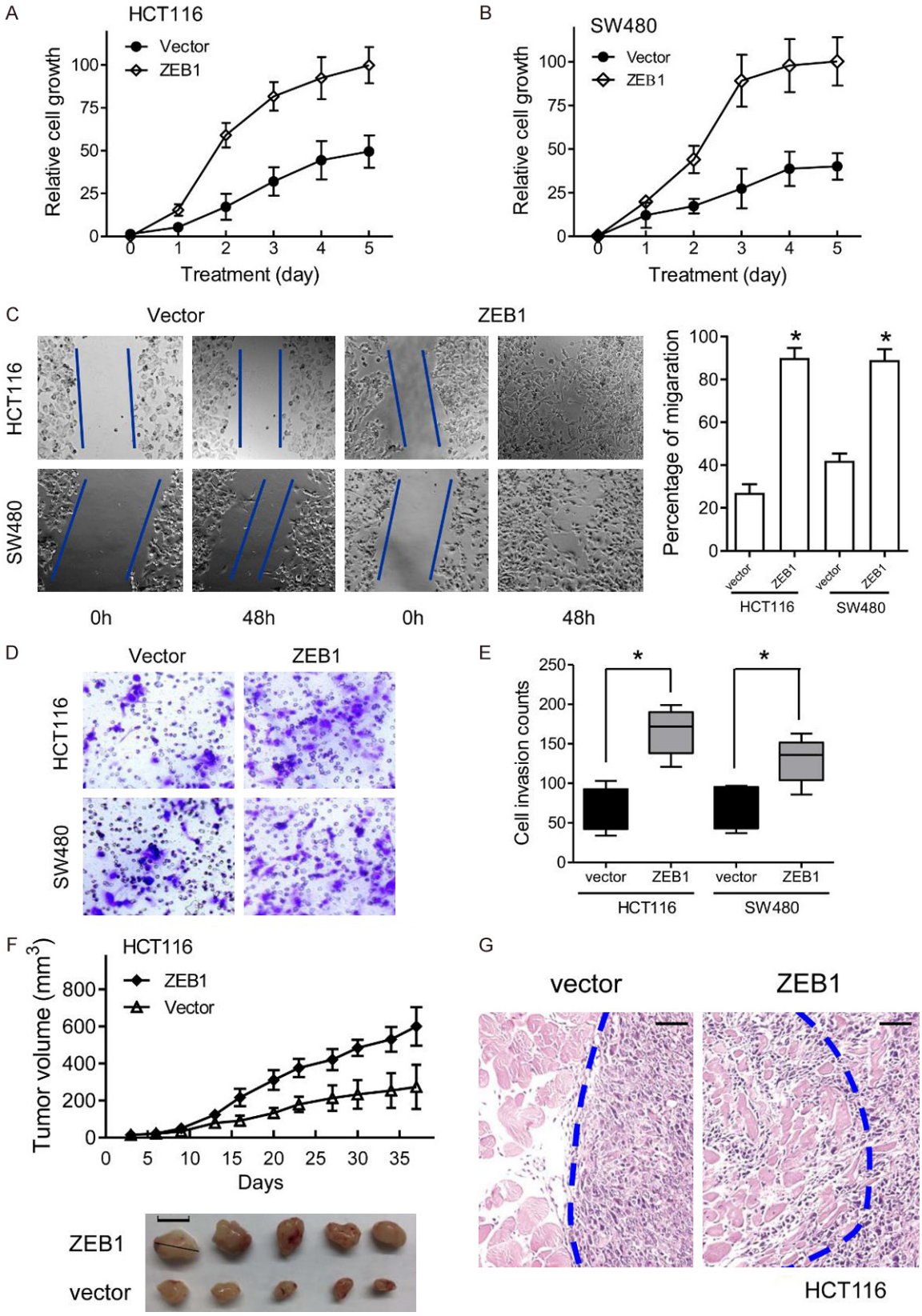


Figure 2. ZEB1 promotes colorectal cancer cell proliferation and invasion in vitro and in vivo. (A, B) The effects of ZEB1 overexpression on HCT116 (A) and SW480 (B) cell proliferation were assessed with CCK-8 assays. The proliferation ability of ZEB1-overexpressed HCT116 and SW480 cell were increased compared with the corresponding

control group. (C) The migratory capacity of ZEB1 overexpressed cells was determined by wound-healing assays. The percentage of migration was determined at 48 h after the initial scratch wound. *P < 0.05. (D, E) The invasion capability was evaluated in ZEB1 overexpressed CRC cells and corresponding control cells. Scale bars represent 50 μm. Results were obtained from three independent experiments, and bar graphs represent cell number per image field (mean ± SD). (F) HCT116 cells with stable ZEB1 overexpression were planted in nude mice for subcutaneous tumor (n = 5 each group). Tumor volume was measured every three days with a caliper (volume = 1/2 (length * width^2)). (G) Microscopic images of tumor tissues with H&E staining. The boundary between tumor and normal tissue was shown with a blue dotted line. Poorly-defined margins were observed in HCT116-ZEB1 tumors. Bar, 100 μm.

caliper at the indicated time. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Jiangjin District Central Hospital.

Amplex red assay

LOX family enzyme activity was measured with cell culture media. Cells were pretreated as indicated and then measured with the Amplex Red protocol (Thermo) and 2 mM benzylamine (Sigma), which was performed as previously reported [22].

Clinical data collection

Patients who underwent surgical resection for CRC in 2010 and 2013 at Jiangjin District Central Hospital were included in this study. Clinicopathologic data, such as age, gender, tumor size and location, and follow-up information were obtained from medical records. Pathologic information, including histologic grade and pathologic TNM staging were determined by the 7th American Joint Committee on Cancer criteria. Our study was approved by the Institutional Review Board of Jiangjin District Central Hospital, and the study was conducted according to the principles expressed in the Declaration of Helsinki.

IHC staining and analysis

Four-micrometer large tissue sections were prepared for IHC staining, which was performed using the Ventana Discovery XT automated staining system (Ventana Medical Systems, Inc., Tucson, AZ). ZEB1 and LOXL2 antibody (Abcam) were used for IHC staining. A semiquantitative scoring system was used to determine the expression levels of ZEB1 and LOXL2, which was measured by the staining intensity and the proportion of stained cells [23]. The score >1 was positive and ≤1 was negative by a statistical analysis. IHC evaluation was performed by two pathologists in a blinded manner.

Statistical analysis

SPSS Statistics software v. 24 (SPSS Inc., Chicago, IL) was used for statistical analysis. For measurement data, statistical analyses and the determination of *p*-values were performed by Student's t-test. For the analysis of clinicopathologic characteristics, Fisher's exact test was used for the analysis of various clinicopathologic factors. Kaplan-Meier survival curves with log-rank statistics and Cox regression analysis were applied to evaluate time to survival. *p*-values < 0.05 were considered significant.

Results

ZEB1 transcriptionally elevates LOXL2 expression in colorectal cancer cells

To assess the roles of ZEB1 in CRC progression, ectopic expression of ZEB1 was performed in HCT116 and SW480, CRC cell lines. The expression of LOXL2 was evaluated in the transfected cell lines, which showed higher protein levels of LOXL2 in ZEB1 overexpressing cells (**Figure 1A**). Furthermore, elevated mRNA levels of LOXL2 in ZEB1 overexpressing cells were consistent with western blot assays (**Figure 1B**). Analyzing the conditioned media from HCT116-ZEB1 cells revealed increased secreted LOXL2 protein (**Figure 1C**) and demonstrated active LOXL2 enzymatic function by Amplex Red assays (**Figure 1D**).

Due to the strong correlation between mRNA and protein levels of LOXL2 and ZEB1, we proceeded to examine whether LOXL2 was transcriptionally regulated by ZEB1. The promoter region of LOXL2 contained two ZEB1 regulatory sites which were predicted by the JASPER transcription factor binding database (**Figure 1E**). We further confirmed direct binding of ZEB1 to the endogenous LOXL2 promoter region by chromatin immunoprecipitation (ChIP) assays with HCT116 cells (**Figure 1F**). Luciferase reporter assays with the wild-type

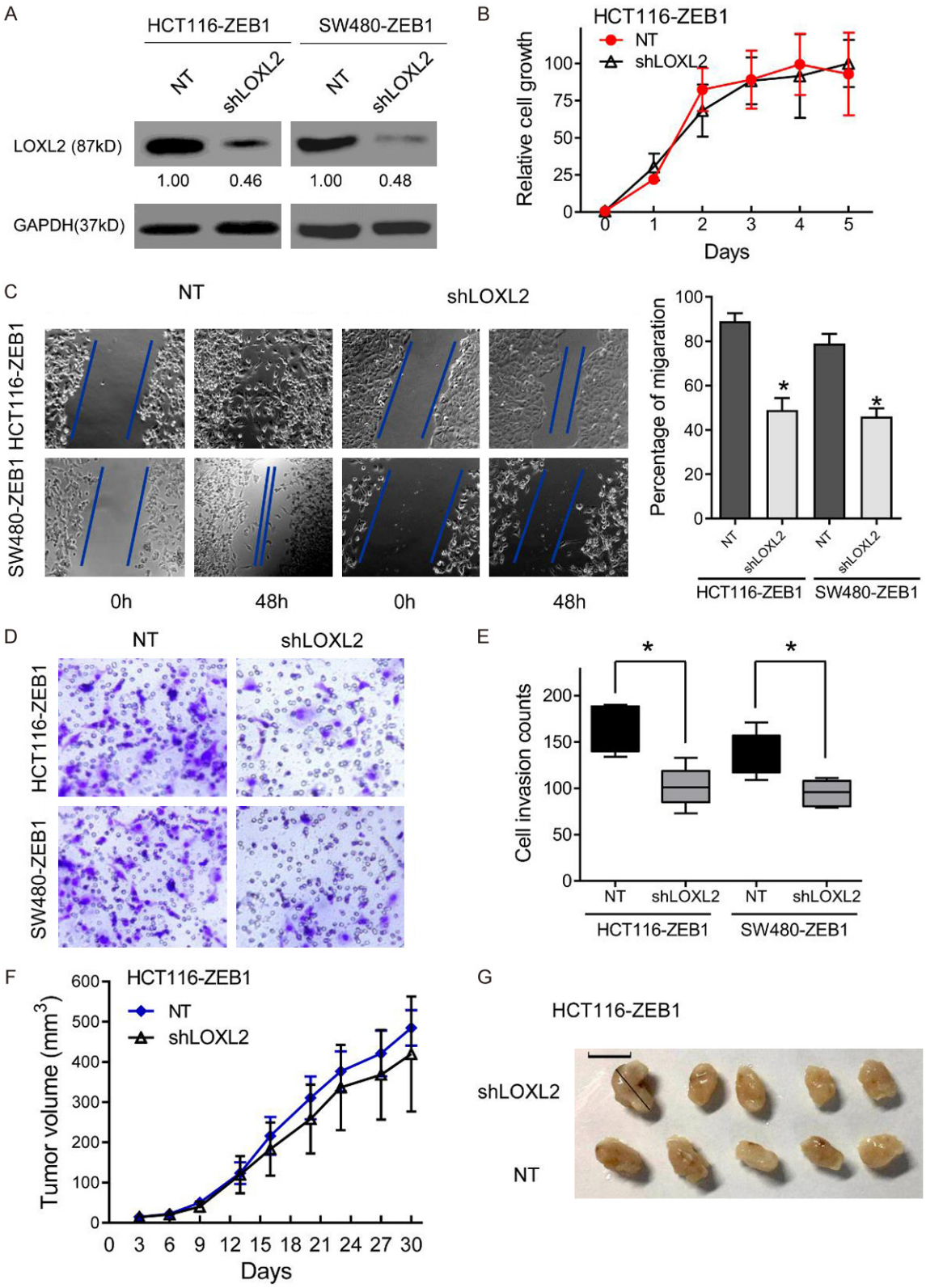


Figure 3. LOXL2 knockdown inhibits ZEB1-induced colorectal cell invasion and migration, but has no effect on cell proliferation. A. LOXL2 knockdown was performed in ZEB1 overexpressing CRC cells. LOXL2 protein levels were evaluated by western blotting. GAPDH was used as a loading control. B. Cell proliferation was assessed with CCK-8 assays, which showed no significant difference in cell proliferation with LOXL2 knockdown in HCT116 cells. C. The

migratory capacity of LOXL2 knockdown CRC cells was determined by wound-healing assays. The percentage of migration was determined at 48 h after the initial scratch wound. D, E. The invasion capability was evaluated in LOXL2 knockdown CRC cells and control cells. F. HCT116-ZEB1 cells with LOXL2 knockdown were planted in nude mice for subcutaneous tumor (n = 5 each group). Tumor volume was measured every three days. G. Subcutaneous tumor pictures are shown with transfected HCT116 cells (LOXL2 knockdown and control cells). NT, scramble control cells. Scale bar = 10 mm.

LOXL2 promoter region further confirmed its transcriptional regulation by ZEB1 (**Figure 1G**), which was verified by the introduction of mutations into each of the ZEB1 binding sites individually and in combination (**Figure 1G**).

ZEB1 promotes colorectal cancer cell proliferation and invasion in vitro and in vivo

Gain-of-function assays were performed to assess the role of ZEB1 in CRC. Forced expression of ZEB1 accelerated the proliferation of HCT116 and SW480 cell lines according to cell viability analysis (**Figure 2A**, **2B**). Wound healing assays indicated increased cell migration ability in ZEB1 overexpressing cells compared to control ones (**Figure 2C**). Transwell assays also showed increased cell invasion ability of ZEB1 overexpressing cells compared to corresponding control cells (**Figure 2D**, **2E**). Furthermore, increased xenograft growth was observed in those with ectopic ZEB1 expressing CRC cells (**Figure 2F**). Subcutaneously implanted tumors of ZEB1 overexpression also showed enhanced tissue infiltration compared to corresponding control tumors (**Figure 2G**). Our data suggested that ZEB1 expression promoted cell proliferation and invasion of CRC cells.

LOXL2 knockdown inhibits ZEB1 induced colorectal cell invasion and migration, but has no effect on proliferation

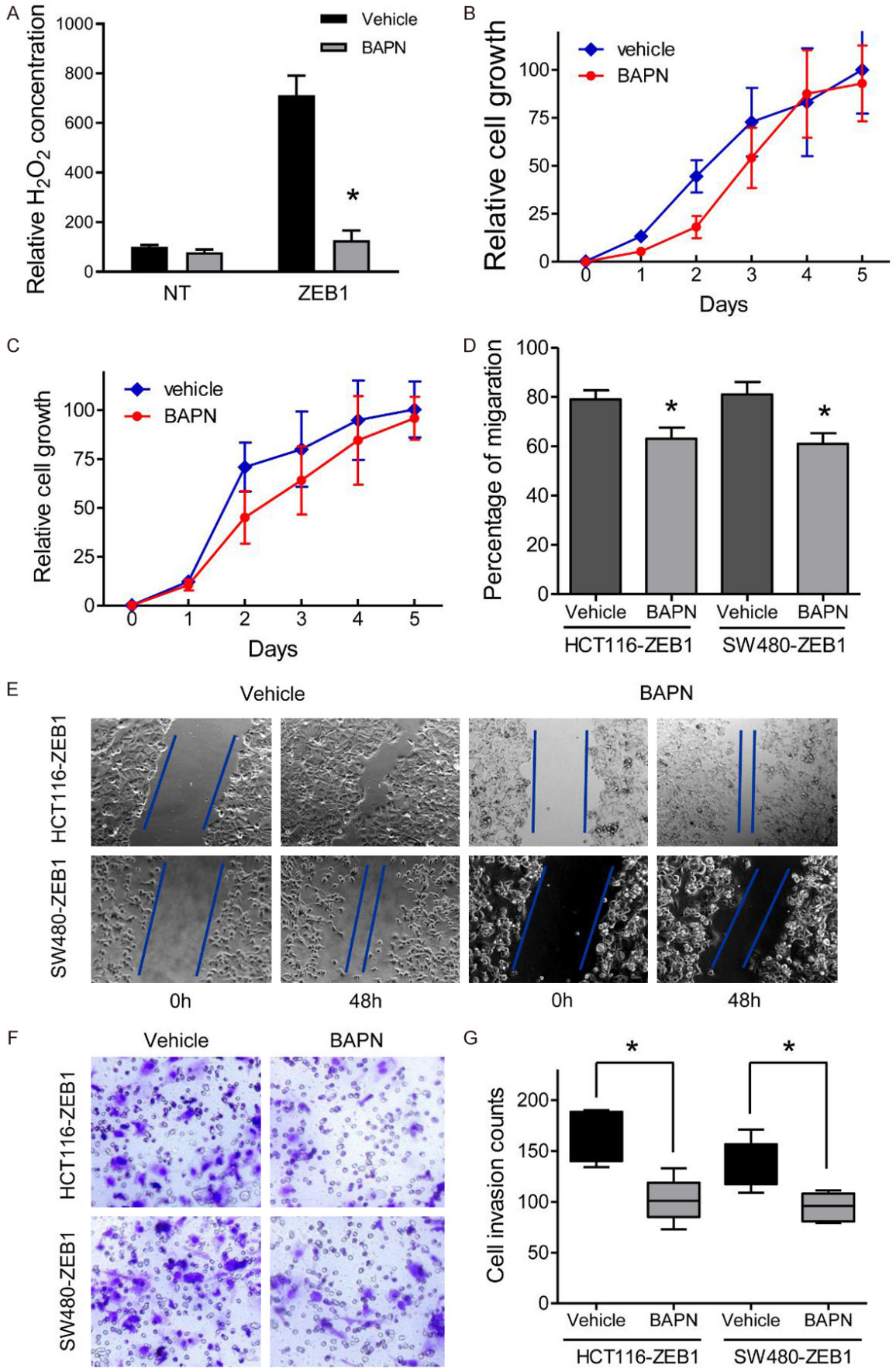
We next assessed the functional relevance of LOXL2 in promoting CRC cell proliferation and invasion. LOXL2 knockdown was performed in ZEB1 expressing cells, which was confirmed with western blot assays (**Figure 3A**). Cell proliferation and viability showed no obvious inhibition in LOXL2 knockdown cells compared to control cells (**Figures 3B** and S1A). Wound healing assays showed decreased cell migration ability in LOXL2 knockdown cells vs. control cells (**Figure 3C**). Transwell invasion analysis also indicated decreased cell invasion in LOXL2 knockdown cells (**Figure 3D**, **3E**). We next evaluated the effects of LOXL2 silencing on tumor growth of HCT116-ZEB1 cells *in vivo*. The xenografts from LOXL2 knockdown cells showed no significant decrease in tumor size (**Figure 3F**, **3G**). Furthermore, mRNA expression of EMT-related molecules was examined, which showed decreased SNAI1 and VIM and increased CDH1 in LOXL2-silenced ZEB1 overexpressing cells (Figure S1B). These results indicated LOXL2 expression participated in the cell invasion and migration process, but was not involved in cell proliferation induced by ZEB1 overexpression.

LOXL2 inhibition by BAPN attenuates colorectal cancer cell invasion and migration

Pan-LOX enzymatic activity was inhibited with BAPN, which showed significantly reduced enzymatic activity in the conditioned media of ZEB1 overexpressing and control HCT116 cells (**Figure 4A**). Cell viability assays also indicated no obvious inhibitory effects of cell proliferation of BAPN-treated ZEB1 expressing cells (**Figure 4B**, **4C**). Wound healing assays showed decreased cell migration ability in BAPN-treated cells (**Figure 4D**, **4E**). Transwell invasion of ZEB1-overexpressed HCT116 and SW480 cells, showed significant decrease in invasion through laminin-rich Matrigel with BAPN treatment (**Figure 4F**, **4G**). These results are consistent with LOXL2 knockdown analysis, suggesting LOXL2 is a promising target to attenuate CRC progression.

Positive correlation between ZEB1 and LOXL2 expression in colorectal cancer specimens

IHC staining for ZEB1 and LOXL2 was performed with CRC specimens, which was evaluated with the semi-quantitative scoring system [24]. Stained ZEB1 protein was located in the cell nuclei (**Figure 5A**), whereas LOXL2 was arranged in cytoplasm (**Figure 5B**). About 29.28% of specimens were positive for ZEB1 (77/263), and 65.02% positive for LOXL2 (171/263). A higher percentage of LOXL2 expression was observed in ZEB1 positive specimens than negative ones (87.01% vs. 55.91%,


A
Relative H_2O_2 concentration
1000
800
600
400
200
0
Vehicle
BAPN
*
NT
ZEB1
B
Relative cell growth
100
75
50
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0h
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Vehicle
BAPN
HCT116-ZEB1
SW480-ZEB1
G
Cell invasion counts
200
150
100
50
0
*
*
Vehicle
BAPN
Vehicle
BAPN
HCT116-ZEB1
SW480-ZEB1

Figure 4. LOXL2 inhibition by BAPN attenuates colorectal cancer cell invasion and migration. (A) Amplex Red assays were performed to determine LOXL2 enzymatic activity in conditioned media of transfected HCT116 cells with or without 500 μM BAPN treatment. (B, C) Relative cell viability of HCT116-ZEB1 (B) or SW480 (C) cells with different treatment was evaluated with CCK-8 assays, which showed BAPN treatment had no significant inhibitory effect on cell proliferation. (D) Migration ability of infected CRC cells with or without BAPN treatment *in vitro* was determined in a wound healing assay. Images were obtained 72 h after seeding. The percentage of migration was determined at 48 h after the initial scratch wound. (E) Representative figures obtained in three independent experiments are shown. (F, G) The invasion capability was evaluated in ZEB1-overexpressing cells with different treatments by transwell assays. Results were obtained from three independent experiments, and bar graphs represent cell number per image field (mean ± SD). *, P < 0.05.

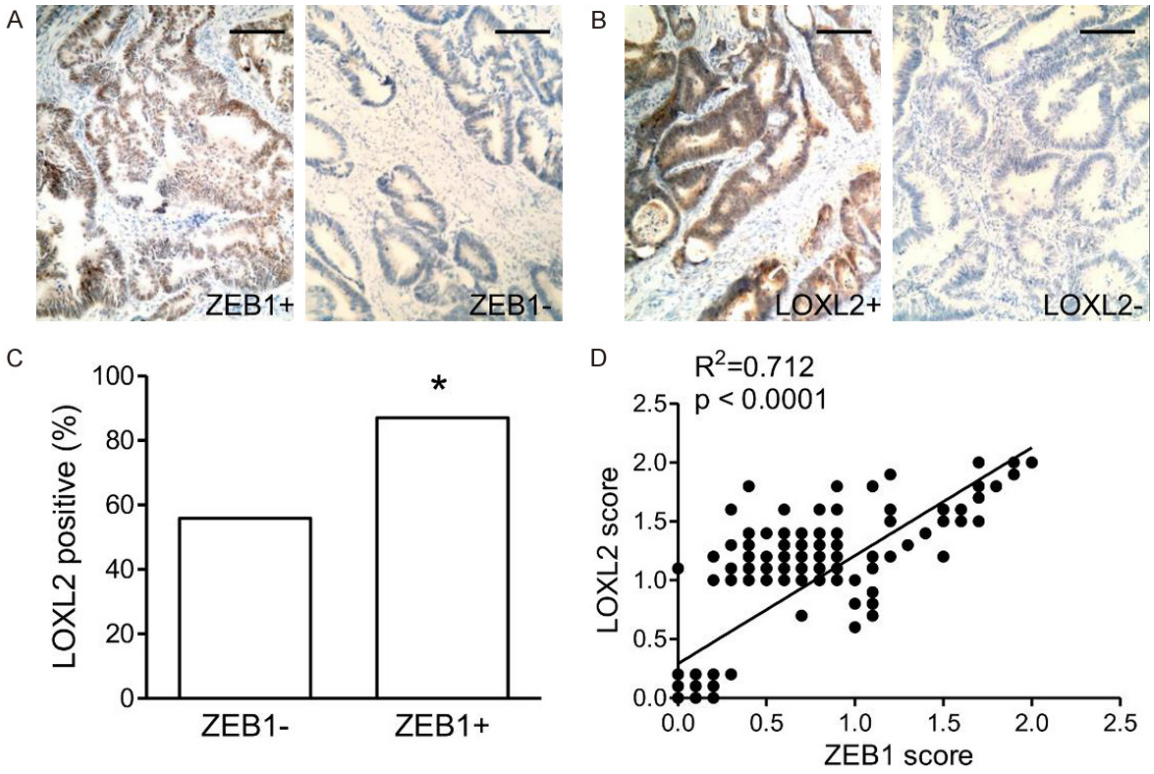

Figure 5. Positive correlation between ZEB1 and LOXL2 expression in colorectal cancer specimens. (A, B) IHC analysis of ZEB1 (A) and LOXL2 (B) expression in CRC tissues. Representative images of CRC tissue with negative and positive expression are presented. Scale bars represent 100 μm. (C) The percentage of LOXL2-positive specimens was compared between ZEB1-positive or negative CRC specimens. (D) Positive correlation of ZEB1 and LOXL2 expression scores in CRC specimens according to linear regression.

P < 0. 01, **Figure 5C**). Moreover, significant positive correlation was observed between ZEB1 scores and LOXL2 scores (P < 0. 0001, **Figure 5D**). We further evaluated the correlation between ZEB1/LOXL2 expression status and related features of CRC (**Table 1**). Positive ZEB1/LOXL2 expression correlated with lymph node metastasis and TNM stage (P < 0.001 and P = 0.002, respectively). ZEB1/LOXL2 co-expression showed a positive correlation with increased lymph node metastasis and later clinical stage. However, ZEB1/LOXL2 expression was not found to be associated with age, gender, ECOG score, ortumor location (P >0.05).

Increased ZEB1/LOXL2 expression predicts poor prognosis in colorectal cancer patients

Based upon these findings, we wanted to determine the prognostic value of ZEB1/LOXL2 expression in CRC patient survival estimation. Significantly worse disease-free survival (DFS) was observed in ZEB1 or LOXL2-positive pa-

Table 1. Correlation of ZEB1/LOXL2 expression with clinicopathologic characteristics of CRC patients

Characteristic	Cases	ZEB1/LOXL2	
		Positive (%)	*P*-value
Total	263	67 (25.48)	
Gender			
Male	165	46 (27.88)	0.246
Female	98	21 (21.43)	
ECOG			
0	159	34 (24.26)	0.060
1, 2	104	33 (31.73)	
Age			
≤59	136	33 (24.26)	0.641
>59	127	34 (26.77)	
Tumor location			
RCC	44	10 (22.73)	0.295
LCRC	219	67 (30.59)	
Histologic grade			
Low	43	9 (20.93)	0.455
Medium-High	220	58 (26.36)	
LN metastasis			
Yes	89	38 (32.58)	< 0.001
No	174	29 (21.84)	
TNM stage			
I-II	152	28 (18.42)	0.002
III-IV	111	39 (35.14)	

tients than negative ones (P = 0.017 and P < 0.001, **Figure 6A**, **6C**). However, no significant difference was observed in overall survival (OS) between two groups (P = 0.752 and P = 0.084, **Figure 6B**, **6D**). Further survival analysis revealed that ZEB1+/LOXL2+ patients showed significant worse prognosis in both OS and DFS compared to other patients (P = 0.001, P = 0.011. **Figure 6E**, **6F**). To evaluate the independent prognostic significance of ZEB1/LOXL2, COX-regression analysis was performed. The results showed that ZEB1/LOXL2 expression was a significant detrimental factor for DFS (HR = 1.513, 95% CI: 1.096-2.089, P = 0.012) and OS (HR = 2.005, 95% CI: 1.302-3.086, P = 0.002. **Table 2**). Multivariate analyses also indicated that ZEB1/LOXL2 co-expression was significantly associated with poor DFS (HR = 1.601, 95% CI: 1.148-2.233, P = 0.006) and OS (HR = 2.172, 95% CI: 1.384-3.408, P = 0.001. **Table 2**). Our results indicated that ZEB1/LOXL2 co-expression was an independent detrimental factor for CRC patients.

Discussion

High proportions of CRC patients suffer disease progression after curative surgery, leading to poor therapeutic response and shorter survival [25]. It is urgently needed to identify molecular mechanisms in disease progression of CRC. In this study, we investigated the expression of ZEB1 and LOXL2 in CRC cells and tumor tissues, which indicated that the transcriptional regulation of LOXL2 by ZEB1 participates in cell migration and invasion of CRC cells. IHC evaluation of ZEB1 and LOXL2 provided predictive evidence for the prognosis of CRC patients.

Previous published studies showed that aberrant ZEB1 expression enhanced malignant biologic behavior and disease progression in CRC cell lines [4, 26, 27], such as increased epithelial-mesenchymal transition by suppressing the expression of basement membrane components [28-30]. ZEB1 is a member of the zinc finger family, and works as a transcription factor in molecular regulation [30, 31]. The transcriptional regulation of multiple genes, such as IL-2, CD4, GATA-3 and epithelial cadherin (E-cadherin) [10, 32], plays an important role in carcinogenesis, progression, invasion, and metastasis of a variety of tumors [8, 33-35]. Our study provided further evidence that ZEB1 promoted cell growth, migration, and invasion of CRC cells. Molecular mechanism studies identified the transcriptional activity of ZEB1 in LOXL2 expression in CRC cells. Detailed mechanistic studies are still needed to show the molecules involved in this procession.

The LOX family catalyzes the covalent interchain crosslinking of collagen, enhancing fibrotic matrix crosslinking and stabilization in the extracellular matrix [36]. Other than fibrotic diseases, LOXL2 also plays a crucial role in cancer by the modification of extracellular matrix [37]. Elevated LOXL2 expression in fibroblasts contributes to the formation of a metastatic niche in hepatocellular carcinoma [15]. LOXL2 inhibition with allosteric antibody reduced cancer-associated fibroblasts in human tumors [38, 39]. Furthermore, LOXL2 overexpression in cancer cells contributes to cancer invasion by silencing matrix metalloproteinase-9 and -1 to inhibit extracellular protein formation [40]. More importantly, LOXL2 induced epithelial-mesenchymal transition (EMT) of CRC cells

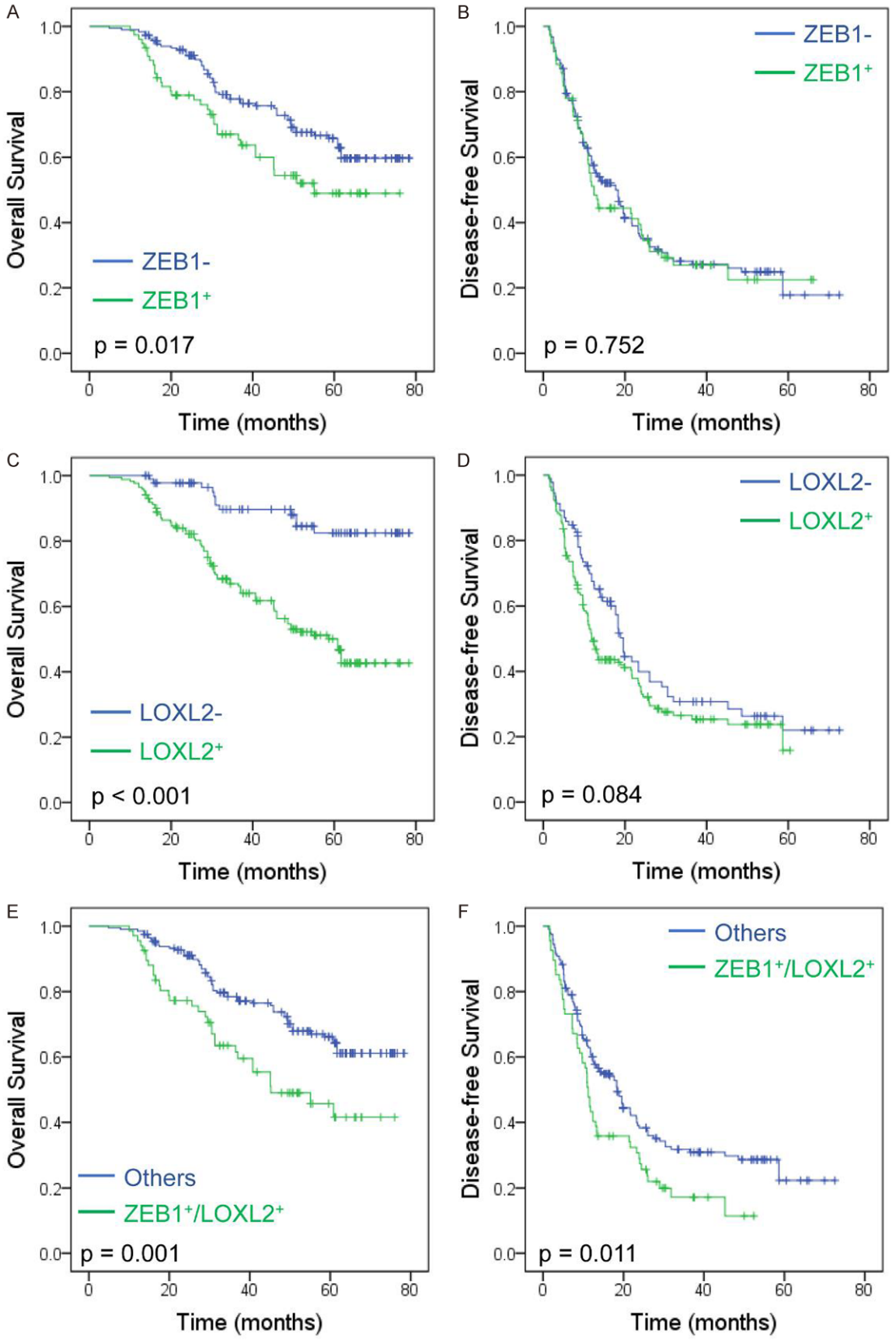
A
Overall Survival
ZEB1-
ZEB1+
p = 0.017
Time (months)
B
Disease-free Survival
ZEB1-
ZEB1+
p = 0.752
Time (months)
C
Overall Survival
LOXL2-
LOXL2+
p < 0.001
Time (months)
D
Disease-free Survival
LOXL2-
LOXL2+
p = 0.084
Time (months)
E
Overall Survival
Others
ZEB1+/LOXL2+
p = 0.001
Time (months)
F
Disease-free Survival
Others
ZEB1+/LOXL2+
p = 0.011
Time (months)

Figure 6. Increased ZEB1/LOXL2 expression predicts poor prognosis in colorectal cancer patients. A, B. The OS and DFS of CRC patients with different ZEB1 expression status. Positive ZEB1 expression was correlated with poor OS survival (P = 0.017), but no significant difference in DFS (P = 0.752). C, D. The OS and DFS of CRC patients with different ZEB1 expression status. Positive ZEB1 expression was correlated with poor OS survival (P < 0.001), but there was no significant difference in DFS (P = 0.084). E, F. Correlation between OS or DFS of CRC patients and ZEB1/LOXL2 expression. High ZEB1/LOXL2 expression was correlated with poor patient survival (P = 0.001, P = 0.011, respectively).

Table 2. Univariate and multivariate analyses of ZEB1/LOXL2 expression in disease-free survival and overall survival of colorectal cancer patients

Variable analysis	Disease-Free Survival			Overall Survival		
	HR	95% CI	P	HR	95% CI	P
Univariate		N = 263			N = 263	
ZEB1/LOXL2	1.513	1.096-2.089	0.012	2.005	1.302-3.086	0.002
Multivariate		N = 263			N = 263	
Age	0.943	0.696-1.277	0.704	0.685	0.449-1.045	0.079
Gender	0.936	0.714-1.226	0.629	0.78	0.53-1.147	0.207
ECOG	1.479	1.009-2.168	0.045	0.733	0.402-1.336	0.311
Location	0.859	0.58-1.274	0.451	0.899	0.496-1.628	0.725
Grade	0.908	0.571-1.444	0.684	1.234	0.657-2.319	0.514
Stage	0.907	0.581-1.417	0.667	0.606	0.322-1.142	0.121
LNM	0.914	0.662-1.262	0.587	0.831	0.529-1.306	0.422
ZEB1/LOXL2	1.601	1.148-2.233	0.006	2.172	1.384-3.408	0.001

CI = confidence interval; HR = hazard ratios; LNM = lymph node metastasis. The variables were compared in the following ways: Age, ≤59 years vs. >59 years; Gender, male vs. female; ECOG, 0 vs. ≥1; Location, left vs. right; Grade, low vs. middle and high; Stage, I-II vs. III-IV; LNM, no vs. yes; ZEB1/LOXL2 expression, both positive vs. others.

through the activation of FAK/Src pathway and upregulation of SNAII [36]. Increased cancer metastasis was also observed in LOXL2-positive CRC cells which was correlated with EMT [37, 41]. Our data also supported that LOXL2, as a downstream factor of ZEB1, participated in cell migration and invasion of CRC cells, but had no obvious effects on cell proliferation. The inhibition of LOXL2 impaired the invasion and migratory abilities in ZEB1-overexpressing CRC cells, which was correlated with the regulation of EMT-related molecules. Thus, ZEB1/LOXL2 is a promising biomarker to attenuate disease progression of CRC. An in-depth study of clinical trials for ZEB/LOXL2 inhibitors may shed light on the administration to CRC patients.

Clinical significance of ZEB1 and LOXL2 in CRC was also investigated in this study. Previous studies indicated that ZEB1 overexpression was crucial for the tumorigenesis of prostate cancer, gastric cancer, osteosarcoma, and hepatocellular carcinoma [8, 42]. Increased ZEB1 expression was also observed in CRC versus adjacent normal tissues [34], and was associated with poor clinical outcome. Increased LOXL2 expression was also observed in CRC tissues versus adjacent noncancerous tissues [43]. Retrospective clinical analysis also supported LOXL2 as a prognostic marker in CRC [44]. Here in this study, significant clinical correlation was observed in ZEB1/LOXL2 with serious clinicopathologic factors, including TNM stage and lymph node metastasis. In support of this, we provided further evidences that ZEB1/LOXL2 co-expression predicted disease progression in CRC according to Kaplan-Meier analysis and Cox regression models, and is an independent adverse factor for CRC patients.

Conclusions

In summary, our data support a model in which ZEB1 enhances LOXL2 transcription and expression through direct binding to its promoter. Gain of function assays indicate that LOXL2 expression is involved in increased cell migration and invasion, but did not affect cell prolif-

eration. IHC evaluation of ZEB1/LOXL2 provides important prognostic information for CRC patients. These findings provide a molecular basis for the promotion of invasive cancer phenotype by ZEB1-LOXL2 overexpression.

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Disclosure of conflict of interest

None.

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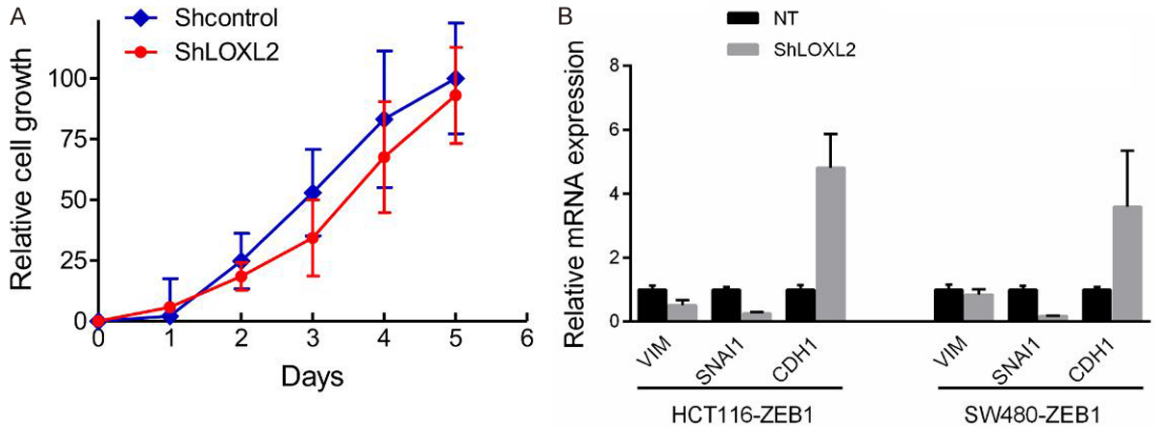

Figure S1. LOXL2 knockdown inhibits ZEB1-induced colorectal cell invasion, but has no effect on cell proliferation and migration (A) Cell proliferation was assessed with CCK-8 assays, which showed no significant difference in proliferation ability with LOXL2 knockdown in SW480 cells. (B) Expression of EMT-related molecules was examined in LOXL2-silenced ZEB1 overexpressing cells. VIM (vimentin), SNAI1 (Snail), CDH1 (E-cadherin). GAPDH was used as an internal control.