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Original Article

DEC1 is involved in TGF-β1-induced epithelial-mesenchymal transition of gastric cancer

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Abstract: DEC1 is a helix-loop-helix (bHLH) transcription factor, whose deregulation has been observed in several tumors. However, the effects of the dysregulation of this gene on epithelial-mesenchymal transition (EMT) are controversial, with its roles in gastric cancer (GC) remaining unclear. In the present study, we focused on the impact of DEC1 on EMT and cell mobility in gastric cancer. We found that DEC1 expression positively correlated with TGF-β1 and EMT markers in tumor issues, and that DEC1 facilitated TGF-β1-induced EMT in gastric cancer. In addition, gastric cancer cell migration potential was reduced after DEC1 knockdown. Using murine metastasis models, we confirmed that DEC1 promoted GC metastasis and further explored the correlation of DEC1 with TGF-β1 and E-cadherin *in vivo*. Chromatin immunoprecipitation (ChIP) assays revealed that DEC1 could directly interact with the promoter region of TGF-β1. These results suggest that DEC1 functions as a tumor enhancer that partially participates in TGF-β1-mediated EMT processes in GC, thus contributing to tumor metastasis.

Keywords: DEC1, TGF-β1, EMT, gastric cancer, migration

Introduction

DEC1 is a basic helix-loop-helix (bHLH) transcription factor and closely linked to lineage commitment and cellular differentiation [1]. DEC1 has been implicated in a variety of biological phenomena, including differentiation, regulation of molecular clock, immune response, and biological metabolism [2-5]. Recent studies have shown that DEC1 is an important player in the progression and metastasis of various tumors [6-10]. DEC1 increases the migratory and invasive abilities of pancreatic tumor cells *in vitro*, but inhibits the invasion and EMT process in human endometrial cancers. Although DEC1 plays an important role in tumor metastasis, the underlying mechanism remains unclear.

Epithelial-mesenchymal transition (EMT) is a genetically controlled and evolutionarily conserved process that allows epithelial cells to acquire mesenchymal characteristics and thereby gain increased motility and invasiveness [11, 12]. In this process, immotile epithelial cells transition into migratory mesenchymal cells, losing their epithelial characteristics of cell-cell adhesion and apical-basal polarity, while concomitantly displaying increased expression of mesenchymal adhesion proteins and markers [13]. In tumors, EMT provides malignant cells with enhanced metastatic capabilities, regulated by complex networks between tumor cells and the tumor microenvironment [14, 15]. Several pathways, including TGF-β, Notch, Wnt/β-catenin, Ras, and integrin signaling, have been reported to participate in the EMT process [8, 12, 16]. A previous study found DEC1 to function downstream of TGF-β1 [17]. Accumulating evidence suggests that DEC1 plays an important role in the EMT process in human pancreatic and endometrial cancers [8, 9]. However, no studies have focused

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on the influence of DEC1 on EMT in gastric cancers. Our previous research found that downregulation of DEC1 could inhibite GC cell proliferation *in vitro* and tumorigenicity *in vivo*, and that positive correlations between DEC1 and Survivin expression were observed in tissue sections from GC patients [18]. Here we aim to further elucidate the important role that DEC1 plays in gastric cancer metastasis.

In the present study, we focused on the impact of DEC1 on EMT and cell mobility in gastric cancer. We found that DEC1 expression was positively correlated with EMT marker in tumor issues, and DEC1 facilitated TGF-β1-induced EMT. In addition, the migration ability of gastric cancer cells decreased following the knockdown of endogenous DEC1. Using murine metastasis models, we confirmed that DEC1 promoted GC metastasis and further explored the correlation of DEC1 with TGF-β1 and E-cadherin. ChIP assays revealed that DEC1 could directly interact with the promoter region of TGF-β1. These results reveal that DEC1 is a player involved in TGF-β1-mediated EMT processes in GC, thus contributing to tumor metastasis.

Materials and methods

Cell culture

The human gastric cancer cell lines BGC823, MGC803, HGC27 and MKN-45 were obtained from the Cell Bank of Shanghai (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). All cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (FBS; Gibco). Cells were maintained at 37°C with 5% CO_2 in humidified incubator. These cells were incubated with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA).

Cell transfection

DEC1 vector (Genechem) was formed by linking human full length DEC1 CDS to GV218 vector. According to manufacturer's instructions, plate 1×10^5 cells in 500 μl of growth medium without antibiotics in each 24-well format. DEC1 vector was transfected into HGC27 and BGC823 cells using lipo2000 reagents. Cells transfected with empty GV218 vector served as negative control. In order to silence DEC1 expression, small interfering RNA against DEC1 (siDEC1) (Ribobio) was transfected within MGC803 and MKN-45 cells. siRNA sequences: siDEC1-1: GCAGTGGTTCTTGAACTTA, siDEC1-2: GATCGGCGCAATTAAGCAA, siDEC1-3: CCTCAGTGCCAGTGCTATA.

As we have described [18], tumor cell lines with stable DEC1 overexpression or knockdown were established by transfection of lentivirus containing DEC1-overexpression plasmid (pGV-puro vector, GeneChem, China) or DEC1 short hairpin RNA (shRNA) (pGC-LV vector, GeneChem, China), respectively, followed by selection with puromycin (5 μg/ml, Sigma).

Protein extraction and western blot assay

Cells were lysed with RIPA buffer (Beyotime, Jiangsu, China) supplemented with protease inhibitor PMSF. Total protein was quantified by BCA protein assay (Thermo Scientific, Rockford, USA). Proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Boston, MA, USA). Subsequently, the membrane was blocked and probed overnight at 4°C with primary antibodies including anti-DEC1 (1:500; Santa), anti-N-cadherin (1:10000; Epitomics), anti-E-cadherin (1:1000; Epitomics), anti-Vimentin (1:2500; Epitomics), anti-TGF-β1 (1:500; Abcam) and anti-GAPDH (1:2500; Proteintech Group, Inc., Wuhan, China). After incubation with HRP-conjugated secondary antibodies (1:10000, Proteintech Group, Inc., Wuhan, China), the blots were exposed to FluorChemE system (Cell Biosciences, Santa Clara, USA).

qRT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) following the manufacturer's instructions. First strand complementary DNAs were prepared using the Reverse Transcription Reaction Kit (Takara). qRT-PCR was performed using SYBR Premix Ex Taq (Takara) and analyzed on the Applied Biosystems 7300 Real-Time PCR System. The relative amount mRNA was calculated using the 2(-ΔΔCt) method after normalization to GAPDH mRNA levels. All the samples were performed in triplicates in each experiment.

PCR reaction was performed using DEC1 primers: 5'-ACTTACCTTGAAGCATGTGAAAGCA-3' (forward), 5'-CATGTCTGGAAACCTGAGCAGAA-3'

(reverse); TGF-β1 primers: 5'-GAGGGGAAATTGAGGGCTTT-3' (forward), 5'-CGGTAGTGAACCCGTTGATG-3' (reverse); GAPDH primers: 5'-AGAAGGCTGGGGCTCATTTG-3' (forward), 5'-AGGGGCCATCCACAGTCTTC-3' (reverse).

Enzyme-linked immunosorbent assay (ELISA)

The TGF-β1 production from different cancer cells was measured by a quantitative sandwich enzyme immunoassay technique using a Quantikine human TGF-β1 ELISA kit, according to the manufacturer's instruction (R&D systems). Cancer cells were incubated under normoxia for 24 h, then the medium was replaced to 3 ml serum free RPMI-1640. Cells were incubated for additional 24 h. Conditional medium (CM) was collected from each dish and centrifuged at 1000 g for 5 min. TGF-β1 level of serum free CM was measured using ELISA kit.

Wound-healing assay

To investigate their metastatic ability, cells (2×10^5 per well) were seeded in 24-well plates and cultured for 12 h until the confluency of monolayers reached 90-100%. Further, a 200-μl sterile pipette tip was used to scratch across the center of each well before the medium was replaced by new medium supplemented with 2% FBS. After incubating for 0 h, 24 h and 48 h, images of cells in the wells were captured by an inversion microscope (Olympus, Japan). The wound area was calculated to estimate the cell's migration efficacy.

Transwell assay

Cell migration was performed in 24-well transwell plates with 8 μm-pore polycarbonate membranes (Costar, Corning, MA, USA). The transfected cells (1×10^5 cell/ml) suspended in serum-free medium were added to the upper chamber and incubated for 24 h at 37°C for migration assay. Migrated cells were fixed with 100% methanol for 15 min, then stained with 0.1% Crystal Violet-Solution for 20 min, and five representative fields from sample were counted under a light optic microscope (Olympus). Each experiment was performed in triplicate.

Patients and ethics statement

A total of 63 specimens undergoing surgical resection were collected from GC patients admitted to Jinan Central Hospital affiliated to Shandong First Medical University from 2007 to 2014. All patients had not received treatment before sample collection. The tumor stage was clinically and histologically categorized basing on the guidelines described by the sixth edition of The American Joint Committee on Cancer. The clinicopathological features of patients are shown in **Table 1**. The research protocol and consent program were approved by institutionnal review board of Qilu Hospital of Shandong University (KYLL-2019(KS)-157).

Immunohistochemistry

IHC staining was performed using a standard immunoperoxidase staining procedure. Briefly, paraffin sections were cut to a thickness of 4 μm and mounted on silanized slides. Next, slides were dewaxed in xylene and hydrated in graded alcohol solutions. After antigen retrieval with heat treatment in 10 mM sodium citrate buffer (PH 8.0), the slides were incubated with the primary antibodies against anti-DEC1 (1:150, Genetex), anti-E-cadherin (1:500, Epitomics). Following a final wash, the slides were incubated with secondary antibody (KIT-5010, Max Vision, Maixin, Bio, China), and visualized by incubation with 3, 3-diaminobenzidine solution. Slides incubated with normal mouse or rabbit IgG instead of primary antibodies were used as negative control. The nucleus was counterstained with hematoxylin.

Immunohistochemical analysis was performed by two independent investigators concurrently. The percentage of stained cells was recorded from at least 5 random fields at 400× magnification. Cell staining was evaluated following the immunoreactive score (IRS) proposed by Remmele and Stegner with slight modifications [19]: IRS = SI (staining intensity) × PP (percentage of positive cells). SI was assigned as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0, negative; 1, 1-10% positive cells; 2, 11-50% positive cells; 3, 51-80% positive cells; 4, > 80% positive cells. For statistical analyses, cases with an IRS of 0-3 were defined as negative, otherwise as positive.

In vivo experiments

Male BALB/c nude mice (5 weeks old) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Animal studies were approved

Table 1. Association of DEC1, TGF-β1 or E-cadherin expression with the clinicopathological characteristics of GC

Characteristics	Case (63)	DEC1 expression		*P* value	TGF-β1 expression		*P* value	E-cadherin expression		*P* value
		High (43)	Low (20)		High (36)	Low (27)		High (18)	Low (45)	
Age (years)										
≤ 50	9	6	3	0.912	7	2	0.177	3	6	0.707
> 50	54	37	17		29	25		15	39	
Gender										
Female	21	13	8	0.444	13	8	0.589	10	11	0.018*
Male	42	30	12		23	19		8	34	
Tumor size (diameter)										
≤ 4	30	16	14	0.015*	17	13	0.942	11	19	0.175
> 4	33	27	6		19	14		7	26	
Differentiation										
I	39	22	17	0.01*	18	21	0.025*	14	25	0.101
II-III	24	21	3		18	6		4	20	
Tumor invasion (AJCC)										
Tis.-T2	18	7	11	0.002*	9	9	0.469	11	7	0.000*
T3-T4	45	36	9		27	18		7	38	
Lymphatic metastasis										
Absent	26	14	12	0.039*	11	15	0.046*	13	13	0.002*
Present	37	29	8		25	12		5	32	
TNM stage										
I-II	28	14	14	0.005*	13	15	0.124	13	15	0.005*
III-IV	35	29	6		23	12		5	30	

*Statistically significant difference, P < 0.05; AJCC, American Joint Committee on Cancer.

by the Institutional Animal Care Committee at Shandong University and conducted according to the NIH animal usage guidelines and Chinese regulations and standards for laboratory animal usage. A total of 5×10^5 HGC27 or 2×10^6 MKN-45 stably transfected cells suspended in 100 μl of medium were injected into the tail vein (n=6 per group). Mice were killed after 4 weeks (MKN-45 groups) or 8 weeks (HGC27 groups), and their lungs were harvested for further study. Visible lung surface macro-metastatic spots were counted using a dissecting microscope (Nikon). Then, the lung tissues were embedded in paraffin and sectioned. Hematoxylin-eosin (HE) staining was performed to determine the number of metastatic lesions, and IHC staining was conducted to detect DEC1, TGF-β1, E-cadherin levels.

ChIP

ChIP was conducted using an enzymatic ChIP kit (Cell Signaling Technology, #9003) according to the manufacturer's instructions. Chromatin samples were incubated with anti-DEC1 (Novus Biological, NB100-1800) antibody. Rabbit IgG (Cell Signaling Technology, #2729) was used as negative controls. A non-immunoprecipitated sample (2%) was used as the input control. The purified DNA was then detected by qPCR. The ChIP-qPCR was performed using TGF-β1 primers: 5'-ATTGGGGACAGTAAATGTATGG-3' (forward), 5'-GGGAAACAAGGTAGGAGAAGAG-3' (reverse).

Statistical analysis

SPSS version 19.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± SEM and tested by KS normality test to examine whether they were normally distributed. Student's t-test was employed for two-group comparisons if the data was fit for Gaussian distribution. The Mann-Whitney test was used for non-normally distributed data. The associations between the

expression of DEC1 and clinicopathologic features were analyzed using Pearson's chi-squared test. The spearman correlation analysis was used to assess the correlation between DEC1 expression and E-cadherin and TGF-β1 expression. $P < 0.05$ was considered statistically significant.

Results

DEC1 levels are associated with EMT in gastric cancer cells and enhance cell migration

EMT is an initial and critical step in the metastasis of many human cancers [20]. Our previously study indicates that downregulation of DEC1 inhibits gastric cancer (GC) cell proliferation *in vitro* and tumorigenicity *in vivo* [18]. However, its roles in the metastasis of GC remain unclear. To assess the potential impact of DEC1 on EMT in gastric cancer, we examined the expression levels of DEC1 in various GC cell lines, including HGC-27, MGC803, BGC823 and MKN-45 cells. Interesting, higher DEC1 expression correlated with increased levels of TGF-β1 and mesenchymal-related proteins (**Figure 1A**). Additionally, a positive correlation was observed between the mRNA levels of DEC1 and TGF-β1 across these different GC cell lines (Figure S1A, S1B). These findings suggest that DEC1 is involved in the EMT process, potentially contributing to tumor metastasis. Consistent with stronger DEC1 expression, MGC803 cells exhibited greater migration in wound healing assays than DEC1-lower BGC823 cells (**Figure 1B**).

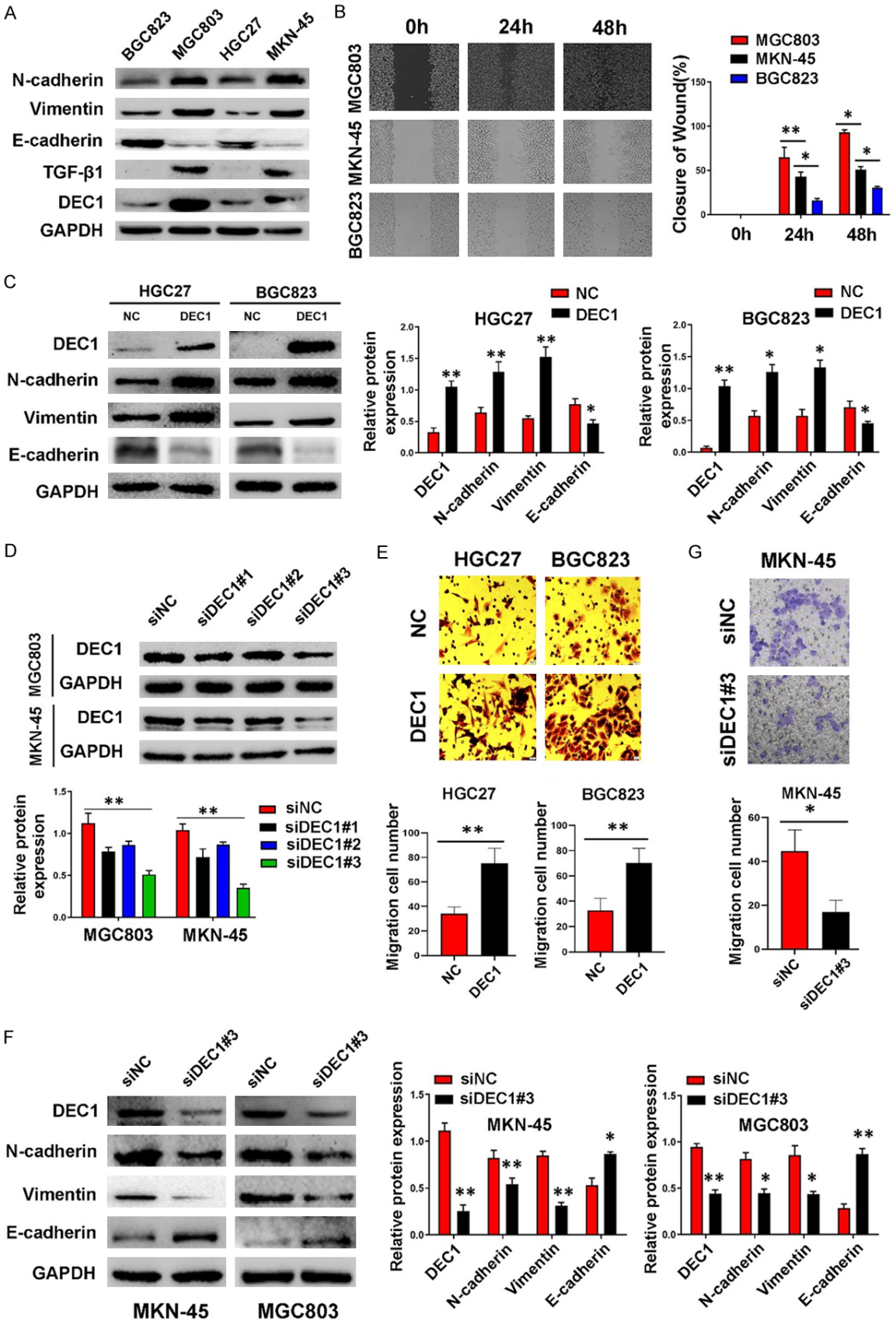
DEC1 promotes EMT process in gastric cancer cells

To investigate whether DEC1 could trigger the EMT of gastric cancer cells, we used a Western blot assay to examine the expression levels of EMT biomarkers following manipulation of DEC1 expression. HGC-27 and BGC823 cells with endogenous low DEC1 expression were selected for DEC1 up-regulation by DEC1 vector transfection. DEC1/HGC-27 and DEC1/BGC823 cells transfected with DEC1 vector showed increased expression of mesenchymal-related proteins (N-cadherin and Vimentin) compared to cells with corresponding vector transfection (**Figure 1C**). Additionally, the increased migration in HGC-27 and BGC823 cells transfected with DEC1 vector was observed (**Figure 1E**).

Next, DEC1 down-regulation was performed through expressing targeted, small interfering RNA. DEC1-siRNA and control-siRNA were transfected into MGC803 and MKN-45 cells with endogenous high DEC1 expression to achieve significant known-down (**Figure 1D**). DEC1 siRNA significantly suppressed the expression of N-cadherin and Vimentin, while increasing the expression of E-cadherin (**Figure 1F**). Moreover, DEC1 knockdown significantly reduced the migration of MKN-45 cells, as analyzed by Transwell assays (**Figure 1G**). Therefore, DEC1 appears to increase mesenchymal-like phenotypes in GC cells and promotes cell migration.

DEC1 is involved in TGF-β1-induced EMT

EMT, an initial and critical step in metastasis, can be induced by various signals from the tumor microenvironment, including TGF-β1 [21-23]. To determine whether DEC1 is induced by TGF-β1 in gastric cancer cells, we treated DEC1-high MGC803 cells and DEC1-low BGC823 cells with selected concentrations of TGF-β1. As shown in **Figure 2A**, **2B**, DEC1 was upregulated in both cell lines after treated with TGF-β1. However, TGF-β1-treated MGC803 cells exhibited minor changes in N-cadherin, Vimentin, E-cadherin, while more pronounced changes were seen in BGC823 cells. This suggests that while DEC1 expression can be upregulated by TGF-β1 induction, DEC1-low tumor cells are more susceptible to TGF-β1-induced EMT. Subsequently, we assessed TGF-β1 production from gastric cancer cells using ELISA. The production of TGF-β1 from DEC1-high MGC803 cells was significantly increased compared to DEC1-low BGC823 cells (**Figure 2C**). To ascertain whether the abolishment of TGF-β1-induced EMT in DEC1-siRNA cells resulted from dysregulation of EMT-related proteins, we analyzed the expression of the epithelial marker E-cadherin and mesenchymal markers N-cadherin and Vimentin. As expected, TGF-β1 treatment reversed the inhibition of N-cadherin and Vimentin and also reversed the upregulation of E-cadherin in siDEC1/MGC803 cells. Moreover, pretreatment of cells with TGF-β1 further increased the expression of N-cadherin and Vimentin and further inhibited the expression of E-cadherin in DEC1/BGC823 cells (**Figure 2D**, **2F**, **2G**). These findings suggest that DEC1 has inducible effects on EMT following TGF-β1 treatment in gastric cancer.


A
BGC823
MGC803
HGC27
MKN-45
N-cadherin
Vimentin
E-cadherin
TGF-β1
DEC1
GAPDH
B
0h
24h
48h
MGC803
MKN-45
BGC823
MGC803
MKN-45
BGC823
Closure of Wound(%)
0h
24h
48h
C
HGC27
BGC823
NC
DEC1
DEC1
N-cadherin
Vimentin
E-cadherin
GAPDH
NC
DEC1
HGC27
Relative protein expression
DEC1
N-cadherin
Vimentin
E-cadherin
NC
DEC1
BGC823
Relative protein expression
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N-cadherin
Vimentin
E-cadherin
D
siNC
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siDEC1#2
siDEC1#3
MGC803
DEC1
GAPDH
MKN-45
DEC1
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Relative protein expression
siNC
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siDEC1#2
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BGC823
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HGC27
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NC
DEC1
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Migration cell number
NC
DEC1
G
MKN-45
siNC
siDEC1#3
MKN-45
Migration cell number
siNC
siDEC1#3
F
siNC
siDEC1#3
siNC
siDEC1#3
DEC1
N-cadherin
Vimentin
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GAPDH
MKN-45
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MKN-45
Relative protein expression
DEC1
N-cadherin
Vimentin
E-cadherin
siNC
siDEC1#3
MGC803
Relative protein expression
DEC1
N-cadherin
Vimentin
E-cadherin

Figure 1. High DEC1 expression increased mesenchymal-like phenotypes in GC cells and promoted cell mobility. A. Expression of DEC1 and EMT-related proteins assessed by western blotting in the HGC-27, MGC803, BGC823, MKN-45 gastric cancer cell lines. MGC803 cells showed greater expression of DEC1 as well as Vimentin, β-catenin and N-cadherin, HGC-27 and BGC823 cells showed weaker DEC1 and lower mesenchymal-related proteins, but express relatively higher E-cadherin. B. DEC1-high MGC803 and MKN-45 cells exhibited a greater ability to migrate than DEC1-low BGC823 cells (Magnification ×4). C. Expression of DEC1 and EMT-related proteins assessed by western blotting in the HGC-27 and BGC823 cells transfected with DEC1 vector and empty vector, respectively. E. Comparison of transfer ability of HGC-27 and BGC823 cells transfected with DEC1 vector and empty vector by the migration assay (Magnification ×200). D, F. Expression of DEC1 and EMT-related proteins assessed by western blotting in the MGC803 and MKN-45 cells transfected with siDEC1 and siNC, respectively. G. Comparison of transfer ability of MKN-45 cells transfected with DEC1-siRNA and control-siRNA by the migration assay (Magnification ×200). The error bars indicate ± SEM. *P < 0.05; **P < 0.01 by Student's t-test. All the results were repeated thrice.

DEC1 promotes TGF-β1-induced migration in gastric cancer cells

To further explore the contribution of DEC1 to tumor cell migration, we examined its effect on the migration of MGC803 and BGC823 cells using a Transwell migration assay. As illustrated in **Figure 2H**, DEC1-high MGC803 cells were significantly more migratory than DEC1-low BGC823 cells. Additionally, TGF-β1 stimulation, compared with no treatment, significantly increased the migration of siDEC1/MGC803 and DEC1/BGC823 cells.

DEC1 transcriptionally regulates TGF-β1 expression

DEC1, a basic helix-loop-helix (bHLH) transcription factor, binds to specific sequences in the target gene promoter and enhances the transcription of downstream genes [9]. Our previous results demonstrated that TGF-β1 upregulates the expression of DEC1, which plays an important role in TGF-β1-induced EMT. Additionally, we observed a correlation between DEC1 and TGF-β1 expression at both protein and mRNA levels (**Figures 1A**, **2A**, S1A, S1B). To ascertain whether DEC1 can regulate TGF-β1 transcription, we conducted Bioinformatic analysis and ChIP assays. DEC1 was predicted to bind with the TGF-β1 promoter sequence by the JASPAR database, suggesting DEC1 might transcriptionally regulate TGF-β1 expression (Figure S1C). Further ChIP experiments confirmed that DEC1 directly interacts with the promoter region of TGF-β1 (**Figure 2E**).

Expression of DEC1, TGF-β1, and E-cadherin in human gastric cancer tissues

The expression levels of DEC1, TGF-β1 and E-cadherin were compared in primary lesion of 63 patients with gastric cancer using immunohistochemical staining (**Figure 3A**). High expressions of DEC1 and TGF-β1 were detected in 68.3% (43/63) and 57.1% (36/63) of primary gastric cancer tissues, respectively, while lower expression of E-cadherin was detected in 71.4% (45/63) tissues (**Table 1**). Consistent with the results in GC cell lines, significant TGF-β1 immunoreactivity was detected in DEC1-high GC tissues, but E-cadherin expression was lower (**Figure 3B**). We then evaluated the correlations between clinicopathological features of patients with gastric cancer and expression levels of DEC1 and EMT-related proteins in primary lesions (**Table 1**). The expression levels of DEC1 were closely correlated with tumor Size (P=0.015) and lymphatic metastasis (P=0.039). TGF-β1 expression was related to differentiation (P=0.025). E-cadherin expression was significantly correlated with invasion and lymphatic metastasis (P=0.000 and P=0.002, respectively). Spearman relation analysis further examined the correlation between DEC1, TGF-β1, and E-cadherin. The results indicated that the expression levels of DEC1 was positively related to TGF-β1 (r=0.305, P=0.015), but negatively related to E-cadherin expression (r=-0.399, P=0.002) in GC tissues (**Table 2**).

DEC1 facilitated GC metastasis in vivo

Our *in vitro* studies indicated that DEC1 enhanced cell motility by inducing EMT. To gain insight into the involvement of DEC1 in metastasis *in vivo*, DEC1-overexpressing HGC27 cells or DEC1-knockdown MKN-45 cells were injected into nude mice. As shown in **Figure 4A**, overexpression of DEC1 in HGC27 cells increased the number of metastatic nodules both on the surface of and inside the lungs in these animals. Consistent with this observation, DEC1 knockdown in MKN-45 cells reduced lung metastasis (**Figure 4B**). We also performed IHC

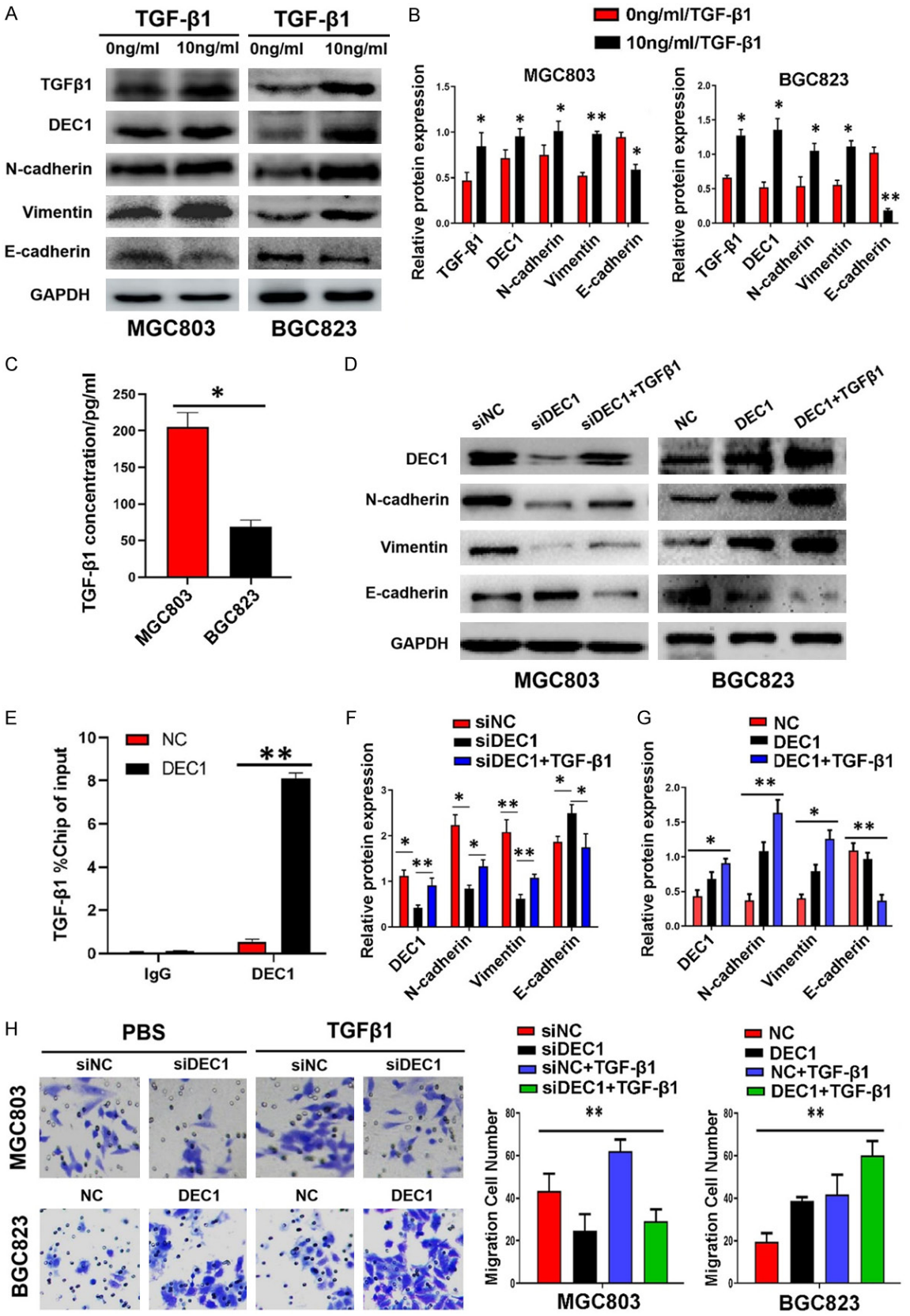

Figure 2. DEC1 was induced by TGF-β1 and promoted TGF-β1-induced migration in gastric cancer cells. A, B. Western blot results of DEC1 expression and EMT-related protein derived from DEC1-high MGC803 and DEC1-low BGC823

cells after treated with 10 ng/ml TGF-β1. C. TGF-β1 production in DEC1-high MGC803 cells was significantly higher than DEC1-low BGC823 cells. D, F, G. Western blot results of EMT-related proteins derived from siDEC1/MGC803 and DEC1/BGC823 cells alone or combined with TGF-β1 (10 ng/ml). E. ChIP assay revealed that DEC1 directly interacts with the promoter region of TGF-β1 gene. DEC1 antibody was used for IP, and qPCR of the TGF-β1 promoter in output was performed. H. TGF-β1 stimulation significantly increased the migration of siDEC1/MGC803 and DEC1/BGC823 cells, compared with no TGF-β1 treatment. The error bars indicate ± SEM. *P < 0.05; **P < 0.01 by Student's t-test. All the results were repeated thrice.

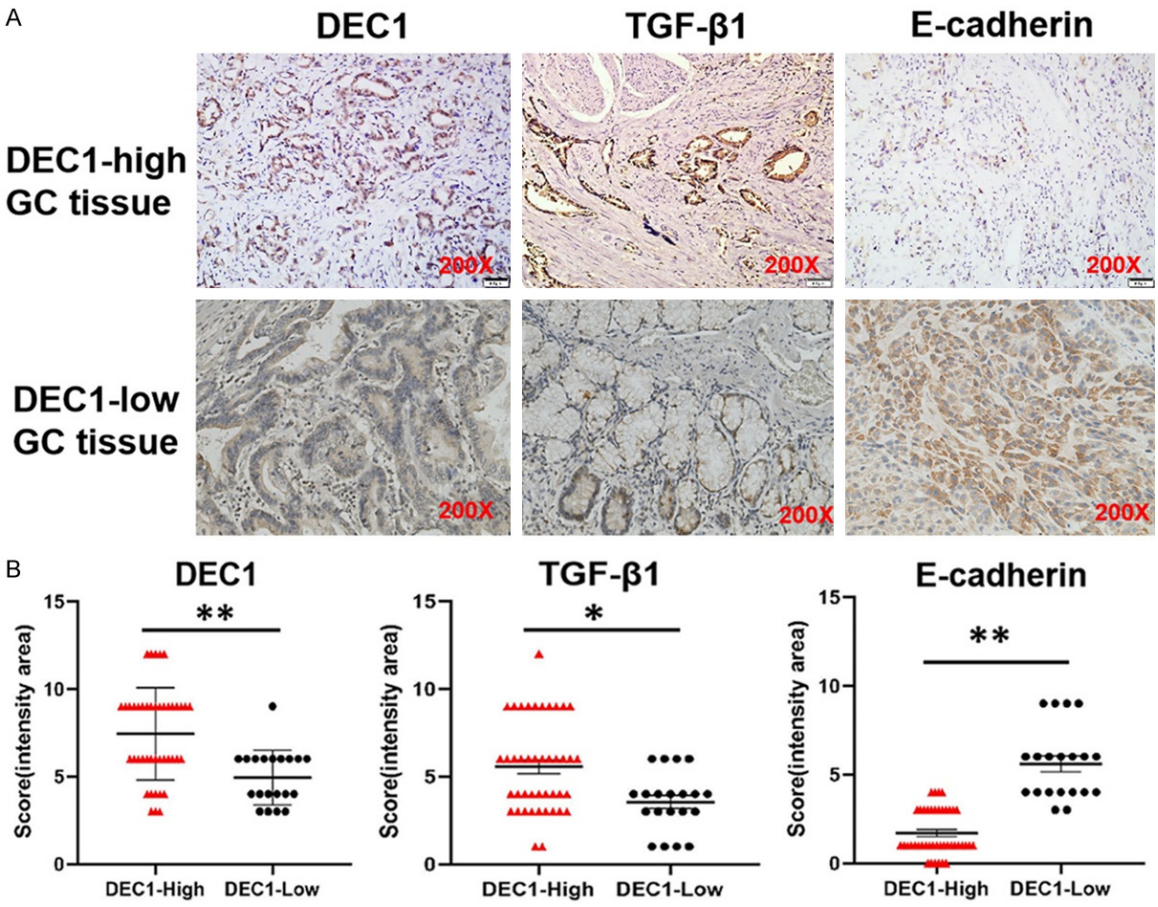

Figure 3. Expression of DEC1, TGF-β1 and E-cadherin in GC tissues. DEC1 expression in GC tissues was positively correlated with TGF-β1 levels but negatively correlated with E-cadherin levels. Representative images of cells expressing high or low levels of DEC1 were displayed in (A) and the statistical results were displayed in (B). The cutoff score for positive staining of DEC1, TGF-β1 and E-cadherin were ≥ 4. *P < 0.05; **P < 0.01.

to elucidate the expression of DEC1, EMT markers, and TGF-β1. As expected, metastatic tumors in the DEC1-overexpression group showed dramatically elevated TGF-β1 levels as well as lower E-cadherin levels (**Figure 4C**). In contrast, DEC1-knockdown had the opposite effect on the expression of these proteins (**Figure 4D**). Taken together, our data point to a model in which DEC1 facilitates GC metastasis via TGF-β1-induced EMT *in vivo*, while inhibiting DEC1 suppresses GC metastasis.

Discussion

Metastasis is a leading cause of cancer-related death [24]. There is a critical need to study the mechanism of GC metastasis and to identify potential molecular targets to further improve the treatment and survival of GC patients. In our current study, we found that DEC1 is involved in GC migration and, both *in vivo* and *in vitro*. First, we revealed that higher DEC1 expression correlated with increased levels of

Table 2. Spearman relation analysis among expressions of DEC1, TGF-β1 and E-cadherin in gastric cancer tissues by immunohistochemistry

	DEC1 expression		*P* value	r value
	High (43)	Low (20)		
TGF-β1 expression				
High	29	7	0.015*	0.305
Low	14	13		
E-cadherin expression				
High	7	9	0.002*	-0.399
Low	36	11		

*Statistically significant difference, P < 0.05.

mesenchymal-related proteins in corresponding GC cells. Furthermore, consistent with higher DEC1 expression, MGC803 cells demonstrated greater mobility in wound healing assays compared to DEC1-low BGC823 cells, aligning with previous findings [8]. In addition, Transwell assay results showed that the migration ability of MGC803 cells was remarkably attenuated due to knockdown of DEC1, whereas overexpression of DEC1 dramatically increased the number of migrating MGC823 cells. Together, these data suggest that increased expression of DEC1 may be involved in promoting metastasis of GC.

Moreover, we explored the molecular mechanism by which DEC1 influence GC cell metastasis. The dynamic EMT process mediates the transition from immotile epithelial to mobile mesenchymal cells, initiating and accelerating metastasis [12, 25, 26]. Through this process, epithelial cells lose their epithelial characteristic of cell-cell adhesion and acquire a mesenchymal phenotype [27]. Growing evidence has demonstrated that EMT plays a crucial role in the metastasis of cancer cells across various tumor types, including gastric cancer [28-30]. Our previous study found that DEC2 could inhibit EMT-associated metastasis via the inactivation of the ERK/NF-κB pathway in gastric cancer [31]. Here, we showed that overexpression of DEC1 upregulated mesenchymal markers N-cadherin and Vimentin, but downregulated the epithelial marker E-cadherin. Conversely, downregulation of DEC1 led to opposite effects. As such, we concluded that DEC1 promotes EMT in gastric cancer cells as up-regulation of EMT in DEC1-high tumor cells appears to be one of the reasons for the higher metastatic potential compared to DEC1-low tumor cells.

TGF-β signaling is initiated upon interaction of TGF-β with transmembrane kinase receptors, TGF-βRI and TGF-βRII [32]. TGF-β serves as a potent inducer of EMT by activating both Smad and non-Smad signaling pathways [21, 33, 34]. A previous study has demonstrated that down-regulation of DEC1 inhibits the Smad3 phosphorylation and the expression of TGF-βRI after TGF-β treatment in pancreatic cancer [8]. Furthermore, a recent report suggested that DEC1 inhibits EMT by competing with SP1 for binding to the same proximal region of the TWIST1 promoter [9]. Zawel L et al. identified DEC1 as a downstream target of TGF-β with sequence-specific transcriptional repressor activities [17]. Based on these findings, we hypothesized that DEC1 plays a crucial role in EMT process. We then investigated whether DEC1 participates in TGF-β1-induced EMT in gastric cancer. Transwell migration assays were used to evaluate the effect of DEC1 on cell motility with or without TGF-β1 treatment. Our results showed that after treatment with selected concentrations of TGF-β1, the expression of DEC1 in MGC803 and BGC-823 cells increased. DEC1 further increased the expression of N-cadherin induced by TGF-β1 in these cells while decreasing the expression of E-cadherin. Intriguingly, we noticed that MGC803 cells with high DEC1 expression were not sensitive to TGF-β1 stimulation, contrary to DEC1-low tumor cells, which were more susceptible to TGF-β1-induced EMT. Tumor cells can produce TGF-β1 through either autocrine or paracrine signaling, resulting in higher concentrations of TGF-β1 in tumor tissues than physiological levels. We also examined the secretion levels of TGF-β1 in different gastric cancer cell lines and found that TGF-β1 production from DEC1-high MGC803 cells was significantly higher than from DEC1-low BGC823 cells. Therefore, this high basal level of TGF-β1 in MGC803 cell might be one of the key reasons of the differential response to TGF-β1 stimulation regarding DEC1 induction. TGF-β1 in the tumor microenvironment, secreted by tumor-related fibroblasts, platelets, and tumor cells, plays a vital role in EMT activation [22, 35]. Our results showed that TGF-β1 induced EMT in GC cells, increased DEC1 expression, and revealed a correlation between DEC1 and TGF-β1 expression at both protein and mRNA levels. ChIP assay confirmed that DEC1 directly interacts with the promoter

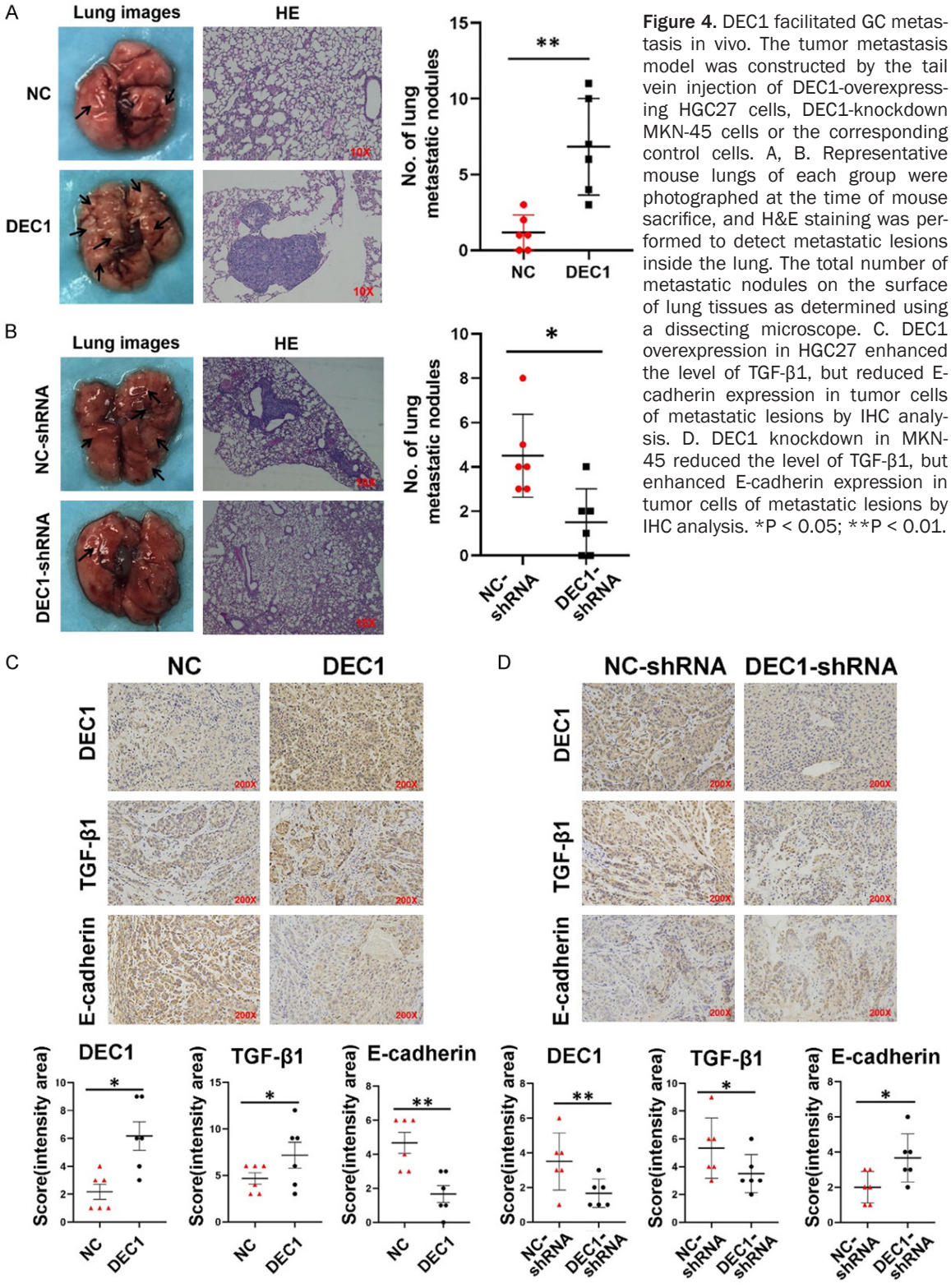

Figure 4. DEC1 facilitated GC metastasis in vivo. The tumor metastasis model was constructed by the tail vein injection of DEC1-overexpressing HGC27 cells, DEC1-knockdown MKN-45 cells or the corresponding control cells. A, B. Representative mouse lungs of each group were photographed at the time of mouse sacrifice, and H&E staining was performed to detect metastatic lesions inside the lung. The total number of metastatic nodules on the surface of lung tissues as determined using a dissecting microscope. C. DEC1 overexpression in HGC27 enhanced the level of TGF-β1, but reduced E-cadherin expression in tumor cells of metastatic lesions by IHC analysis. D. DEC1 knockdown in MKN-45 reduced the level of TGF-β1, but enhanced E-cadherin expression in tumor cells of metastatic lesions by IHC analysis. *P < 0.05; **P < 0.01.

region of TGF-β1 gene. In summary, DEC1 can trigger TGF-β1-induced EMT process.

Additionally, we also examined the expression of DEC1, TGF-β1, and E-cadherin in gastric cancer tissues and analyzed their correlations. As expected, significant DEC1 and TGF-β1 immunoreactivity was observed in cancer tissues, while the E-cadherin expression was lower. DEC1 expression was significantly correlat-

ed with TGF-β1 (P=0.015) and E-cadherin (P=0.002), respectively. Using murine metastasis models, we further confirmed that DEC1 promoted GC metastasis and explored the correlation of DEC1 with TGF-β1 and E-cadherin *in vivo*.

In conclusion, our results demonstrated that DEC1 expression positively correlated with TGF-β1 and EMT markers in tumor issues. As far as we know, this is the first study to show that DEC1 could promote EMT in gastric cancer cells, establishing a positive feedback loop between DEC1 and TGF-β1. Furthermore, we found that reducing DEC1 expression could inhibit gastric cancer cell migration by suppressing the EMT process induced by TGF-β1. These findings help contribute to our understanding of the role of the TGF-β1-DEC1-EMT axis in gastric cancers.

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Disclosure of conflict of interest

None.

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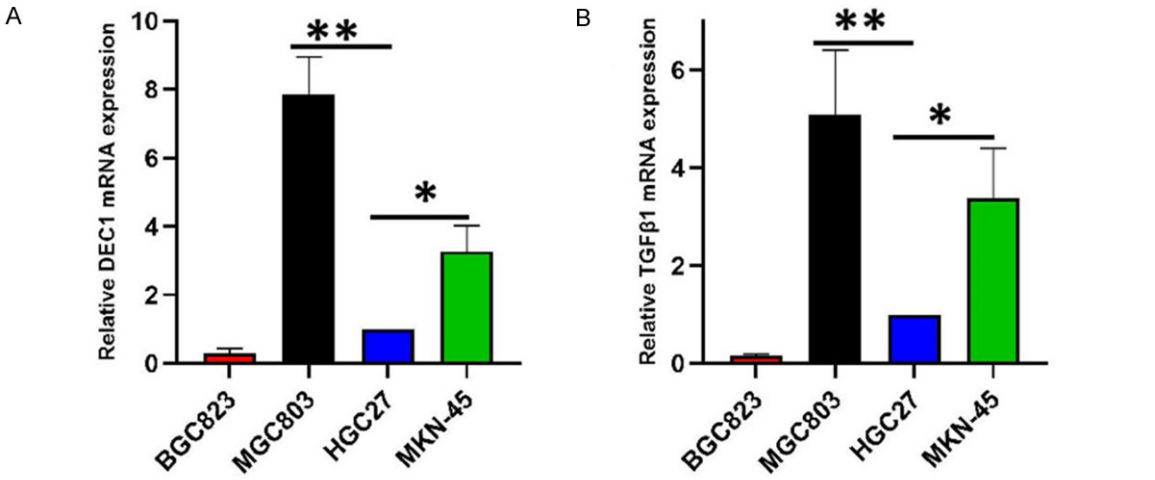

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	Name	Score	Relative score	Sequence ID	Start	End	Strand	Predicted site sequence
MA0464.2	MA0464.2.BHLHE40	6.374133	0.853791742674188	NC_000019.10:c41332323-41330323	1362	1371	+	CACCCGTGCC

Figure S1. A, B. The mRNA levels of DEC1 and TGF-β1 were examined by real-time PCR analyses across the different GC cell lines including BGC823, MGC803, HGC27 and MKN-45. The expression levels of DEC1 and TGF-β1 were normalized to GAPDH. C. DEC1 was predicted to bind with the TGF-β1 promoter sequence by the JASPAR database. *, $P < 0.05$; **, $P < 0.01$.