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Original Article

Krüppel-like factor 9 upregulates E-cadherin transcription and represses breast cancer invasion and metastasis

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Abstract: Aberrant expression of Krüppel-like factor 9 (KLF9) is frequently found in some types of cancer and is implicated in cancer initiation and progression. However, the effects of KLF9 on cancer metastases and the underlying mechanisms still need to be understood. Here, we found that KLF9 evidently inhibited the capabilities of migration and invasion of breast cancer cells. The expression of KLF9 was markedly decreased in breast cancer patients compared with benign tumors, and was positively correlated with the expression of E-cadherin in the tissues of breast cancer patients. Mechanistically, chromatin immunoprecipitation combined with site-directed mutagenesis-luciferase assay revealed that KLF9 activated the *E-cadherin* promoter by binding to GT-box elements located +84 bp and -143 bp from the *TSS* in the *E-cadherin* promoter, leading to elevated expression of E-cadherin mRNA and protein. *In vivo* experiments confirmed that KLF9 strongly inhibited the lung metastasis of breast cancer and increased mouse E-cadherin expression in 4T1 mouse breast cancer cells. Taken together, our findings demonstrated that KLF9 could suppress breast cancer invasion and metastasis by upregulating E-cadherin, which provided new insight into aggressive treatment of breast cancer by targeting the KLF9/E-cadherin axis.

Keywords: Krüppel-like factor 9, E-cadherin, metastasis, breast cancer

Introduction

Breast cancer is the most common malignancy and the major etiology of cancer-related mortality in women throughout the world. Cancer metastasis remains the major cause of cancer recurrence and tumor-associated death [1]. Metastasis, a complex biological process comprising multiple steps, includes loss of cell-cell adhesion, increased motility and invasiveness, degradation and remodeling of extracellular matrix (extracellular matrix, ECM), entry and survival in the lymph or blood circulation, spread to distant anatomic sites, and colonization at some of those sites [2].

E-cadherin, which is the classical member of the type-1 classical cadherins, is a well-known tumor metastasis suppressor protein. It is a single-span transmembrane glycoprotein that forms the core structure of adherens junctions (adherens junctions, AJs) with opposing E-cadherin molecules on adjacent epithelial cells [3]. The cytoplasmic domain of E-cadherin interacts with β-catenin, which link the actin cytoskeleton via α-catenin. The integration of AJs and the cytoskeleton by E-cadherin promotes the intercellular adhesion of epithelial cells [4]. Owing to the well-established function of E-cadherin in maintaining AJs, loss of E-cadherin or its inactivation promotes metastasis by enabling the dissociation of tumor cells from neighboring cells, which is an early step in metastatic progression [5, 6]. Additionally, E-cadherin loss or reduction may lead to the activation of several downstream signaling pathways, thereby promoting the expression of prometastatic genes [2]. In detail, E-cadherin loss may liberate β-catenin and subsequently activate Wnt/β-catenin signaling [7], affect the expression of cell matrix adhesion receptors, and induce the "cadherin switch" by changing the gene expres-

sion profile involved in epithelial-to-mesenchymal transition (epithelial-to-mesenchymal transition, EMT), which is associated with the phenotypic shift during cancer metastasis [8, 9], all of which may contribute to different stages of the metastatic cascade. However, recent studies have demonstrated that many metastatic tissues continue to express E-cadherin [10], and E-cadherin is involved in collective cell behaviors facilitating epithelial cell metastasis. This controversial phenomenon might be attributable to the existence of two forms of E-cadherin: membrane-anchored E-cadherin and soluble E-cadherin [8]. Thus, some investigators suggest that changes in the functional activity of E-cadherin expressed on the tumor cell surface in response to environmental factors are an important determinant of the ability of cancer cells to metastasize [11]. Additionally, a recent meta-analysis involving 33 studies showed that reduced E-cadherin expression might be a predictor of a poorer prognosis and promote breast cancer metastasis [12]. Therefore, the significance of E-cadherin inactivation for metastasis should be further recognized, and the mechanism by which E-cadherin expression is regulated in cancer metastasis still needs to be understood.

Krüppel-like factor 9 (Krüppel-like factor 9, KLF9), which belongs to the KLF transcriptional factor family, can bind GC-rich elements or GT-boxes (CACCC) through three C_2-H_2 zinc finger domains and regulate diverse biological processes, including neuronal development, cell differentiation, growth, proliferation, and apoptosis. Recently, emerging evidence has revealed that KLF9 is downregulated in many cancer types and associated with cancer metastasis, including breast cancer [13], gastric cancer [14], lung cancer [15], and renal cell carcinoma [16]. Our previous study showed that KLF9 was downregulated in breast cancer cells and inhibited cancer metastasis by regulating MMP9 expression, which is associated with ECM degradation [13]. This is the first study aimed at determining the function of KLF9 in cancer metastasis and the corresponding mechanisms through which KLF9 regulates the metastatic process. One study performed in 2019 indicated that KLF9 suppressed gastric cancer cell metastasis by inhibiting MMP28 transcription [14]. Another study showed a contradictory result: THBS4 promoted gastric cancer metastasis by increasing KLF9 expression, indicating an opposite effect of KLF9 in cancer metastasis [17]. Furthermore, the miRNA-140-5p/KLF9/KCNQ1 pathway was detected to regulate tumor cell metastasis in renal carcinoma [16]. A clinical study of KLF9 in breast cancer patients indicated that reduced expression of KLF9 was related to lymph node metastasis, low differentiation, high clinical stage, and affected the survival of breast cancer patients [18]. Considering the critical role of KLF9 in breast cancer metastasis, we therefore speculated that in addition to the ECM/MMP9 signaling pathway, KLF9 might inhibit breast cancer metastasis through other molecular pathways, which could explain the effect of KLF9 on metastasis in depth.

In the current study, we indicated that KLF9 could repress breast cancer metastasis by upregulating E-cadherin expression. The *in vitro* results demonstrated that KLF9 transcriptionally upregulated E-cadherin expression by binding to GT-boxes on the promoter of *E-cadherin*. In malignant tissues of human breast, relatively low expressions of KLF9 were found in aggressive breast cancers, and the protein level of KLF9 were positively associated with E-cadherin expression.

Materials and methods

Cell culture and transfection

The cell lines involved in this study were human HEK 293T, breast cancer cells (MCF-7, T47D), and 4T1 murine breast cancer cells. Cells were cultured at 37°C in a 5% CO_2 atmosphere with appropriate humidity. When cells were growth 70-80% confluence, they were transfected with plasmid using Lipofectamine 3000 (Invitrogen) according to the standard protocol.

Plasmids construction

The human KLF9 cDNA sequence was inserted into the pcDNA3.1-Flag vector at multiple clone sites of *EcoRI* and *XhoI* as previously described [13]. Two synthesized sequences used for knocking down endogenous KLF9 (shKLF9#1, 5'-acagtctggaaagtccagat-3'; shKLF9#2, 5'-tgaggagtgaccacctcacaaagcacgcc-3') were inserted into the pRNATU6.1/Hygro (GenScript, Piscataway, NJ) vector at the *BamHI-HindIII* site to construct interference plasmids.

Scratch wound healing and transwell assays

Scratch wound healing was conducted in a culture dish with the diameter of 35 mm [19]. Briefly, cells in dishes were scratched vertically with a 200 μl pipette tip and photographed in a microscopic field, which was recoded as a 0-hour scratched area with a mark around the field. After twenty-four hours, the same field was photographed according to the mark and recorded as a 24-hour scratched area. The distance between the scratched cells at 24 hours and 0 hours was recorded to analyze the wound healing rate. Transwell invasion assays were conducted in chambers covered with Matrigel (BD, Bioscience). Cells were cultured in the top chamber with Matrigel-coated membrane. After 24 hours of incubation, cells translocating to the lower surface of the membrane were stained and counted.

RNA extraction and reverse transcription-PCR

Extraction of total RNA and reverse transcription to cDNA were performed as described previously [13]. Primers used for RT-PCR were as follows: *KLF9*: 5'-tcccatctcaaagcccatta-3' (sense) and 5'-aactgcttttccccagtgtg-3' (antisense); *E-cadherin*: 5'-cttcggaggagagcggtg-3' (sense) and 5'-ctagtcgtcctcgccgcc-3' (antisense); and *GAPDH*: 5'-ggtgtgaaccatgagaagt-3' (sense) and 5'-gactgtggtcatgagtcct-3' (antisense). All PCR products were electrophoresed on a 1% agarose gel.

Real-time PCR assay

Isolation of total RNA and reverse transcription reaction were performed as described previously [13]. We applied Bio-Rad CFX Connect Real-Time Detection System with TB Green Premix Ex Taq II (Takara) to detect mRNA levels of target genes. Data outputted from the PCR system were normalized to the product level of reference gene *GAPDH*. The primers used in this assay were as follows: *KLF9*, 5'-tcccatctcaaagcccatta-3' (sense) and 5'-aactgcttttcccagtgtg-3' (antisense); *CDH1*, 5'-tgcccagaaaatgaaaaagg-3' (sense) and 5'-gtgtatgtggcaatgcgttc-3' (antisense); *CDH2*, 5'-gacaatgcccctcaagtgtt-3' (sense) and 5'-ccattaagccgagtgatggt-3' (antisense); *Vimentin*, 5'-gagaactttgccgttgaagc-3' (sense) and 5'-gcttcctgtaggtggcaatc-3' (antisense); *Fibronectin*, 5'-ccaacctacggatgactcgt-3' (sense) and 5'-gctcatcatctggccatttt-3' (antisense); *MMP1*, 5'-ccaaatgggcttgaagct-3' (sense) and 5'-gtagcacattctgtccctaa-3' (antisense); *MMP2*, 5'-tggcaagtacggcttctgtc-3' (sense) and 5'-ttcttgtcgcggtcgtagtc-3' (antisense); *MMP9*, 5'-tactgtgcctttgagtccg-3' (sense) and 5'-ttgtcggcgataaggaag-3' (antisense); *MMP28*, 5'-caatgaacaggtccccaaag-3' (sense) and 5'-ggccgcataactgttggtat-3' (antisense); *GAPDH*, 5'-aggtcggagtcaacggattt-3' (sense) and 5'-tagttgaggtcaatgaaggg-3' (antisense).

Western blot and antibodies

Western blotting was conducted as described previously [13]. Mouse monoclonal anti-Flag, anti-GAPDH, and anti-β-actin antibodies were purchased from Sigma (Sigma, Saint Louis, MO, USA). Rabbit anti-E-cadherin and anti-KLF9 antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA). The secondary antibodies of anti-mouse or anti-rabbit IgG were obtained from goats (ZSGB-BIO, Beijing, China).

Luciferase reporter gene assay

Promoter activity was assessed by a luciferase reporter system. In detail, twenty-four hours after transfection, cells expressing luciferase were lysed and subjected to luciferin (Promega, Madison, WI, USA), which is the reactive substrate of luciferase. Immediately, the amount of bioluminescence representing the luciferase activity was detected using a Microplate Luminometer (Centro LB 960, Berthold Technologies GmbH & Co. KG, Germany) and normalized to the activity of β-galactosidase, which was cotransfected with the pGL3-Luc reporter vector.

Chromatin immunoprecipitation assays

The primers for the *E-cadherin* promoter used in chromatin immunoprecipitation (ChIP) PCR analyses were as follows: region 1, 5'-gcggtacgggggggcggt-3' (sense) and 5'-acgccgagcgagggcaggcg-3' (antisense); region 2, 5'-gtcttagtgagccaccggcggg-3' (sense) and 5'-gttcacctgccggccacagcc-3' (antisense); and region 3, 5'-gccccgacttgtctctctacaa-3' (sense) and 5'-tggagatggggtctcactctttc-3' (antisense).

Construction of wild-type and mutated E-cadherin promoter reporters

The regulation of E-cadherin transcription is mainly determined by 400 bp upstream of the *E-cadherin TSS* (transcription start site, *TSS*),

which contains sufficient response elements for transcriptional regulation [20]. Hence, a DNA chain from 639 bp upstream of the *E-cadherin TSS* to +124 bp downstream of the *E-cadherin TSS* was cloned and inserted into the pGL3-Luc reporter vector to construct the luciferase reporter plasmid pE-cadherin-Luc (-639, +124). The primers used for cloning the *E-cadherin* promoter were 5'-ggggtaccctgaaatcctagcactttgg-3' (sense, underlined bases indicate the *KpnI* site) and 5'-ccgctcgagtgaactgacttccgcaagctc-3' (antisense, underlined bases indicate the *XhoI* site). Mutations of the GT and GC boxes in the *E-cadherin* promoter were carried out using pE-cadherin-Luc (-639, +124) as a template. Primers used to construct point mutations of GT-boxes were 5'-gcccgacccgaccgaaaaaggcgcctgccctcgc-3' for disrupting GT-box 1 (+84 bp from *TSS* of *E-cadherin* gene), 5'-ggctgcagccacgaaaaacctctcagtggcgtc-3' for disrupting GT-box 2 (-13 bp from *TSS* of *E-cadherin* gene), and 5'-gtctatgcgaggccaaaaaggcgggccgtcagc-3' for disrupting GT-box 3 (-143 bp from *TSS* of *E-cadherin* gene) (underlined bases indicate mutant nucleotide acids). Additionally, the primers 5'-aatcagcggtacgggaaaaggtgcctccggggc-3' for disrupting GC-box 1 element and 5'-cgggccgtcagctccaaaatggggaggggtccg-3' for disrupting GC-box 2 element (underlined bases indicate mutant nucleotide acids) were synthesized to construct mutant *E-cadherin* promoter vectors.

Immunohistochemistry

Immunohistochemistry (IHC) assays were performed to detect the relative expression of KLF9 and E-cadherin in breast tissues as described previously [13]. Antibodies against KLF9 or E-cadherin were used to detect protein levels. Positive and negative staining were shown with brown and blue color, respectively. An IHC score from 0 to 8 (0, no expression, negative; 1-2, expression < 10%, weak; 3-5, expression 10-50%, moderate; 6-8, expression > 50%, high) was used to quantify the expression levels of KLF9 and E-cadherin in tissues of breast tumors. Staining samples were classified as protein positive if IHC scores were greater than 2; otherwise, the samples were defined as negative [21]. All patients involved in this study gave written informed consent. Additionally, all IHC analyses were approved by the Ethics Committee for Medical College of Dalian University.

Mouse model of metastasis

Female BALB/c mice (6-8 weeks old) raised under specific pathogen-free (SPF) conditions were purchased from Dalian Medical University. All animal experiments were performed in accordance with approved guidelines of the Ethics Committee for Medical College of Dalian University. A total of 15 mice were used for *in vivo* animal study. Among these, five mice were assigned to negative control which were treated with normal saline (normal saline, NS); five mice were assigned to positive control which were treated with 4T1 cells transfected with pcDNA3.1-Flag control vector; and five mice were assigned to case group which were treated with 4T1 cells transfected with pcDNA3.1-Flag-KLF9. Stable transfectant 4T1 cells with pcDNA3.1-Flag or pcDNA3.1-Flag-KLF9 were established through selecting cells by antibiotic G418. Then, 4T1 cells (about 5×10^6) were dissolved in 100 μl normal saline and injected into the peripheral blood through the tail veins of BALB/c mice. Ten days later, the mice were killed, then the lungs were removed immediately. Mouse lungs were subjected to HE and IHC staining for further analyses. Finally, The carcinoma tissue separated from the rest of the lung tissue was lysed in 200 μl of RIPA lysis buffer for western blotting [22].

Quantitative evaluation of metastatic carcinoma

Hematoxylin and eosin (HE) staining of mouse lungs was conducted as described previously [23]. Metastases were observed by microscopic examination of HE staining, and the metastatic area was calculated as a percentage against the overall lung area by ImageJ software [13].

Microarray datasets analysis

We obtained two mRNA expression datasets of human breast tumors ("TCGA breast" and "Curtis breast") from the Oncomine website (https://www.oncomine.org). Illumina HumanHT-12 V3.0 R2 platform was used to measure the mRNA expression of human breast. The mRNA expression values of *KLF9* and *CDH1* (gene name of E-cadherin protein) were then retrieved from the Oncomine database, and the correlation of the two genes expression was assessed by Pearson r analysis.

Statistical analysis

Each group contained three samples or more, and each experiment repeated at least three times. Data were shown as the means ± SDs. An unpaired t-test was applied when there were two groups comparison. The one-way analysis of variance with Bonferroni's multiple-comparison correction was used, when there were three or more than three groups comparison. Correlation degree of the two factors from IHC assay was assessed by r-square value in linear regression analysis. Furthermore, Chi-squared test was used to assess E-cadherin expression difference between KLF9 positive and negative IHC groups. A p value less than 0.05 was considered to be statistically significant.

Results

KLF9 inhibits breast cancer cell migration and invasion in vitro

Our previous study indicated that KLF9 expression was downregulated in breast cancer cells, and its expression in T47D cells was higher than that in MCF-7 cells [13]. Hence, MCF-7 cell line was use for KLF9 overexpression and T47D cell line was used for KLF9 knockdown in the present study. To validate the inhibitory effect of KLF9 on the metastases of breast cancer cells, scratch wound healing assay was applied to assess cell migratory ability, and transwell assay was used to determine cell invasive ability when KLF9 was overexpressed or knocked down in breast cancer cells. As expected, when Flag-KLF9 was transfected into MCF-7 cells, the migratory ability of MCF-7 cells was downregulated compared with that of cells in the control group (**Figure 1A**). Similarly, KLF9 overexpression in MCF-7 cells markedly decreased the migratory ability of cells in transwell membrane coated with Matrigel (**Figure 1B**). In contrast, knockdown of endogenously expressed KLF9 with two independent shRNA interference plasmids (sh-KLF9#1 and sh-KLF9#2) in T47D cells obviously increased the migration and invasion of breast cancer cells, as shown in **Figure 1C** and **1D**. Therefore, these findings indicated that KLF9 may repress the abilities of mobility and invasion of breast cancer cells.

Concerning the role of KLF9 in the malignant behaviors of breast cancer cells, our previous study demonstrated that KLF9 could regulate MMP9 expression to inhibit breast cancer metastasis [13]. In the present study, we continued to screen other invasion-related proteins that can be regulated by KLF9. To obtain a view of gene expression changes regulated by KLF9, we synthesized a series of mRNA primers, including MMPs family and epithelial and mesenchymal proteins, to conduct real-time PCR assay in MCF-7 cell line. The results showed that, besides the MMPs family proteins (*MMP1*, *MMP9*, *MMP28*) were downregulated by KLF9, epithelial and mesenchymal markers were also changed following KLF9 overexpression in breast cancer cells (**Figure 1E**). These findings indicated that KLF9 can exert an anti-metastasis function by regulating the gene profiling related to matrix degradation and cell adhesion or morphology.

KLF9 upregulates E-cadherin expression at the transcriptional level

Based on the genes screened from real-time RCR, we selected a putative marker E-cadherin to further study the association of E-cadherin with KLF9 in cancer metastasis. As E-cadherin is sufficient to induce wide-range of transcriptional alteration for cancer invasion and metastasis [2], and also the expressions of KLF9 and E-cadherin exhibit a relatively positive trend in breast cancer cells, we next investigated whether KLF9-mediated inhibition of invasiveness was associated with the alteration of E-cadherin expression. As anticipated, transfection of KLF9 in MCF-7 cells at increasing doses induced a dose-dependent increase in *E-cadherin* mRNA levels (**Figure 2A**), indicating that KLF9 could upregulate *E-cadherin* mRNA. Meanwhile, western blot results showed a dose-dependent increase in E-cadherin protein following transfection of MCF-7 cells with gradiently increased KLF9, which was consistent with the mRNA changes of KLF9 shown as **Figure 2A**, **2B**. These results demonstrated that KLF9 upregulated E-cadherin expression at the transcriptional level.

To confirm this speculation, a luciferase reporter assay was applied to test *E-cadherin* promoter activity. As expected, overexpression of KLF9 in HEK 293T cells caused a significant increase in *E-cadherin* promoter activity (**Figure 2C**). Similarly, *E-cadherin* promoter activity exhibited a dose-dependent increase when KLF9 was expressed in an ascending gradient

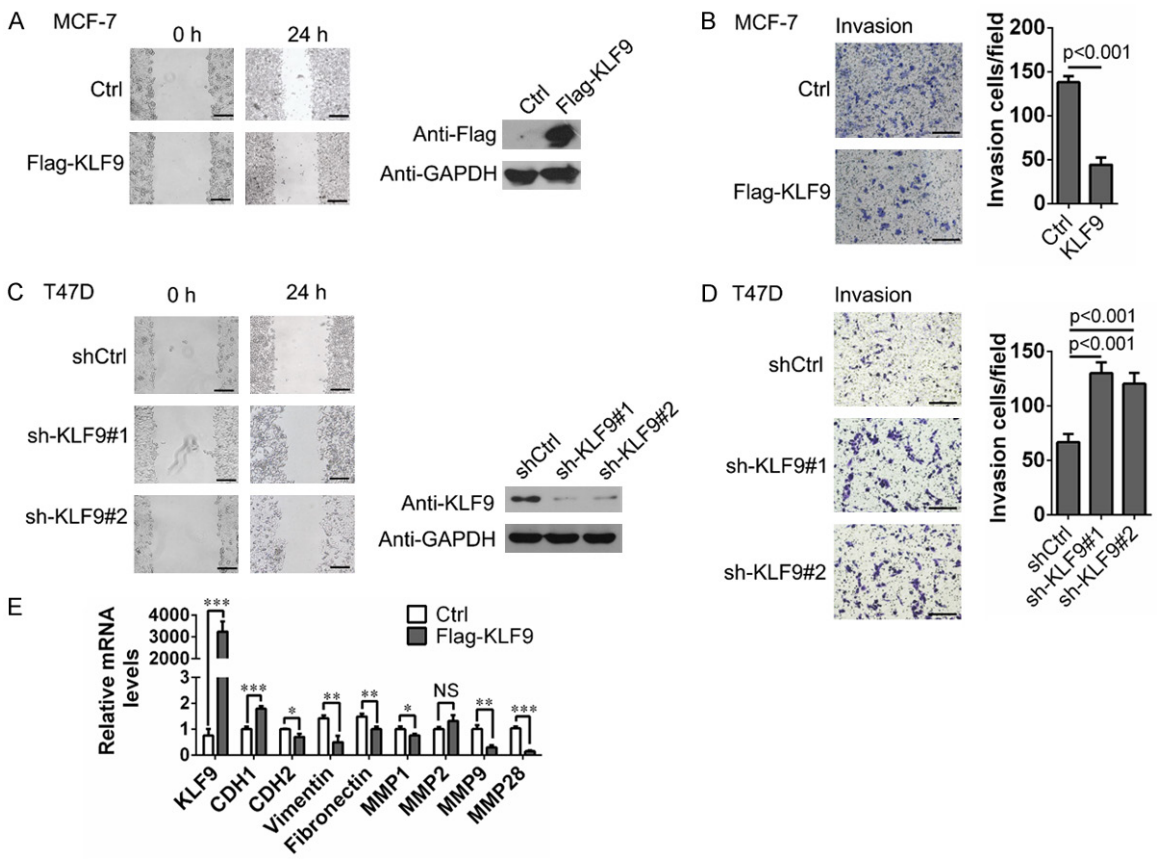


Figure 1. KLF9 suppresses migration and invasion of breast cancer cells. (A) Scratch wound healing assays of MCF-7 cells transfected with control vector or Flag-KLF9 (left); western blotting of Flag-KLF9 in MCF-7 cells (right). Scale bar: 100 μm. The western blotting images were cropped, and the original images were supplied in supplementary files named as "(A) (right)-01" and "(A) (right)-02". (B) Transwell invasion assays of MCF-7 cells transfected with control vector of Flag-KLF9 (left). Scale bar: 50 μm. Data quantification of the invasive cells (right). Each group repeated three times. (C) Scratch wound healing assays of T47D cells transfected with control vector or shKLF9s (sh-KLF9#1 and sh-KLF9#2) (left); western blotting of Flag-KLF9 in T47D cells (right). Scale bar: 100 μm. The western blotting images were cropped, and the original images were supplied in supplementary files named as "(C) (right)". (D) Transwell invasion assays of T47D cells transfected with control vector of shKLF9s (sh-KLF9#1 and sh-KLF9#2) (left). Scale bar: 50 μm. Data quantification of the invasive cells (right). (E) Real-time PCR of some invasion-related genes (epithelial and mesenchymal markers or MMPs family members) in KLF9-transfected MCF-7 cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant. Each group repeated three times. Data are shown as the means ± SDs ($P < 0.05$, significant).

in MCF-7 cells (**Figure 2D**). In contrast, the luciferase activity driven by the *E-cadherin* promoter was significantly decreased when KLF9 was knocked down in T47D cells (**Figure 2E**). Taken together, these results suggested that KLF9 upregulated E-cadherin expression by activating the promoter of the *E-cadherin* gene.

Identification of KLF9 binding sites in the E-cadherin promoter

As a transcription factor, KLF9 could upregulate *E-cadherin* transcriptional activity, so we wondered whether there are some response elements in the *E-cadherin* promoter that bind KLF9. First, the consensus sequence was identified as the most enriched KLF9 binding motif predicted by the JASPAR database which is an online website to predict transcription factor binding sites (http://jaspar.genereg.net) (**Figure 3A**). Then, the *E-cadherin* promoter sequence was scanned according to the KLF9-binding motif, and the results revealed several GC-rich sites and GT-boxes considered to be the putative binding sites of KLF9, including two GC-boxes and three GT-boxes. These were found at nucleotides +84 to +90 (GT-box 1, core sequence: CACCC), nucleotides -13 to -7

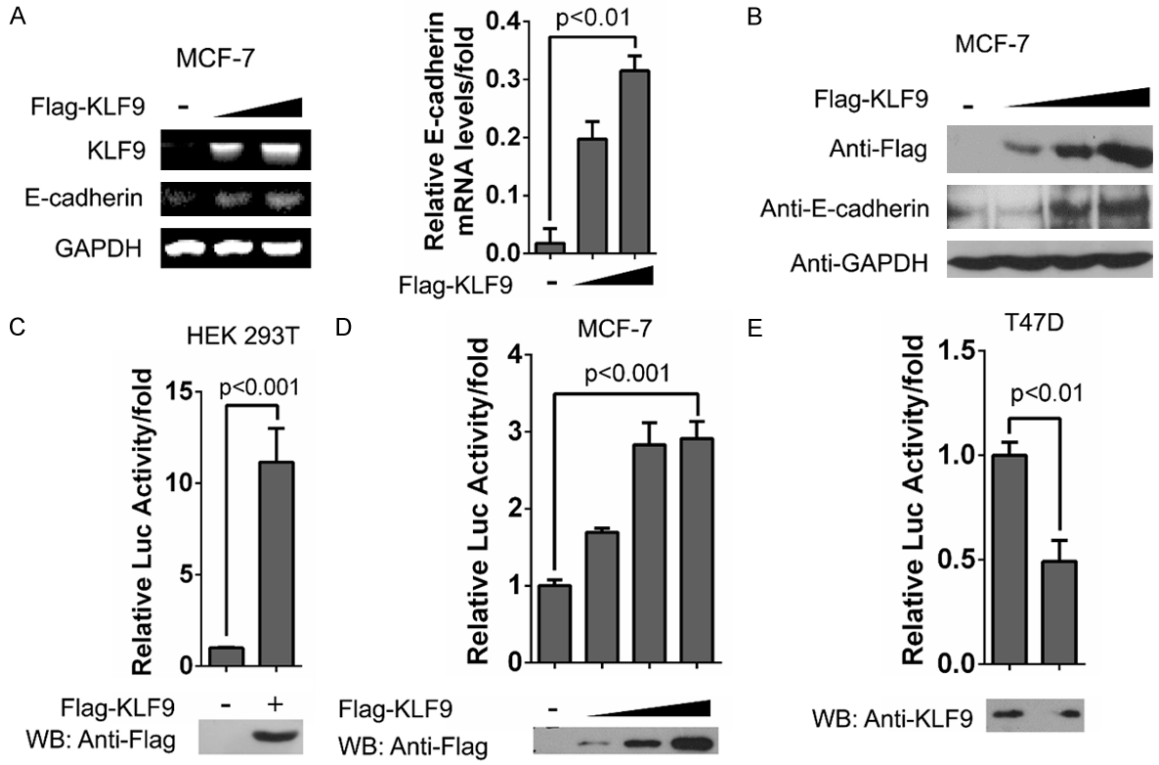


Figure 2. KLF9 upregulates E-cadherin expression and promoter activity. (A) RT-PCR of *E-cadherin* and *KLF9* mRNA in MCF-7 cells transfected with gradient doses of KLF9 (left). The DNA gels were cropped, and the original images were supplied in supplementary files named as "(A) (01)", "(A) (02)" and "(A) (03)". Quantification of the relative mRNA levels of *E-cadherin* normalized against GAPDH (right). (B) Western blotting of E-cadherin and KLF9 in MCF-7 cells transfected with gradient doses of KLF9. The western blotting images were cropped, and the original images were supplied in supplementary files named as "(B) (01)" and "(B) (02)". (C-E) Luciferase reporter gene assay of E-cadherin promoter activities in HEK 293T cells overexpressing Flag-KLF9 (C) or in MCF-7 cells transfected with gradiently increased doses of Flag-KLF9 (D) or in T47D cells transfected with shKLF9 (E). pE-cadherin-luciferase plasmid was cotransfected into cells, and empty vectors were used for control. The western blotting images included in the columns were cropped, and the original images were supplied in supplementary files named as "(C)" and "(D & E)". Data are shown as the means ± SDs ($P < 0.05$, significant).

(GT-box 2, core sequence: CACCC), nucleotides -49 to -43 (GC-box 1, core sequence: GGGCGG), nucleotides -123 to -117 (GC-box 2, core sequence: CCGCCC), and nucleotides -143 to -137 (GT-box 3, core sequence: GGGTG) in the region of the *E-cadherin* promoter, as listed in **Figure 3B**. To confirm the predictive binding elements of KLF9 in the *E-cadherin* promoter, a ChIP assay was performed in HEK 293T cells. We cloned three fragments orderly from the *E-cadherin* promoter (upstream from the *TSS* of *E-cadherin* gene), including a DNA domain from -58 to +112 (region 1) covering GT-box 1, GT-box 2 and GC-box 1, a DNA domain from -266 to -72 (region 2) covering GC-box 2 and GT-box 3, and a domain from -529 to -337 (region 3) without the putative binding sites of KLF9, as illustrated in **Figure 3C** (top). ChIP assays indicated that KLF9 could interact with the sequences belonging to region 1 and region 2, which contained the putative binding sites of KLF9, consistent with the speculation predicted by the promoter analysis tool (**Figure 3C**, bottom). Collectively, these results indicated that KLF9 could activate *E-cadherin* transcription by binding some response elements, such as GT-boxes and GC-boxes.

Since the KLF9 binding region identified by ChIP assay, nucleotides -266 to +112 in the *E-cadherin* promoter, contained five putative binding sites of KLF9, we then applied site-directed mutagenesis in the *E-cadherin* promoter inserted into the reporter gene construct, combined with a promoter reporter assay to identify the KLF9-binding sits. In the site

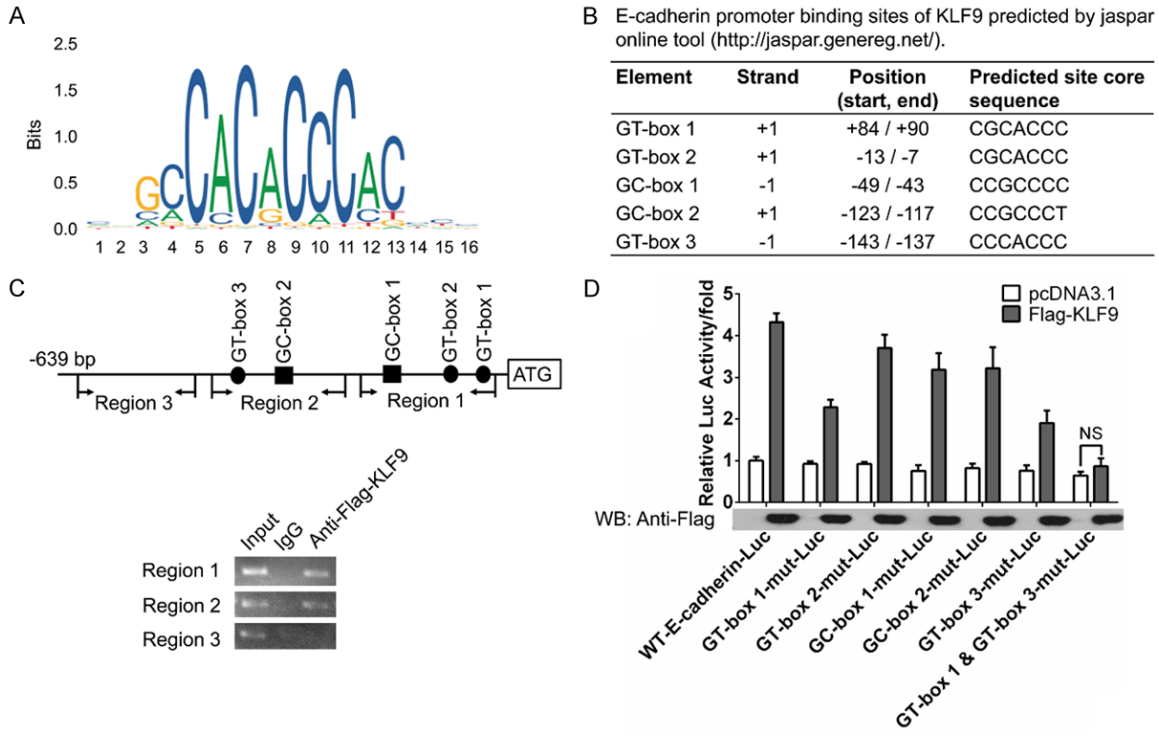
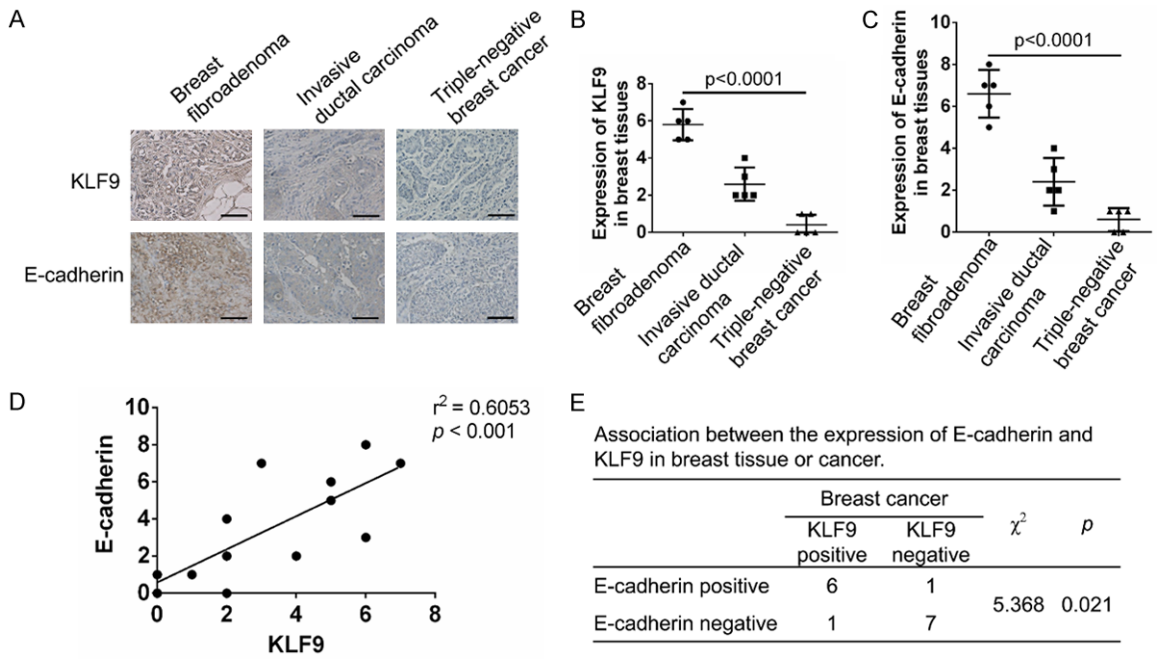


Figure 3. KLF9-binding elements are identified in the *E-cadherin* promoter. (A) KLF9 binding motif identified using the JASPAR database. (B) *E-cadherin* promoter binding sites of KLF9 predicted by the JASPAR database. (C) Top, schematic illustration of the *E-cadherin* promoter; bottom, ChIP PCR reactions of E-cadherin promoter regions in HEK 293T cells transfected with Flag-KLF9. Antibody IgG was used for negative control. The DNA gels were cropped, and the original images were supplied in supplementary files named as "(C) (bottom)". (D) Site-specific mutants for GT or GC boxes in the *E-cadherin* promoter reporter construct combined with luciferase reporter assays determining promoter activities in MCF-7 cells treated with Flag-KLF9 or control vector. The western blotting images were cropped, and the original images were supplied in supplementary files named as "(D)". Data are shown as the means ± SDs ($P < 0.05$, significant; NS, not significant).

mutant *E-cadherin* promoter reporter assay, mutation of GT-box 1 (CACCC to AAAAA) or GT-box 3 (GGGTG to AAAAA) caused an appropriate 50% reduction in KLF9-induced activation respectively, whereas mutation of GT-box 2 (CACCC to AAAAA), GC-box 1 (GGGCGG to AAAAG), or GC-box 2 (CCGCCC to CCAAAA) alone in the core sequences had almost no effect on the activating effect of KLF9 compared to the wild-type promoter construct, indicating that elements of GT-box1 at +84 bp and GT-box 3 at -143 bp might be potential KLF9 binding sites in the *E-cadherin* promoter (**Figure 3D**). Consistent with our speculation, mutations of both GT-box 1 and GT-box 3 in the *E-cadherin* promoter completely abolished the activating effect of KLF9 (**Figure 3D**), suggesting that these two response elements in the promoter might be essential for KLF9-mediated activation of *E-cadherin* transcription. Therefore, we concluded that KLF9 activated *E-cadherin* transcription by binding to GT-box elements at +84 and -143 bp in the *E-cadherin* promoter.

The expression of KLF9 and E-cadherin in human tissues of breast tumors

To validate the consistency of breast cell line data with those in human tissues, an IHC assay was applied to detect the expression of KLF9 and E-cadherin in breast tumor tissues obtained from clinical. Three groups with a total of 15 breast tumor samples (each group included 5 cases) were involved in the IHC staining analysis, including fibroadenomas, invasive ductal cancer (excluded triple-negative breast cancer subtype), and triple-negative breast cancer (TNBC) samples. As shown in **Figure 4A**, compared with benign breast fibroadenoma, KLF9 expression was drastically downregulated in aggressive tissues of breast cancer. Addition-



E

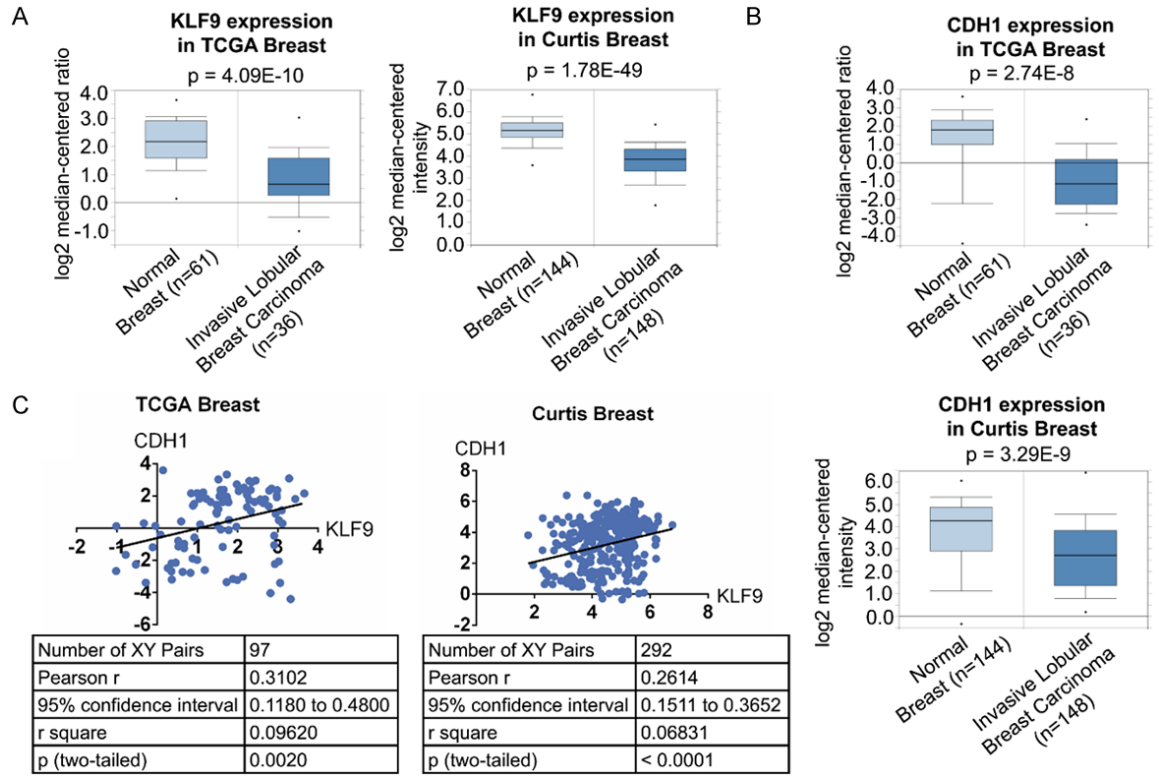
Association between the expression of E-cadherin and KLF9 in breast tissue or cancer.

	Breast cancer		χ^2	p
	KLF9 positive	KLF9 negative		
E-cadherin positive	6	1	5.368	0.021
E-cadherin negative	1	7		

Figure 4. E-cadherin expression is positively correlated with KLF9 in human breast cancer tissues. (A) Representative results of IHC staining showing the protein expressions of KLF9 and E-cadherin in breast tumor tissues. Antibodies against KLF9 or E-cadherin were used to detect protein levels. Scale bar: 100 μm. (B & C) KLF9 (B) and E-cadherin (C) protein levels in tissues of breast fibroadenoma (n = 5), invasive ductal carcinoma (n = 5), and triple-negative breast cancer (n = 5). (D) Linear regression analyses of association between KLF9 and E-cadherin expressions with r-square values evaluating correlation degree. (E) Chi-squared test detecting the correlation of KLF9 with E-cadherin expression. Data are shown as the means ± SDs (P < 0.05, significant).

ally, high expression of E-cadherin was found in fibroadenoma tissues, and loss of E-cadherin was commonly detected in TNBCs, with the same expression trend as KLF9. IHC score-based assessment showed that KLF9 expression in invasive ductal cancers or TNBCs was significantly downregulated compared with that in fibroadenomas (**Figure 4B**). Similarly, E-cadherin expression in invasive ductal cancers or TNBCs was also significantly downregulated compared with that in fibroadenomas (**Figure 4C**). Linear correlation analysis showed a significantly positive correlation between KLF9 and E-cadherin expression in breast tumors (**Figure 4D**, $r^2 = 0.6053$, $P < 0.001$). The association of KLF9 and E-cadherin levels was further analyzed by Chi-squared test in patient breast samples, and a significant correlation between KLF9 and E-cadherin was observed (**Figure 4E**). Taken together, the detection of KLF9 and E-cadherin expression in clinical patients indicated a positive correlation between KLF9 and E-cadherin in breast cancer tissues.

To further confirm the co-expression of KLF9 and E-cadherin in breast cancer patients, we analyzed mRNA expression data in breast tissues from public database. Two independent public datasets from Oncomine (https://www.oncomine.org) were selected, TCGA (The Cancer Genome Atlas, TCGA) dataset and Curtis dataset. As shown in **Figure 5A** (left), in TCGA breast specimens, *KLF9* mRNA expression level was significantly reduced in breast carcinomas compared with normal breast tissues. Additionally, in Curtis breast dataset, we found a dramatically decrease of *KLF9* expression in breast lobular carcinomas when compared with normal breast tissues (**Figure 5A**, right). Similarly, examination of *E-cadherin* mRNA (named *CDH1*) in TCGA or Curtis datasets demonstrated that *CDH1* mRNA expression was markedly downregulated in breast cancer compared with normal breast tissues (**Figure 5B**). Then, we retrieved the expression data of *KLF9* and *CDH1* from TCGA and Curtis breast databases, and applied Pearson r test to analyze the correlation between *KLF9* and *CDH1* expression in breast tumor tissues. As expected, the expression of *KLF9* mRNA level was positively correlated with *CDH1* mRNA expression (**Figure 5C**; $P < 0.01$ in TCGA breast and $P <$



Number of XY Pairs	97
Pearson r	0.3102
95% confidence interval	0.1180 to 0.4800
r square	0.09620
p (two-tailed)	0.0020

Number of XY Pairs	292
Pearson r	0.2614
95% confidence interval	0.1511 to 0.3652
r square	0.06831
p (two-tailed)	< 0.0001

Figure 5. *KLF9* and *CDH1* mRNA expression were downregulated in breast cancer tissues than in normal from two public datasets ("TCGA breast" and "Curtis breast"). A. *KLF9* mRNA levels in normal and invasive lobular breast cancer tissues in the TCGA breast (normal = 61, tumor = 36) (left) and Curtis breast (normal = 144, tumor = 148) (right) datasets from Oncomine database (https://www.oncomine.org). B. *CDH1* mRNA levels in normal and invasive lobular breast cancer tissues in the TCGA breast (normal = 61, tumor = 36) (top) and Curtis breast (normal = 144, tumor = 148) (bottom) datasets from Oncomine database. C. Pearson test showing the correlation of *KLF9* and *CDH1* mRNA levels in TCGA breast (left) and Curtis breast (right). The mRNA expression data were retrieved from Oncomine website. P < 0.05, significant.

0.0001 in Curtis breast), which was in accordance with the results from our IHC study as shown in **Figure 4D** and **4E**.

KLF9 upregulates E-cadherin expression in 4T1 cells and suppresses breast cancer cell metastasis in vivo

4T1 cells, which are basal-like murine breast cancer cells, were used to detect the *in vivo* effect of KLF9 on breast cancer metastasis. BALB/c mice were intravenously administered with 4T1 cells stably transfected with control vector or vector carrying the KLF9 gene, and the metastases of mouse lungs were assessed after 10 days of feeding. Before the *in vivo* animal experiment, we first analyzed whether these two elements in the *E-cadherin* promoter were conserved between human and mouse species to ensure that human KLF9 might work on the mouse *E-cadherin* promoter with the same mechanism described above. As expected, comparison of the *E-cadherin* promoter between human and mouse indicated that the two potential binding sites of KLF9 were also found in the mouse *E-cadherin* promoter, as shown in **Figure 6A**. Elements of the two GT-boxes, which were identified to be the most potential binding sites, appeared to be conserved between human and murine species (**Figure 6A**). Therefore, human KLF9 transfection into 4T1 cells could affect the *E-cadherin* promoter activity of mouse. In the HE staining assay, the lungs removed from mice treated with 4T1 cells that had been transfected with empty vector (4T1-Ctrl, positive control) showed the most metastatic nodules compared to lungs treated with normal saline (NS, negative control), indicating that 4T1 murine breast cancer cells had dramatic metastatic ability. When KLF9 was stably transfected into 4T1 cells, the lungs subjected to cells containing KLF9

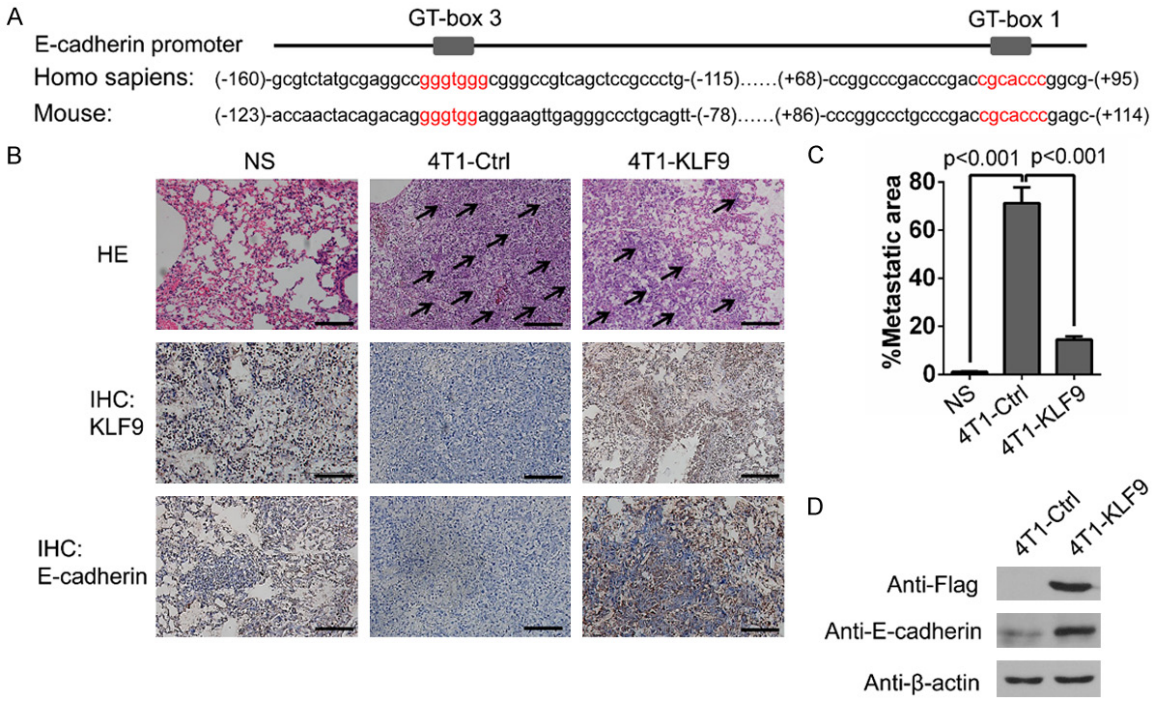


Figure 6. KLF9 inhibits the metastasis of 4T1 cells and upregulates E-cadherin expression in BALB/c mice. (A) Conservation analysis of KLF9 binding sites on the *E-cadherin* promoter in human and murine species. Nucleotides marked in red represent *cis-elements* of GT-boxes predicted to be the potential binding sites of KLF9. (B) HE staining showing the lung metastases of the 3 groups (NS: negative control; 4T1-Ctrl: positive control; and 4T1-KLF9: case group). IHC staining showing the KLF9 (anti-KLF9 antibody, species: human and mouse) and E-cadherin (anti-E-cadherin antibody, species: mouse) expressions in mouse lungs among the three groups. Scale bar: 100 μm. NS, normal saline. (C) The percent lung metastatic area calculated by ImageJ was shown among the three groups. (D) Western blotting assessing Flag-KLF9 and the corresponding mouse E-cadherin expression in lung carcinoma tissues in control and 4T1-KLF9 groups. The western blotting images were cropped, and the original images were supplied in supplementary files named as "(D) (01)" and "(D) (02)". Data are shown as the means ± SDs (n = 5). $P < 0.05$, significant.

(4T1-KLF9, case group) showed fewer metastatic tissues than the positive control group. The black arrows point to the metastatic nodules, as illustrated in **Figure 6B**. In addition, qualification of the metastatic nodules in the lungs among the 3 mouse groups showed the metastatic areas were decreased when KLF9 was transfected into 4T1 cells (**Figure 6C**). IHC staining was performed to detect the KLF9 and E-cadherin protein levels in the 3 groups. Because KLF9 was stably transfected to 4T1 cells in the case group (4T1-KLF9), the KLF9 protein expression in 4T1-KLF9 group was higher than 4T1-Ctrl group. Compared with the positive control group (4T1-Ctrl), the lungs bearing 4T1 cells transfected with KLF9 exhibited upregulation of mouse E-cadherin, suggesting that KLF9 may increase E-cadherin expression in mouse 4T1 cells (**Figure 6B**). Finally, western blotting was used to qualify the protein levels in metastatic tissues of the case and control groups. As shown in **Figure 6D**, when KLF9 was overexpressed in 4T1 cells, the E-cadherin levels were upregulated compared to those in cells transfected with the control vector, which was consistent with the results in human breast cancer cells. Taken together, KLF9 could upregulation E-cadherin expression and suppress breast cancer metastasis *in vivo*, and these results supported the findings from *in vitro* assays using human breast cancer cells.

Discussion

In the current study, we first demonstrated that KLF9 may upregulate E-cadherin expression by activating its transcription, and this finding could provide a novel mechanism by which KLF9 inhibits breast cancer metastasis. First, as a cancer suppressive factor, KLF9 could

inhibit breast cancer cell metastasis. Second, KLF9 upregulated E-cadherin expression by binding to GT-boxes in the *E-cadherin* promoter to activate its transcription. Third, in breast cancer patients, the expression of KLF9 was positively associated with E-cadherin expression through IHC staining analysis. Finally, animal experiments revealed that KLF9 increased E-cadherin expression and inhibited breast cancer metastasis *in vivo*. This work showed the inhibitory effect of KLF9 on cancer metastasis and identified a new mechanism involving E-cadherin, whose functional loss is considered to be a hallmark of EMT and the fundamental event to promote cancer metastasis.

KLF9, a relatively unexplored member of the KLF transcription factor family, can recognize GC-rich or GT-box response elements in promoters or enhancers to regulate target gene expression and participate in various biological processes, including differentiation, proliferation, and apoptosis. A recent study from us showed that KLF9 regulated MMP9 expression by interacting with NF-κB p50/p65 and binding to the GT box at -449 bp from the *TSS* in the *MMP9* promoter to inhibit cancer metastasis [13]. This work first demonstrated the suppressive effect of KLF9 on cancer cell metastasis and precisely described the underlying mechanism involving the NF-κB/MMP9 pathway. In the current study, one important finding was that in addition to the KLF9/MMP9 pathway, KLF9 might regulate E-cadherin expression to inhibit cancer metastasis, exhibiting a novel mechanism related to the KLF9/E-cadherin pathway in the regulation of KLF9 as a crucial metastasis suppressor. Given that the activated MMP9 alone is sufficient to induce E-cadherin and AJ loss [24], and that MMP9 could degrade functional E-cadherin into soluble E-cadherin to promote cancer metastasis in head and neck squamous cell carcinomas [25], ovarian cancer [24], and colorectal cancer [26], it is reasonable to speculate that there might be crosstalk between pathways of KLF9/MMP9 and KLF9/E-cadherin in metastatic inhibition by KLF9. That is, except for the direct upregulation of E-cadherin expression, KLF9 might downregulate MMP9 expression and activity to inhibit the degradation of E-cadherin, both of which may contribute to the increase in E-cadherin levels in cells.

Similar to the other KLF members, KLF9 has a dual effect on transcriptional activity, exhibiting either transactivation or repression, which depends on cell context. For example, KLF9 can bind to the *Notch1* promoter and suppress Notch1 expression in tumor-initiating stem cells [27]. Additionally, frizzled-5 was sharply inhibited by KLF9 through its binding in the promoter region of frizzled-5 in pancreatic ductal adenocarcinoma [28], which exhibits suppressive activity of KLF9 in transcriptional regulation. In contrast, the present study showed that KLF9 may activate *E-cadherin* transcription by binding to GT-boxes of the *E-cadherin* promoter (**Figure 3**). Consistently, KLF9 transcriptionally activated p53 expression by directly binding to GT boxes within the *p53* promoter in hepatocellular cancer [29]. These opposite effects exerted by KLF9 may come from different cancer types, various subtypes from the same cancer, or complex microenvironments around transcriptional reactions. Due to KLF9 binding to different promoters in the above processes, the transcription factors next to KLF9 are not the same, thereby possibly facilitating the opposite transcriptional activity of KLF9 in the regulation of promoter activity. Therefore, KLF9 may exhibit transcriptionally activating or repressing effects dependent on the cellular context in tumor cells.

Recently, the notion that loss of E-cadherin may promote cancer metastasis has become controversial. Previously, the majority of studies in this field suggested that loss of E-cadherin may markedly increase cell metastatic ability in many cancer types [19, 30-33]. A gene expression analysis of breast cancer cells revealed that loss of E-cadherin promoted metastasis through multiple downstream pathways, and loss of E-cadherin was sufficient to contribute to breast cancer cell metastasis [2]. However, a recent study showed that E-cadherin was still expressed in metastatic tissues and facilitated the survival of cancer cells [10]. Moreover, in the process of collective cell migration, E-cadherin is expressed in metastatic clusters of epithelial cells [34]. Based on these investigations, some researchers explained that the functional state of E-cadherin is an important determinant of metastatic progression other than whether E-cadherin is expressed [11]. In the study, activating mAbs for E-cadherin were used to detect the effect of E-cadherin on the

regulation of cancer metastasis, and transmembrane E-cadherin was shown to reduce cell invasion and migration in primary tumors [11]. Indeed, E-cadherin present in tissue exhibits two forms. One is functional E-cadherin translocated to the cell membrane, and the other is soluble E-cadherin with no function in migration. Lack of E-cadherin cell surface regulation may contribute to cancer invasion [35]. In our study, the detection of E-cadherin by western blot was carried out in breast cancer cells but not in the cell matrix, so it is the functional form expressed on the tumor cell surface. Thus, E-cadherin upregulation induced by KLF9 could act as a tumor suppressor to repress human breast cancer invasion.

Conclusions

In conclusion, we found a novel target gene for the transcription factor KLF9. As a tumor suppressor, KLF9 could bind to GT-boxes in the *E-cadherin* promoter to upregulate E-cadherin expression, thereby inhibiting the metastasis of human breast cancer. These findings were also reproduced in clinical patients and *in vivo* experiments, providing a novel molecular mechanism related to breast cancer metastasis. Therefore, targeting KLF9 to induce KLF9 expression might be a potential choice for the inhibition of breast cancer metastasis.

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Disclosure of conflict of interest

None.

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