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CDCA7 promotes TGF-β-induced epithelial–mesenchymal transition via transcriptionally regulating Smad4/Smad7 in ESCC

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Abstract

Cell division cycle associated 7 (*CDCA7*) is a copy number amplification gene that contributes to the metastasis and invasion of tumors, including esophageal squamous cell carcinoma (ESCC). This present study aimed at clarifying whether high expression of CDCA7 promotes the metastasis and invasion of ESCC cell lines and exploring the underlying mechanisms implicated in epithelial–mesenchymal transition (EMT) of ESCC. The role of CDCA7 in the regulation of ESCC metastasis and invasion was evaluated using ESCC cell lines. Expression of EMT-related markers including E-cadherin, N-cadherin, Vimentin, Snail, and Slug, transforming growth factor β (TGF-β) signaling pathway including Smad2/3, p-Smad2/3, Smad4, and Smad7 were detected in *CDCA7* knockdown and overexpressed cell lines. Dual-luciferase reporter assay and rescue assay were used to explore the underlying mechanisms that CDCA7 contributed to the metastasis and invasion of ESCC. High CDCA7 expression significantly promoted the metastasis and invasion of ESCC cell lines both in vivo and in vitro. Additionally, the expression of CDCA7 positively correlated with the expression of N-cadherin, Vimentin, Snail, Slug, TGF-β signaling pathway and negatively correlated with the expression of E-cadherin. Furthermore, CDCA7 transcriptionally regulated the expression of Smad4 and Smad7. Knockdown of *CDCA7* inhibited the TGF-β signaling pathway and therefore inhibited EMT. Our data indicated that CDCA7 was heavily involved in EMT by regulating the expression of Smad4 and Smad7 in TGF-β signaling pathway. CDCA7 might be a new therapeutic target in the suppression of metastasis and invasion of ESCC.

KEYWORDS
CDCA7, EMT, ESCC, Smad4/Smad7, TGF-β signaling pathway

Abbreviations: CDCA7, cell division cycle associated 7; EMT, epithelial–mesenchymal transition; ESCC, esophageal squamous cell carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; TGF-β, transforming growth factor β.

Hongyi Li and Shaojie Wang contributed equally to this work.

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1 | INTRODUCTION

A concise overview on current cancer epidemiology obtained from the official databases of the World Health Organization and American Cancer Society suggests that cancer poses the highest clinical, social, and economic burden among all human diseases.[1] It is the second cause of death worldwide after ischemic heart disease, causing 8.97 million deaths and is likely to become the first in 2060, causing 18.63 million deaths.[1] Tumor metastasis, one of the most marked biological characteristics of malignant tumors, is the biggest cause of human death.[2] Tumor invasion and metastasis is a complex and multifactorial dynamic process. The development of tumor metastasis is dependent on the interaction between tumor cells and their microenvironment and complex signal pathways.[3,4] Research on the mechanism of tumor metastasis and invasion contributes to a deep understanding of the process of metastasis, identifying the therapeutic targets and laying the foundation for diagnosis and treatment.

A key event in promoting carcinoma metastasis and invasion is the EMT program.[5] In the past decades, EMT has been increasingly recognized to play intricate and pivotal roles in promoting stationary tumor metastasis and invasion.[2] During the EMT process, tumor cells acquire molecular alterations that enable partial loss of epithelial features and partial gain of mesenchymal phenotypes.[6,7] This process is very complex and controlled by various intrinsic signals (e.g. gene mutations), extrinsic signals (e.g. growth factor signaling) as well as various families of transcriptional regulators through different signaling pathways.[2] Among the growth factors known to induce EMT are TGF-β, hepatocyte growth factor, members of the epidermal growth factor family, insulin-like growth factor, and fibroblast growth factor.[8–13] The microenvironment stimulation initiates EMT and controls cancer metastasis through regulating signaling pathways including the Wnt signaling pathway,[14–16] TGF-β signaling pathway,[17–19] Hedgehog signaling pathway,[20] Notch signaling pathway[21] and NF-κB signaling pathway.[22,23]

CDCA7 is one of the copy number amplified genes found by our laboratory.[24] The previous study showed that CDCA7 was a DNA-binding protein that could function as a transcription regulator and high expression of it promotes the proliferation and colony formation of ESCC cells.[25] In addition, it has been reported that CDCA7 causes tumor metastasis and invasion in other cancers including breast cancer, hepatocellular carcinoma, and lymphoma.[26–28] However, the mechanism by which CDCA7 contributes to tumor metastasis and invasion in ESCC has seldom been reported. In this study, we report the effect of CDCA7 on the migration and invasion of ESCC cells and reveal the potential mechanism by which CDCA7 promotes the EMT process in ESCC.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

The *CDCA7* knockdown cell line KYSE150 and the *CDCA7* overexpressed cell lines KYSE150 and KYSE180 used in this study were constructed and preserved by our laboratory. The construction method of the stable cell lines was described in our previous study.[25] The cell lines used in this study were cultured in HyClone™ RPMI-1640 (GE Healthcare Life Sciences, HyClone Laboratories) medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). All of the cell lines were cultured at 37°C, in 5% CO_2. The culture medium was replaced every other day and when the cell fusion was ~80%–90%, subculture was carried out.

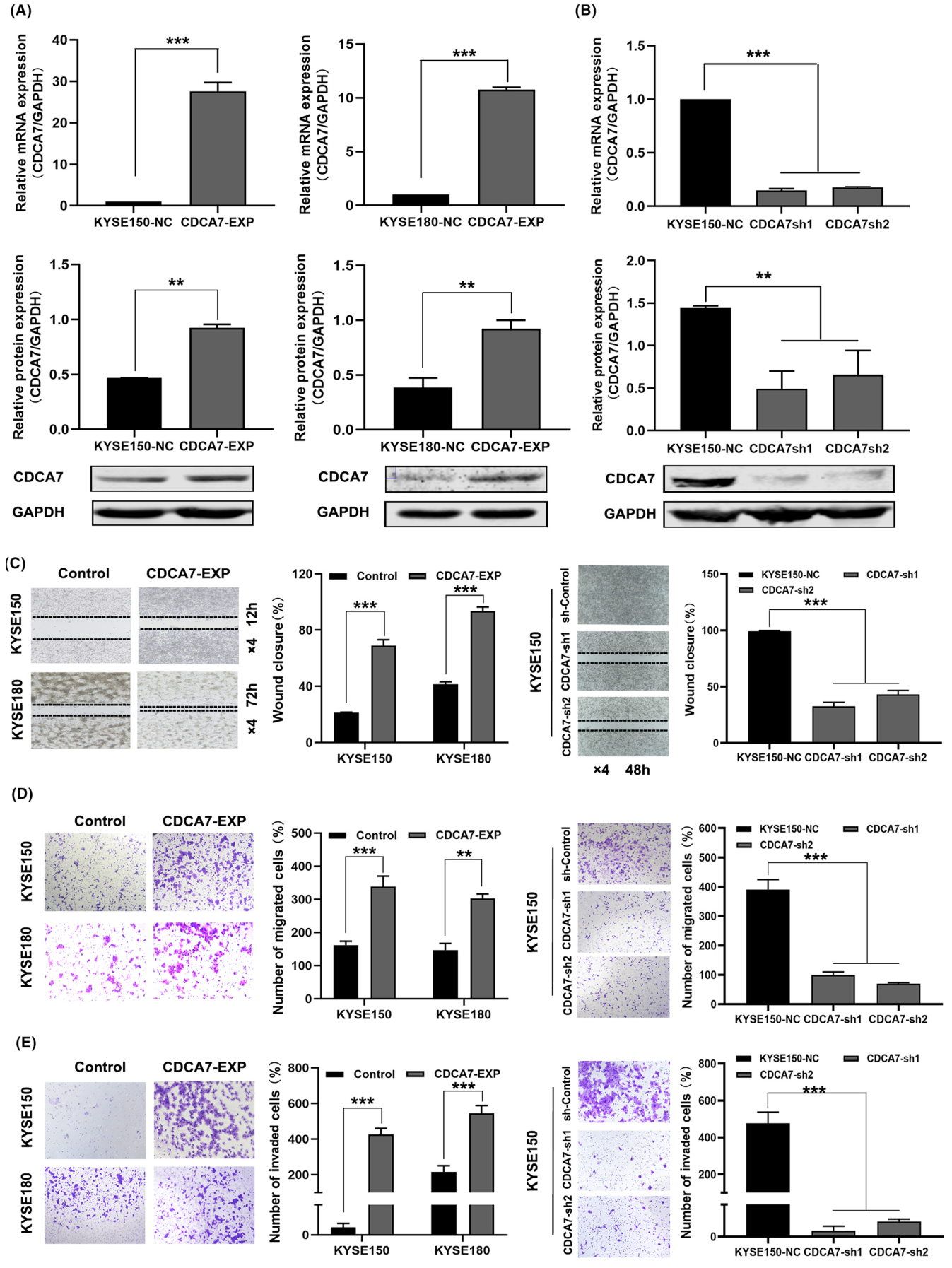
2.2 | Plasmids and siRNA

The plasmids used in this study, such as SBE4, TOP, FOP, pGL3-basic, pGL3-promoter, and pRL-TK, were purchased from Addgene and Promega and preserved in our laboratory. The pGL3-basic, pGL3-promoter, and pRL-TK plasmids were used for the dual-luciferase reporter assay. The gDNA of KYSE150 cells was used as the template for amplification of the DNA fragments of target genes that were obtained from ChIP-seq. The primers used for the PCR reaction are listed in Table S1. TransIntro™ EL Transfection Reagent (TransGen Biotech) was used for the transfection of plasmids. Small interfering RNAs (RiboBio) were used to knock down Smad7. The Smad7 siRNA sequences are 5′-GGTAGTTCCGAAAGCTGAT-3′ (Smad7-siRNA1) and 5′- CCTATAGAAGATACTAGAT-3′ (Smad7-siRNA2). The riboFECT CP Transfection Kit (RiboBio) was used for the transfection of siRNA.

2.3 | RNA extraction and real-time PCR

RNAiso plus (TaKaRa) were used for purifying the total RNA of ESCC cells. Prime Script™ RT Master Mix (TaKaRa) was used for cDNA synthesis. Real-time PCR (RT-PCR) was conducted using the TB Green™ Premix Ex Taq™ II kit (TaKaRa) according to the manufacturer's instruction. The reactions were performed with an Applied Biosystems Step One Plus (ABI) instrument. The expression level of GAPDH was used to normalize the relative expression levels of the target genes. The primers synthesized by Sangon Biotech are listed in Table S2.

FIGURE 1 CDCA7 promotes the migratory and invasive capacities of ESCC cells in vitro. (A) The *CDCA7* overexpression efficiency in KYSE150 cells and KYSE180 cells. (B) The *CDCA7* knockdown efficiency in KYSE150 cells. (C) CDCA7 overexpression promotes wound healing of ESCC cells and CDCA7 knockdown inhibits wound healing of ESCC cells. According to the cell migration ability, photographs were taken at 0, 12, 48 and 72 h respectively. (D) CDCA7 overexpression increased the ability of cell migration and CDCA7 knockdown inhibited the ability of cell migration in ESCC cell lines. (E) CDCA7 overexpression increased the ability of cell invasion and CDCA7 knockdown inhibited the ability of cell invasion in ESCC cell lines. CDCA7, cell division cycle associated 7; ESCC, esophageal squamous cell carcinoma. ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.


(A)
(B)
Relative mRNA expression (CDCA7/GAPDH)
KYSE150-NC CDCA7-EXP
KYSE180-NC CDCA7-EXP
KYSE150-NC CDCA7sh1 CDCA7sh2
Relative protein expression (CDCA7/GAPDH)
CDCA7
GAPDH
(C)
Control
CDCA7-EXP
KYSE150
KYSE180
×4 12h
×4 72h
Wound closure (%)
sh-Control
CDCA7-sh1
CDCA7-sh2
×4 48h
KYSE150-NC
CDCA7-sh1
CDCA7-sh2
(D)
Number of migrated cells (%)
(E)
Number of invaded cells (%)

2.4 | Western blot

The cells were lysed as described in our previous study.[25] In total, 50 μg of protein were separated by 10% or 12% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes (Millipore) at 100 V for 2 h. Then the membranes were blocked using 5% BSA and incubated with primary antibody overnight at 4°C. The primary antibodies used in this study are listed in Table S3. The IRDye 800CW secondary antibody (1:10,000, Abcam) and HRP secondary antibody (1:5000; Abmart) were used to detect the blot. A relative amount of protein was normalized to GAPDH or LaminB level.

2.5 | Wound healing assay

Cells were seeded into six-well plates with 10% FBS. 12 h later, when the cells adhered and the fusion reached 100%, the cells were scratched with yellow tips. Then the cells were washed with PBS twice and serum-free medium was added into the plates. The recovery rate was expressed as follows: [1 − (Wound width at a given time/wound width at $t = 0$)] × 100%.

2.6 | Invasion and migration assay

A trans-well assay was used to detect the invasion and migration ability of ESCC cells. A 8-μm trans-well plate (Corning, Inc.) was used for the trans-well assay. For the migration assay, 5×10^4 KYSE150 ESCC cells or 15×10^4 KYSE180 ESCC cells were seeded into the upper compartment of each trans-well chamber with 200 μl FBS-free medium. The lower compartment of the trans-well chamber was filled with 600 μl medium containing 10% FBS and cultured at 37°C, 5% CO_2 for 48 h or 72 h. Then the cells on the upper compartment were removed and the cells that passed through the membrane were fixed in 4% formaldehyde for 30 min. After washing with PBS for three times, the cells were stained using 0.1% crystal violet. For the invasion assay, 1×10^5 KYSE150 ESCC cells or 2×10^5 KYSE180 ESCC cells were seeded into the upper compartment of each trans-well chamber. The upper compartment was pre-coated with 100 μl Matrigel (1:6 mixed with FBS-free medium, BD Biosciences). The remained operation proceeded as the migration assay described above. Five fields were chosen at random to count the number of transmigrated cells.

2.7 | In vivo experiments

To establish the metastasis model, 10 4–6-week-old BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. and kept in a pathogen-free animal facility under standard conditions. The 10 mice were divided into two independent groups and five mice in each group for the experiments. Next, 2.0×10^6 KYSE150-NC cells or CDCA7-EXP cells were injected respectively into the tail vein of the mice in each group to establish a metastatic tumor model. At 6 weeks later, all the mice were narcotized and ^{18}F-FDG was injected into each mouse's tail vein for PET/CT (Invisacn, IRIS) subsequently. After PET/CT, the mice were sacrificed and whole lung of each mouse was removed. Then the lungs were fixed with 10% formalin, embedded with paraffin, and sectioned. Paraffin sections were stained with H&E in order to identify tumor tissue. Animal studies were approved by the Shanxi Medical University Institutional Animal Care and Use Committee.

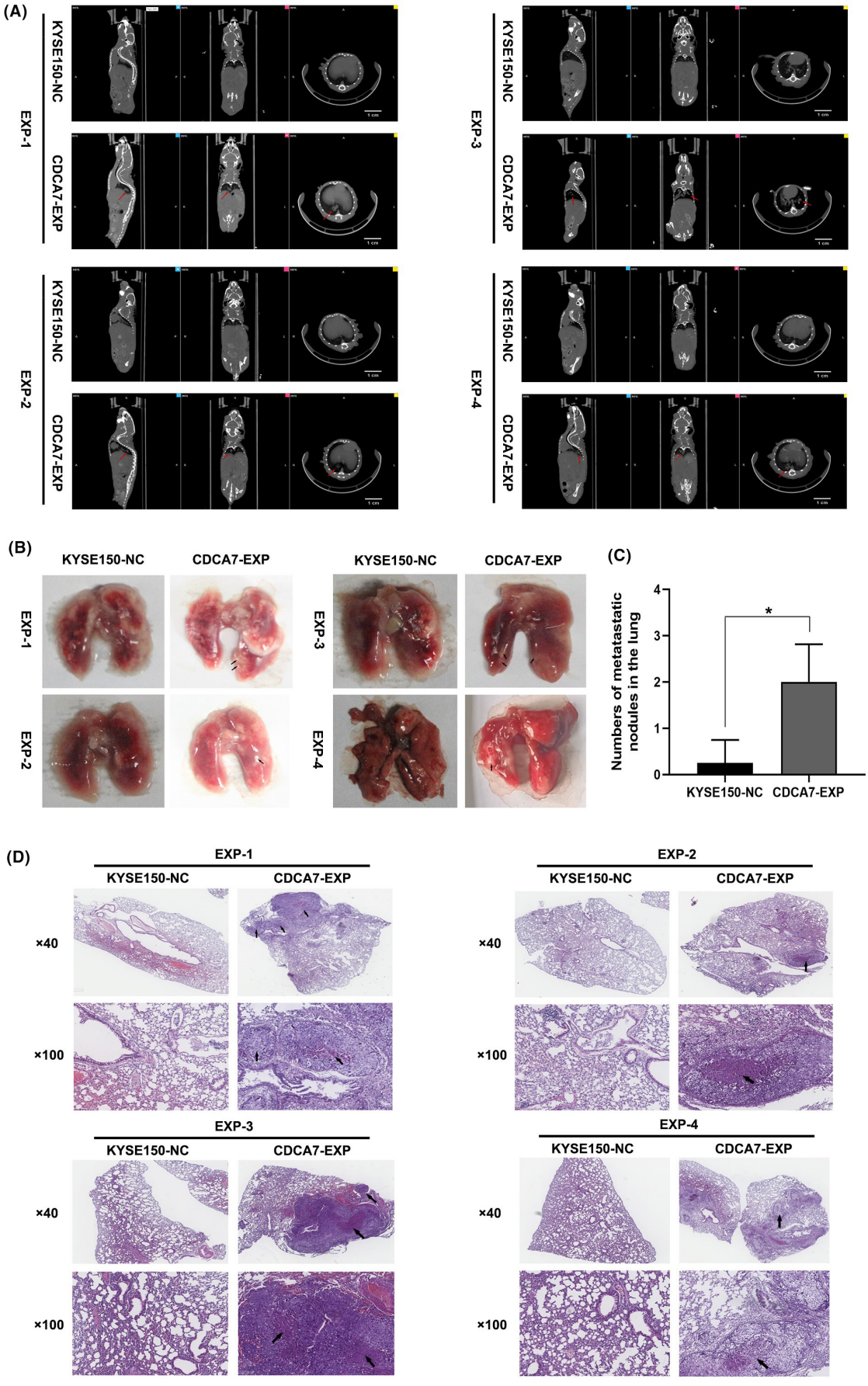
2.8 | Immunofluorescence

Cells were seeded on slides in 12-well plates. After culture for 12–24 h, the cells were fixed in 4% formaldehyde for 30 min at room temperature. Then the cells were washed with PBS and permeabilized with 0.25 Triton X-100 for 30 min. The slides were blocked with 0.5% BSA in PBS for 1 h at room temperature after being washed with PBS four times. The cells were incubated with rabbit antibody against E-cadherin (Proteintech, Cat no. 20874-1-AP, 1:100) overnight at 4°C. Alexa Fluor 594 goat anti-rabbit IgG antibody (Thermo Fisher) was used for 1 h at room temperature. Then the cells were washed with PBS four times and stained with 0.5 μg/mL DAPI, followed by imaging using a confocal microscope (Leica TCS SP8).

2.9 | Dual-luciferase reporter assay

Cells were seeded in 24-well plates in triplicate. At 12–24 h later, when the cells fusion reached 40%–50%, the luciferase reporter plasmid and the pRL-TK plasmid were transiently co-transfected into the cells. After transfection for 48 h, Renilla luciferase and firefly luciferase signals were measured according to the instructions of the Dual-Luciferase Reporter Assay Kit (TransGen Biotech).

FIGURE 2 CDCA7 promotes the migratory and invasive capacities of ESCC cells in vivo. (A) Representative images median sagittal (left), coronal (middle), and transverse (right) sections of CT images of nude mice. Red arrows show water-density nodule lung in CT. The four nude mice with lung metastatic nodules were named EXP1, EXP2, EXP3, and EXP4 respectively. (B) Representative images of metastatic tumor nodules in the lung of nude mice injected with KYSE150 cells stably overexpressing CDCA7 (CDCA7-EXP) or control vector (KYSE150-NC). Black arrows show metastatic tumor nodules in the lung. (C) Number of metastatic tumor nodules in the lung of nude mice injected with KYSE150 cells stably overexpressing CDCA7 (CDCA7-EXP) or control vector (KYSE150-NC). All data are presented as the mean ± standard deviation. (D) Representative images of H&E staining in metastatic tumor nodules in the lung section of nude mice. CDCA7, cell division cycle associated 7; ESCC, esophageal squamous cell carcinoma. * $0.01 < p \leq 0.05$.


(A)
EXP-1
KYSE150-NC
CDCA7-EXP
EXP-2
KYSE150-NC
CDCA7-EXP
EXP-3
KYSE150-NC
CDCA7-EXP
EXP-4
KYSE150-NC
CDCA7-EXP
(B)
KYSE150-NC
CDCA7-EXP
KYSE150-NC
CDCA7-EXP
EXP-1
EXP-2
EXP-3
EXP-4
(C)
Numbers of metatastatic nodules in the lung
4
3
2
1
0
*
KYSE150-NC
CDCA7-EXP
(D)
EXP-1
KYSE150-NC
CDCA7-EXP
×40
×100
EXP-2
KYSE150-NC
CDCA7-EXP
×40
×100
EXP-3
KYSE150-NC
CDCA7-EXP
×40
×100
EXP-4
KYSE150-NC
CDCA7-EXP
×40
×100

2.10 | RNA sequencing

CDCA7 knockdown KYSE150 and CDCA7 overexpression KYSE180 cells and their matched control cells were conducted using RNA sequencing. RNA sequencing was performed by Novogene. Total RNA extraction, secondary cDNA strain synthesis, and purification were performed with standard procedures. Gene expression values were presented as fragment per kilobase of transcript sequence per million mapped reads (FPKM). The DEG-Seq method was used to analyze differential gene expression. Pathway enrichment analysis of clustered differential genes was carried out according to the KEGG database.

2.11 | Bioinformatics and data analysis

The mRNA expression data of various genes, including *CDCA7*, *Myc*, *CDH1*, *CDH2*, *Vimentin*, *SNAI1* and *SNAI2*, were downloaded from the Gene Expression Omnibus (GEO) (GSE53625) and The Cancer Genome Atlas (TCGA) via xenabrowser (https://xenabrowser.net/heatmap/) respectively. The correlation of *CDCA7* and these genes were analyzed by GraphPad Prism software (GraphPad Software).

2.12 | Statistical analysis

Each experiment in the study was repeated three times independently and data were presented as the mean ± SEM. The experimental data was analyzed using GraphPad Prism 8. Student's *t*-test was used for two groups comparison and one-way ANOVA was used for more than two groups comparison. The level of significance in the statistical analyzes is indicated as $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

3 | RESULTS

3.1 | CDCA7 promotes migration and invasion of ESCC cells in vitro

Trans-well and wound healing assays were used to verify the effect of CDCA7 on the ability of migration and invasion of ESCC cells. Quantitative RT-PCR (qRT-PCR) and western blot were applied to validate the efficiency of overexpression and knockdown (Figure 1A,B). Then, we detected the changes in cell phenotypes using the *CDCA7* overexpressed and knocked down cell lines. The results showed that *CDCA7* overexpression increased the ability of cell migration and invasion, while knockdown of *CDCA7* significantly inhibited the ability of cell migration and invasion in ESCC cell lines (Figure 1C–E).

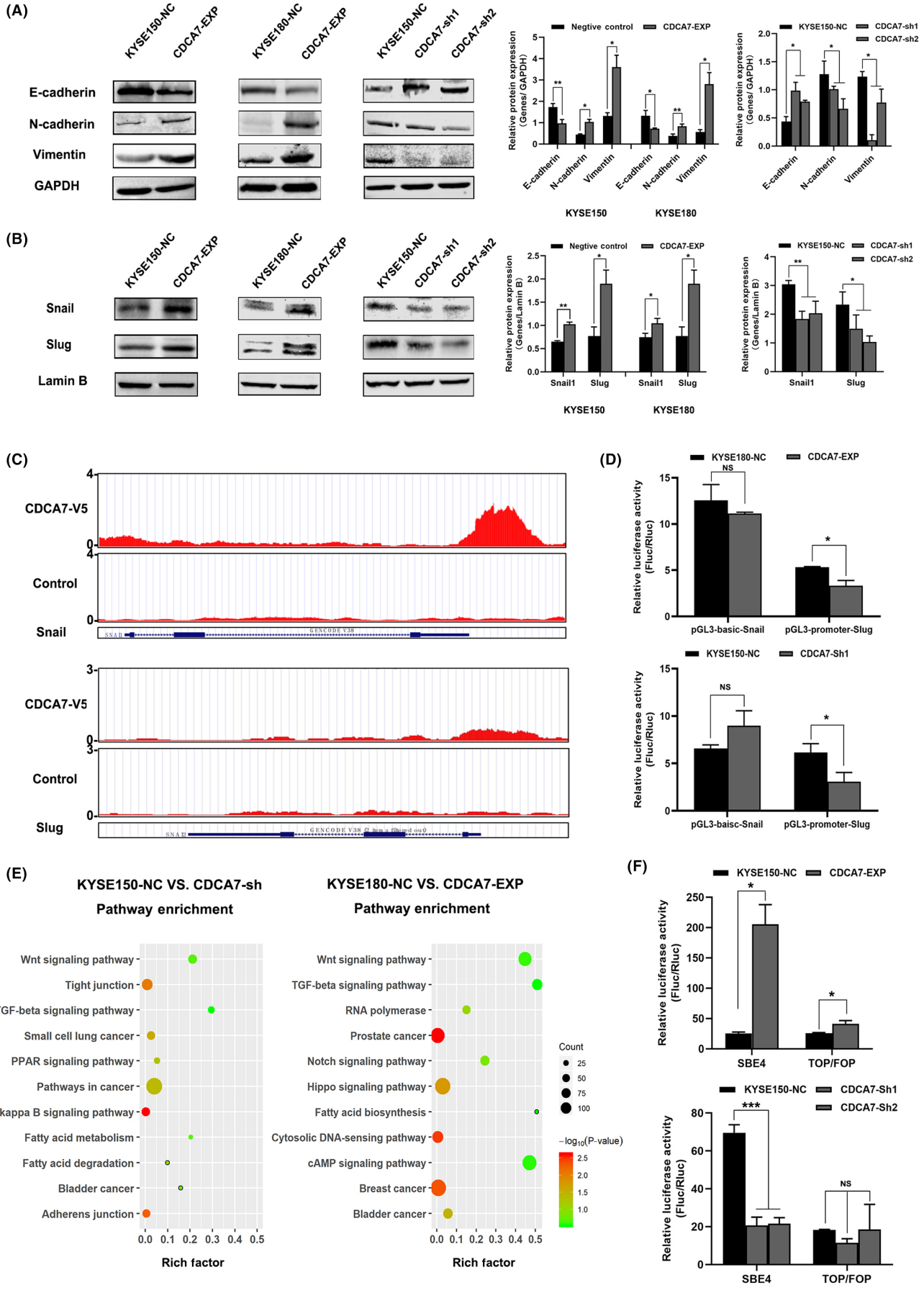
3.2 | CDCA7 promotes migration and invasion of ESCC cells in vivo

To further confirm the role of CDCA7 in promoting metastasis in ESCC in vivo, the lung metastasis model was established. KYSE150-NC cells and CDCA7-EXP cells that overexpressed CDCA7 stably were injected into the tail vein of nude mice. At 6 weeks later, compared with the KYSE150-NC group, the CDCA7-EXP group had worse physical status and one mouse died before small-animal ^{18}F-FDG PET/CT that was used to observe lung metastasis of the two groups. The CT images showed that four out of five nude mice of the CDCA7-EXP group exhibited round and obvious metastatic nodules in the right lung. However, no nodules were formed in the lung of the nude mice in the KYSE150-NC group. The CT images and PET/CT images of the two groups are shown in Figure 2A and Figure S1 respectively. The section indicated by the arrow in the image is the nodules formed. Furthermore, the lung tissues of mice were dissected for observation and quantitative analysis (Figure 2B,C). H&E staining was used to further confirm the metastatic nodules of the two group mice (Figure 2D). These findings demonstrated that *CDCA7* overexpression promoted the migration and invasion ability of ESCC cells in vitro *and* in vivo.

3.3 | CDCA7 may promote the EMT process of ESCC via TGF-β signaling pathway

As biological function experiments for CDCA7 revealed that CDCA7 promoted cell migration and invasion of ESCC cells in vitro and in vivo, western blot was used to verify the expression levels of EMT markers in *CDCA7* overexpression and knockdown cell lines. The results showed that, as the expression of CDCA7 increased, the expression level of E-cadherin decreased and the expression levels of N-cadherin, Vimentin, Snail, and Slug increased. The results reversed in the *CDCA7* knockdown stable cell line (Figure 3A,B; Figure S2). These findings suggested that overexpression of CDCA7 promoted the EMT process of ESCC.

FIGURE 3 CDCA7 may promote the epithelial–mesenchymal transition process of ESCC via the TGF-β signaling pathway. (A) The protein expression levels of E-cadherin, N-cadherin, and Vimentin in CDCA7 knockdown or control KYSE150 cells and CDCA7 overexpression or control KYSE150, KYSE180 cells. (B) The protein expression levels of Snail and Slug in the nucleus of CDCA7 knockdown or control KYSE150 cells and CDCA7 overexpression or control KYSE150, KYSE180 cells. (C) CDCA7 binds with Snail at the position 4857–5968 bp, and binds with Slug at the position −56,077 to −57,016 bp. (D) Luciferase reporter assays showed that CDCA7 had no regulation effect on the expression of Snail and Slug. (E) Pathway enrichment analysis of CDCA7 knockdown cells and CDCA7 overexpression cells both showed that TGF-β signaling pathway and Wnt signaling pathway were significantly enriched. (F) SBE4 flash assay and TOP/FOP flash assay depicted that CDCA7 may regulate SBE4 downstream transcriptional activity. CDCA7, cell division cycle associated 7; ESCC, esophageal squamous cell carcinoma; TGF-β, transforming growth factor β. * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.


(A)
KYSE150-NC
CDCA7-EXP
KYSE180-NC
CDCA7-EXP
KYSE150-NC
CDCA7-sh1
CDCA7-sh2
E-cadherin
N-cadherin
Vimentin
GAPDH
Negtive control
CDCA7-EXP
Relative protein expression (Genes/ GAPDH)
KYSE150
KYSE180
KYSE150-NC
CDCA7-sh1
CDCA7-sh2
(B)
Snail
Slug
Lamin B
Relative protein expression (Genes/Lamin B)
Snail1
Slug
(C)
CDCA7-V5
Control
Snail
GENCODE V38
Slug
(D)
KYSE180-NC
CDCA7-EXP
NS
Relative luciferase activity (Fluc/Rluc)
pGL3-basic-Snail
pGL3-promoter-Slug
KYSE150-NC
CDCA7-Sh1
pGL3-baisc-Snail
(E)
KYSE150-NC VS. CDCA7-sh
Pathway enrichment
KYSE180-NC VS. CDCA7-EXP
Pathway enrichment
Wnt signaling pathway
Tight junction
TGF-beta signaling pathway
Small cell lung cancer
PPAR signaling pathway
Pathways in cancer
NF-kappa B signaling pathway
Fatty acid metabolism
Fatty acid degradation
Bladder cancer
Adherens junction
RNA polymerase
Prostate cancer
Notch signaling pathway
Hippo signaling pathway
Fatty acid biosynthesis
Cytosolic DNA-sensing pathway
cAMP signaling pathway
Breast cancer
Rich factor
Count
−log10(P-value)
(F)
KYSE150-NC
CDCA7-EXP
SBE4
TOP/FOP
KYSE150-NC
CDCA7-Sh1
CDCA7-Sh2

We analyzed the EMT markers in our ChIP-seq data and found that Snail and Slug might be the target genes regulated by CDCA7. As shown in Figure 3C, two DNA fragments related to Snail and Slug were identified. DNA fragment 1 was located at a position 4857–5968 bp to the transcription start site (TSS) of Snail and DNA fragment 2 located at −56,077 to −57,016 bp to the TSS of Slug. Then the two fragments were cloned from the genome of KYSE150 and introduced into the pGL3-basic plasmid and pGL3-promoter plasmid, respectively. However, the results of the dual-luciferase reporter assay showed that CDCA7 did not affect the regulation of Snail and Slug (Figure 3D). Therefore, CDCA7 does not promote the EMT process of ESCC via regulating these transcription factors. To further explore the mechanism of how CDCA7 promotes the EMT process of ESCC, RNA-seq was used to detect the change in the transcriptome in *CDCA7*-overexpressed KYSE180 cells and *CDCA7* knockdown KYSE150 cells. Next, we performed a KEGG pathway enrichment analysis on the data from RNA-seq. The Wnt signaling pathway and the TGF-β signaling pathway, which are related to the EMT process were screened out (Figure 3E). Dual-luciferase reporter plasmids SBE4, TOP and FOP were used to identify the activation of the Wnt signaling pathway and TGF-β signaling pathway. The results showed that the TGF-β signaling pathway was significantly activated as the expression of *CDCA7* increased and vice versa. However, the activation or inhibition of the Wnt signaling pathway was not consistent with the expression level of *CDCA7* (Figure 3F). These results indicated that the *CDCA7* overexpression activated TGF-β signaling pathway and may promote the EMT process of ESCC via TGF-β signaling pathway.

3.4 | CDCA7 promotes EMT process of ESCC via activating TGF-β signaling pathway

To further verify whether CDCA7 overexpression activates TGF-β signaling pathway, protein expression levels of Smad2/3 and p-Smad2/3 were determined in *CDCA7* overexpressed stable cell lines, a *CDCA7* knockdown stable cell line and an ESCC cell line KYSE150/KYSE180 treated with TGF-β1 and the inhibitor of TGF-β signaling pathway LY-364947. As we expected, the expression of p-Smad2/3 increased in the *CDCA7* overexpressed stable cell lines and cells treated with TGF-β1, but decreased in the *CDCA7* knockdown stable cell line and cells treated with LY-364947 (Figures 4A,B; Figure S3A,B).

Additionally, we treated *CDCA7* overexpressed stable cell lines and *CDCA7* knockdown stable cell line with 10 mM LY-364947 and 10 ng/mL TGF-β1 for 48 h respectively. Then wound healing assay and trans-well assay were conducted to detect the invasion and migration ability of the ESCC cell lines treated with LY-364947 and TGF-β1. The results showed that LY-364947 reversed the enhancement of cell invasion and migration caused by CDCA7 overexpression (Figure 4C,E), while TGF-β1 restored the weaken of cell invasion and migration caused by *CDCA7* knockdown (Figure 4D,F). In addition, immunofluorescence results showed that the expression of E-cadherin was increased when cells treated with LY-364947 (Figure 5A) and was decreased when cells treated with TGF-β1 (Figure 5B). The western blot results indicated that KYSE150 cells and KYSE180 cells stably overexpressing CDCA7 treated with LY-364947 inhibited the TGF-β-induced EMT process caused by *CDCA7* overexpression (Figure 5C; Figure S4A). Whereas the EMT process was promoted when *CDCA7* knockdown cells were treated with TGF-β1 (Figure 5D; Figure S4B).

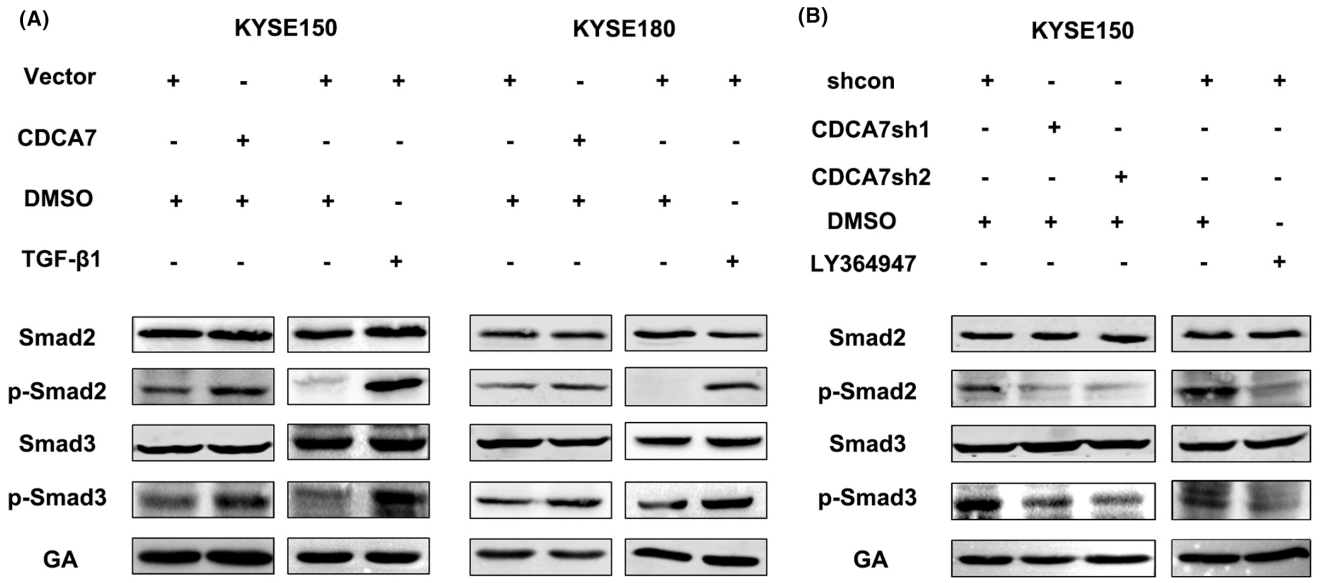
Taken together, these findings suggested that activation of the TGF-β signaling pathway could promote the EMT process in ESCC and the overexpression of *CDCA7* could activate the TGF-β signaling pathway.

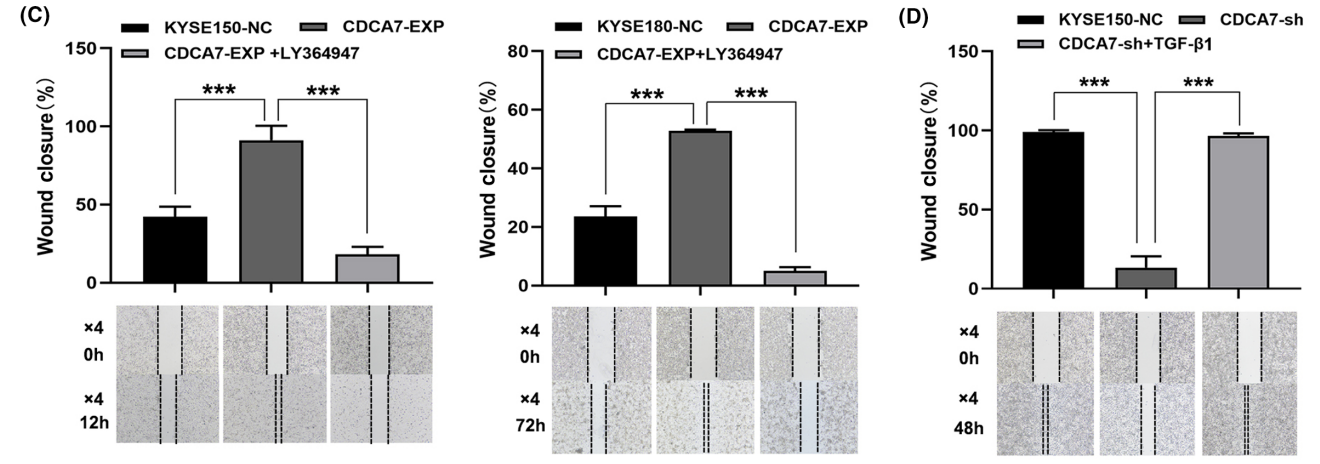
3.5 | CDCA7 activates the TGF-β-related EMT process through regulating the expression of Smad4/Smad7 transcriptionally

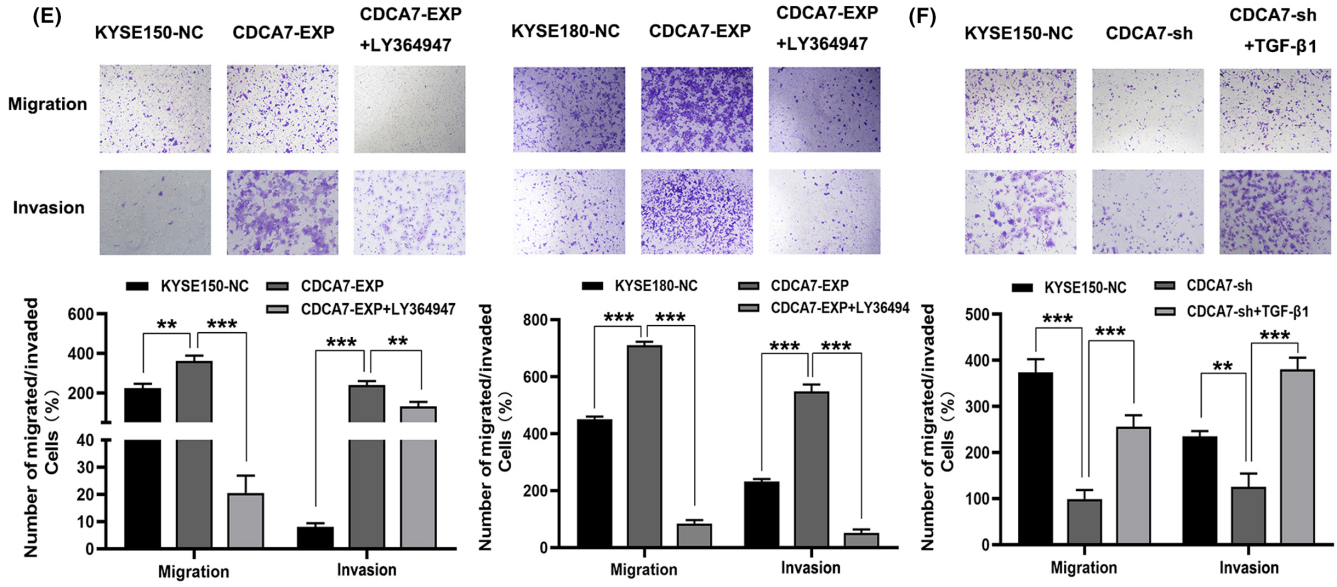
To better understand how CDCA7 activates the TGF-β signaling pathway, we analyzed the genes of the TGF-β signaling pathway in the ChIP-seq results and found that Smad4 and Smad7 might be regulated by CDCA7 (Figure 6C). CDCA7 binds with Smad4 at the position −27 to 846 bp relative to its TSS and binds with Smad7 at the position −973 to 946 bp to its TSS. To confirm this hypothesis, these two DNA fragments were cloned from the genomic DNA of KYSE150 and inserted into the pGL3-basic plasmid for a dual-luciferase reporter assay. As is shown in Figure 6D, CDCA7 positively regulated Smad4 and negatively regulated Smad7. In addition, results of western bolt and qRT-PCR proved this hypothesis. No matter the expression of protein or the expression of mRNA, CDCA7 positively correlated with Smad4 and negatively correlated with Smad7 (Figure 6A,B; Figure S5).

To determine the function of Smad7 in ESCC cells, small interfering RNAs of Smad7 were used for the interference and rescue experiment. Smad7 siRNAs were transfected into *CDCA7* knockdown cells to knock down the expression of Smad7. qRT-PCR and western blot were used to detect the efficiency of knockdown (Figure S6). The results of the wound healing assay and trans-well assay showed that Smad7 silencing restored the invasion

FIGURE 4 CDCA7 promotes the epithelial–mesenchymal transition process of ESCC via activating the TGF-β signaling pathway. (A) CDCA7 overexpression or control KYSE150 and KYSE180 cells treated with TGF-β1 increased the expression levels of p-Smad2 and p-Smad3. (B) CDCA7 knockdown or control KYSE150 treated with LY-364947 decreased the expression levels of p-Smad2 and p-Smad3. (C, D) Wound healing assay was detected after treatment with LY-364947 and TGF-β1 in the CDCA7 overexpression and knockdown cells respectively. According to the cell migration ability, photographs were taken at 0, 12, 48 and 72 h respectively. (E, F) Trans-well assay was detected after treated with LY-364947 and TGF-β1 in the CDCA7 overexpression and knockdown cells respectively. CDCA7, cell division cycle associated 7; ESCC, esophageal squamous cell carcinoma; TGF-β, transforming growth factor β. ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.


(A)
KYSE150
KYSE180
Vector
CDCA7
DMSO
TGF-β1
Smad2
p-Smad2
Smad3
p-Smad3
GA
(B)
KYSE150
shcon
CDCA7sh1
CDCA7sh2
DMSO
LY364947
Smad2
p-Smad2
Smad3
p-Smad3
GA


(C)
KYSE150-NC
CDCA7-EXP
CDCA7-EXP +LY364947
Wound closure (%)
×4 0h
×4 12h
KYSE180-NC
CDCA7-EXP
CDCA7-EXP+LY364947
×4 0h
×4 72h
(D)
KYSE150-NC
CDCA7-sh
CDCA7-sh+TGF-β1
Wound closure (%)
×4 0h
×4 48h


(E)
KYSE150-NC
CDCA7-EXP
CDCA7-EXP +LY364947
KYSE180-NC
CDCA7-EXP
CDCA7-EXP +LY364947
Migration
Invasion
Number of migrated/invaded Cells (%)
Migration
Invasion
(F)
KYSE150-NC
CDCA7-sh
CDCA7-sh +TGF-β1
KYSE150-NC
CDCA7-sh
CDCA7-sh+TGF-β1
Number of migrated/invaded Cells (%)
Migration
Invasion

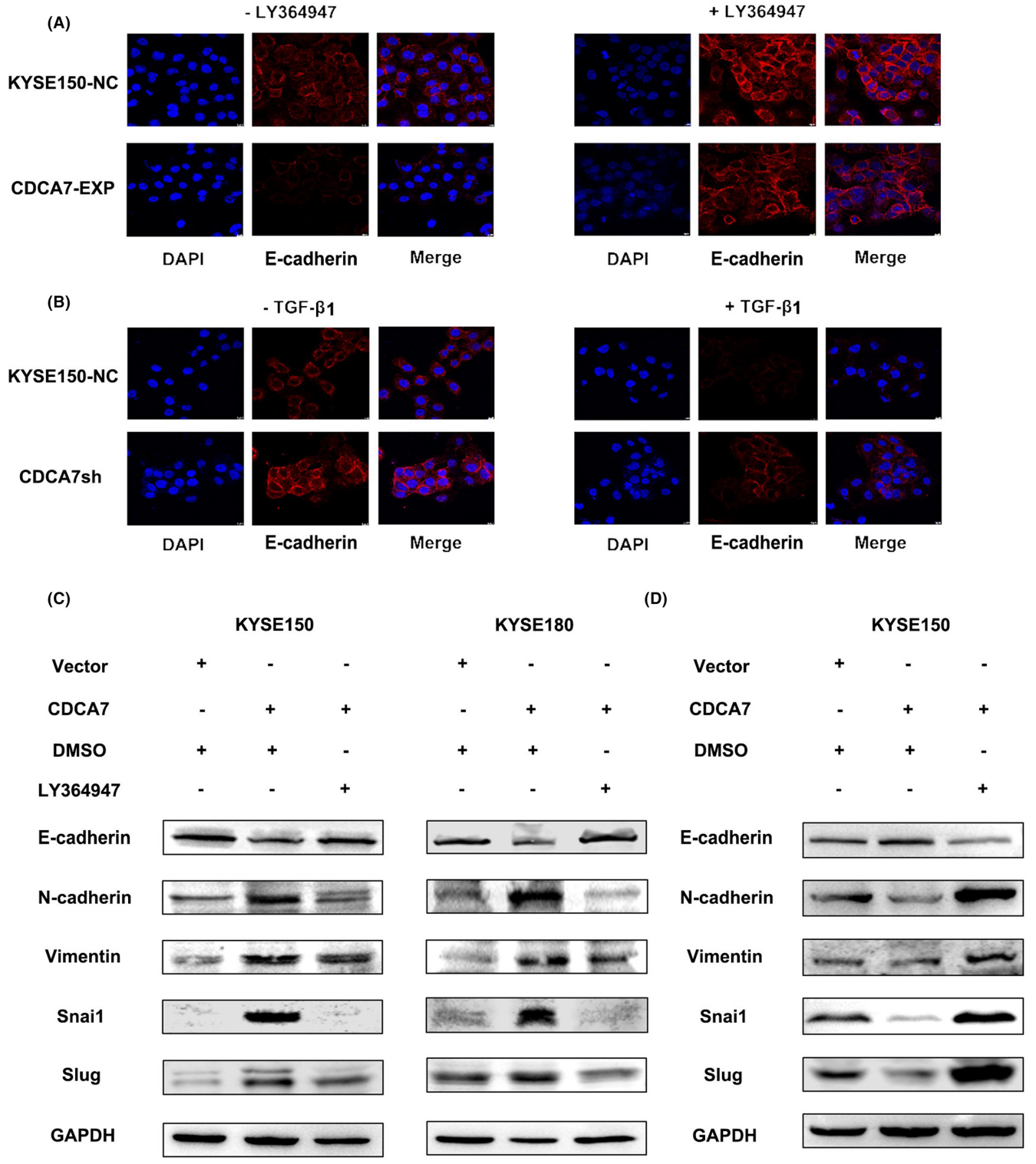


FIGURE 5 Activation of the TGF-β signaling pathway promotes the epithelial–mesenchymal transition process. (A) Immunofluorescent staining of E-cadherin was examined in CDCA7 overexpression or control KYSE150 cells and CDCA7 overexpression or control KYSE150 cells treated with LY-364947. (B) Immunofluorescent staining of E-cadherin was examined in CDCA7 knockdown or control KYSE150 cells and CDCA7 knockdown or control KYSE150 cells treated with TGF-β1. (C) The protein expression levels of E-cadherin, N-cadherin, Vimentin, and Slug were examined in CDCA7 overexpression cells, control KYSE150, and KYSE180 cells and CDCA7 overexpression cells treated with LY-364947. (D) The protein expression levels of E-cadherin, N-cadherin, Vimentin, and Slug were examined in CDCA7 knockdown cells, control KYSE150 cells and CDCA7 knockdown cells treated with TGF-β1. CDCA7, cell division cycle associated 7; TGF-β, transforming growth factor β.

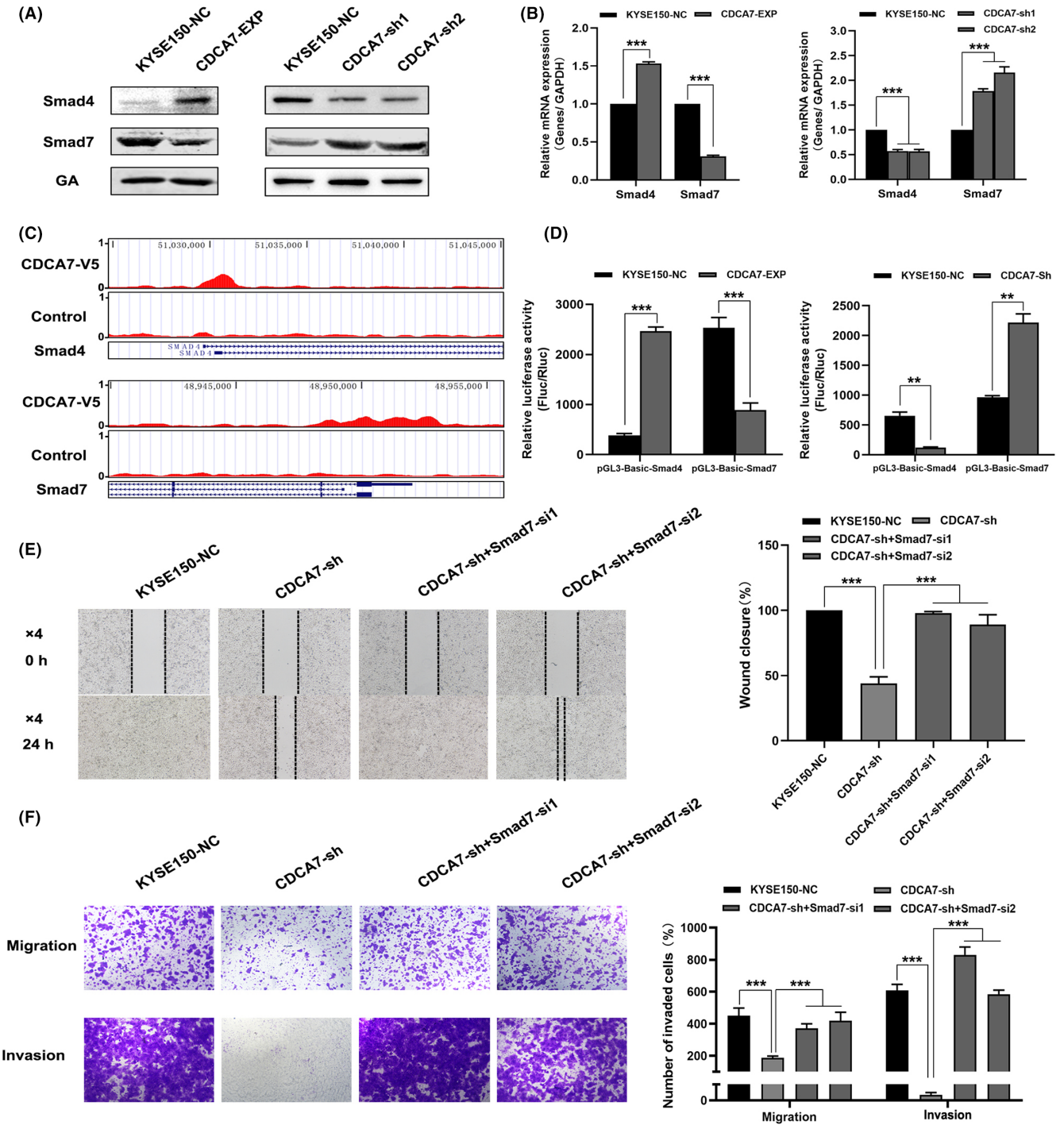


FIGURE 6 CDCA7 promotes the epithelial–mesenchymal transition process of ESCC through activating the transcription of Smad4/Smad7 in the TGF-β signaling pathway. (A) The protein expression levels of Smad4 and Smad7 in CDCA7 knockdown or control KYSE150 cells and CDCA7 overexpression or control KYSE150 cells. (B) The mRNA expression levels of Smad4 and Smad7 in CDCA7 knockdown or control KYSE150 cells and CDCA7 overexpression or control KYSE150 cells. (C) CDCA7 binds with Smad4 at the position −27 to 846 bp and binds with Smad7 at the position −973 to 946 bp respectively. (D) Luciferase reporter assays showed that CDCA7 could activate the transcription of Smad4 and Smad7. (E) Smad7 silencing promoted the wound healing of ESCC cells induced by CDCA7 knockdown. Photographs were taken at 0 and 24 h. (F) Smad7 silencing restored the ability of migration and invasion reduced by CDCA7 knockdown. CDCA7, cell division cycle associated 7; ESCC, esophageal squamous cell carcinoma; TGF-β, transforming growth factor β. ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

and migration ability reduced by *CDCA7* knockdown (Figure 6E,F) and that Smad7 might be an inhibitor of TGF-β signaling pathway. Therefore, overexpression of *CDCA7* promotes the expression of Smad4 and inhibits the expression of Smad7, therefore activating the TGF-β signaling pathway and promoting the EMT process of ESCC (Figure 7).

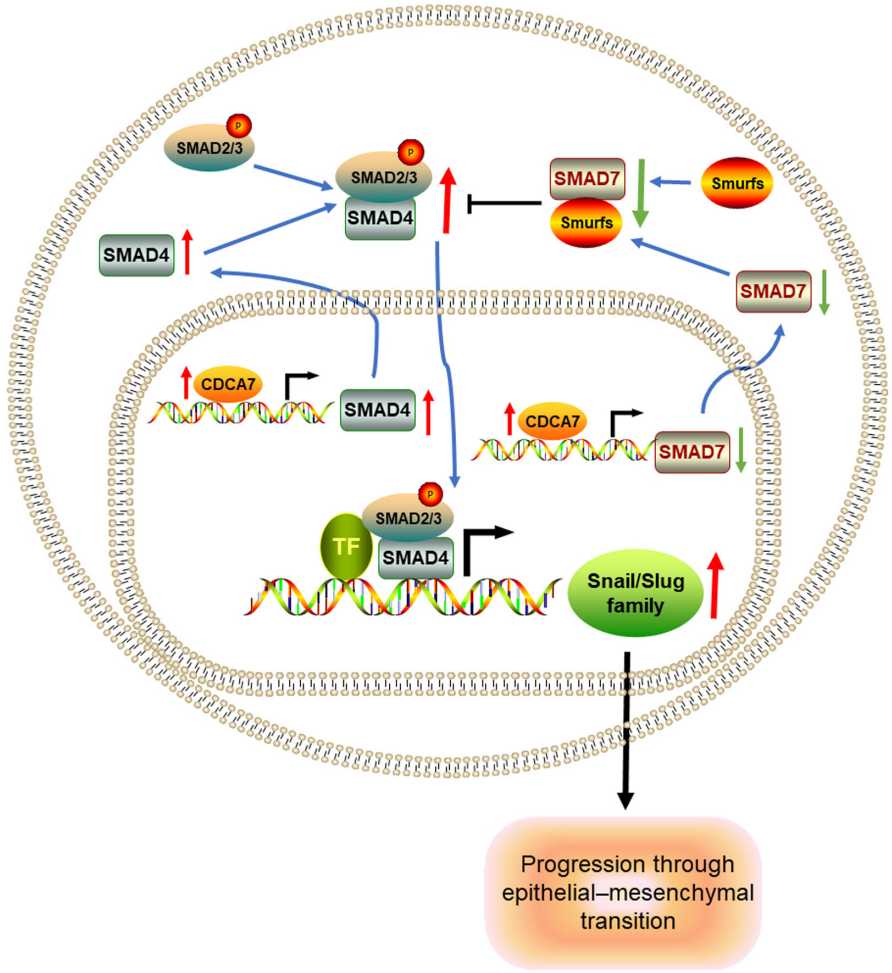


FIGURE 7 CDCA7 activates TGF-β-induced EMT through SMAD pathways. Overexpression of CDCA7 promotes the expression of Smad4 and inhibits the expression of Smad7. Both the increased expression of Smad4 and the decreased expression of Smad7 activate the TGF-β signaling pathway. The activation of the TGF-β signaling pathway promotes the transcription of Snail and Slug, therefore promoting the progression of EMT. CDCA7, cell division cycle associated 7; EMT, epithelial–mesenchymal transition; TGF-β, transforming growth factor β.

4 | DISCUSSION

Esophageal squamous cell carcinoma (ESCC) is one of the high incidence tumors in China[29–31] and the 5-year survival rate is less than 30% due to the lack of effective and specific therapeutic targets and targeted therapeutic drugs.[32] In this study, we found that CDAC7 overexpression has a prometastatic effect on ESCC. Furthermore, CDCA7 was shown to promote the EMT process and ESCC metastasis via activation of the TGF-β signaling pathway through preventing Smad7 mRNA transcription and promoting Smad4 mRNA transcription.

Previous studies have proved that CDCA7 could be used as a transcription regulator to regulate the transcription of downstream genes[25,27] and promote the metastasis and invasion of triple-negative breast cancer via regulating EZH2 transcriptionally.[27] Our study showed the similar results to previous findings that CDCA7 behaves as a prometastatic factor and its upregulation resulted in increased tumor metastasis in ESCC. Both in vivo and in vitro functional studies provided conclusive evidence that CDCA7 promotes migratory and invasive capacities and activates the EMT process in ESCC. CDCA7 positively accelerated the EMT process of ESCC cells, as the key molecular markers of EMT, N-cadherin, Vimentin, Snail, and Slug upregulated and the main invasion suppressor E-cadherin downregulated with *CDCA7* overexpression. The correlation analysis using data from GEO (GSE53625) and TCGA database also supported our study. CDCA7 was positively correlated with Myc, CDH2, SNAI1, SNAI2 and negatively correlated with CDH1 using data from GSE53625 (Figure S7). In TCGA database, CDCA7 was negatively correlated with CDH1 (Figure S8).

To further explain the mechanisms of the CDCA7-induced EMT process in ESCC metastasis, we investigated downstream target genes regulated by CDCA7 in ESCC. Our results further confirmed that CDCA7 could directly regulate the transcription of Smad4 and Smad7, two key factors and indispensable effectors of TGF-β-induced EMT and tumor metastasis.

The TGF-β signaling pathway is well known to have pleiotropic effects on cell proliferation, differentiation, migration, senescence, and apoptosis.[33] In pre-malignant cells and early-stage tumors, the TGF-β signaling pathway inhibits the proliferation of carcinoma cells and induces apoptosis as a tumor suppressor. Whereas, in the later stages of cancer, it has protumoral effects by modulating genomic instability, EMT, immune evasion, cell motility, and metastasis.[33,34] It is commonly accepted that TGF-β-induced EMT is an instrumental step in carcinoma invasion and metastasis.[35,36]

The interaction of TGF-β with TβRI and TβRII, the type I and type II transmembrane kinase receptors, initiates TGF-β signaling.[37] Following ligand binding, the TβRII receptors directly phosphorylate

TβRI receptors to activate them. The phosphorylated TβRI in turn phosphorylates the downstream intracellular mediators Smad2 and Smad3. Two activated Smad proteins (pSmad2/3C) then recruit Smad4 to form trimeric Smad complexes that translocate into the nucleus and regulate TGF-β target gene expression.[38–42] The interactions of Smad complexes with DNA-binding transcription factors, coactivators, and corepressors at regulatory gene sequences activate or represses transcription.[43,44] Collectively, the EMT transcription factors, Snail, and Slug, ZEB1 and ZEB2, and Twist are directly activated by TGF-β signaling[45–47] Subsequently, an active complex formed by Smad3/Smad4 and Snail binds to the regulatory promoter sequences of the gene encoding E-cadherin, leading to TGF-β-induced repression of its expression and therefore modulate cellular adhesion, polarity.[35,48,49] In addition to R-Smads (Smad2 and Smad3) and Co-Smad (Smad4), there is a third Smad protein family, namely the I-Smads (Smad6 and Smad7). Of the two I-Smads, Smad7 could be recruited to the complex of activated TGFβRs or pSmad2/3C to initiate their degradation by Smad specific E3 ligase.[38] Smad7 is a general antagonist of TGF-β family and transcriptionally induced by TGF-β family cytokines and regulates these signaling pathways negatively, therefore establishing an important negative feedback loop.[50]

As the given experimental evidence showed the key role of TGF-β signaling in cancer metastasis, TGF-β and components of TGF-β signaling have indeed been regarded as candidates for anti-metastasis therapies. Clinical trials with several of these agents and multiple therapeutic agents to block TGF-β and its signaling are currently in progress. The development of treatments that can specifically stop or slow down the problem of metastasis is urgently needed.

Our previous study showed that high expression of CDCA7 markedly promoted the proliferation of ESCC cells and the development of ESCC.[25] In this study, we reported that *CDCA7* upregulation significantly promoted the aggressiveness and metastasis of ESCC cells both in vitro and in vivo. In addition, *CDCA7* downregulation markedly inhibited the proliferation and metastasis of ESCC cells. Further research showed that CDCA7 promoted the proliferation, invasion and migration of ESCC through transcriptionally regulating the genes in cell cycle pathway and EMT-related pathway (TGF-β signaling pathway). Therefore, for the future, we wondered whether it is possible to treat ESCC by developing CDCA7 inhibitors and look forward to perform deeper research on CDCA7 to provide more effective therapeutic strategies against ESCC.

AUTHOR CONTRIBUTIONS

Yongping Cui and Xiaolong Cheng designed the study. Hongyi Li, Shaojie Wang, Xiubo Li acquired the data. Hongyi Li, Dinghe Guo, and Yongjia Weng analyzed these data. Hongyi Li prepared the manuscript. Yanqiang Wang, Pengzhou Kong, and Ling Zhang edited the manuscript. Yongping Cui reviewed the manuscript.

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DISCLOSURE

The authors have no conflict of interest. None of the authors of this manuscript is a current Editor or Editorial Board Member of Cancer Science.

DATA AVAILABILITY STATEMENT

Files of RNA-seq detailed results have been uploaded to GEO database with the GSE number GSE201735.

ETHICS STATEMENT

This work did not involve experiments on human subjects. Animal experiments were approved by the ethics committee of Shanxi Medical University for Research Involving Animals.

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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