PCSK9 promotes tumor cell proliferation and migration by facilitating CCL25 secretion in esophageal squamous cell carcinoma

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Abstract. Proprotein convertase subtilisin/kexin type 9 (PCSK9) serves an important role in maintaining plasma cholesterol levels, and fatty acid metabolism is involved in the progression of various types of cancer. In the present study, the role of PCSK9 in the development of esophageal squamous cell carcinoma (ESCC) was investigated. PCSK9 expression was compared between ESCC and normal esophageal epithelial tissues using reverse transcription-quantitative PCR. In addition, the association between PCSK9 expression and clinical staging and prognosis was assessed by immunohistochemistry. The effects of PCSK9 overexpression or knockdown on cell proliferation was evaluated using Cell Counting Kit-8 and colony formation assays. The invasion and migration of cancer cells was assessed using wound healing and Transwell assays. Western blotting was performed to evaluate changes in the expression levels of epithelial-mesenchymal transition (EMT)-related proteins. ELISA was performed to detect the effects of PCSK9 on chemokine (C-C motif) ligand 25 (CCL25) secretion. The results revealed that PCSK9 was highly expressed in ESCC tissues compared with that in normal esophageal tissues, and the high expression of PCSK9 was associated with a poor prognosis. Furthermore, PCSK9 could promote the proliferation, migration and invasion of ESCC cells *in vitro*. Mechanistically, PCSK9 could promote EMT by secreting CCL25. In conclusion, patients with ESCC may benefit from a novel therapeutic strategy based on these findings.

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Introduction

Esophageal cancer is a common digestive malignant tumor, the main types of which are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (1). China has the highest incidence and mortality rates of ESCC in the world, constituting ~50% of the global prevalence (2). Since early ESCC has no apparent symptoms, the cancer is usually in the middle and late stages when clinical symptoms appear. Notably, the 5-year survival rate of patients with middle-to-late stage esophageal cancer is as low as 6-15% (3-5). To improve the long-term survival rate of patients with ESCC, it is necessary to explore new biomarkers and molecular mechanisms.

Lipid metabolism serves a vital role in cancer progression, and excessive lipid uptake, storage and lipogenesis result in rapid tumor growth (6). The subtilisin-like proprotein convertase proprotein convertase subtilisin/kexin type 9 (PCSK9) has a critical role in regulating plasma cholesterol homeostasis and fatty acid metabolism (7). PCSK9 regulates not only lipid transport but also viral infection and insulin resistance, as well as tumor progression and apoptosis. PCSK9 has been reported to be associated with tumor progression in various types of cancer (8,9). In hepatocellular carcinoma and colon cancer, PCSK9 has been shown to promote tumor proliferation (10,11). It has also been found to facilitate tumor invasion and suppress apoptosis in gastric cancer (12). However, it is unclear how PCSK9 contributes to ESCC development.

The dynamic interplay between neoplastic cells and their adjacent stromal microenvironment facilitates the initiation, advancement and dissemination of cancer, and the acquisition of resistance to therapeutic agents in solid tumor development (13). Tumor stroma facilitates tumor metastasis; for example, TGF-β secreted by stromal cells surrounding the tumor induces epithelial-mesenchymal transition (EMT), thereby facilitating tumor cell infiltration (14). In cancer cells, EMT is associated with tumor growth, metastatic tumor formation and enhanced resistance to numerous therapeutic regimens. EMT has been associated with tumor development in a number of studies. Breast tumors induced by receptor tyrosine-protein kinase erbB-2 have been shown to spontaneously express Snail and exhibit EMT characteristics; in prostate cancer, tumor progression is significantly associated with

the switch from E-cadherin to N-cadherin (15). In addition, the presence of EMT markers is associated with high-grade and -stage bladder cancer (16). As evidenced by current research, EMT mechanisms are integral components of cancer progression (17,18).

Tumors express high levels of chemokines and their receptors, which function as growth factors for tumor cells, promote their development and facilitate their metastasis (19). Previous studies have reported that chemokine (C-C motif) ligand 25 (CCL25) is upregulated in various malignancies, where it is associated with organ-specific tumor metastasis. In non-small cell lung cancer, breast cancer and hepatocellular carcinoma, CCL25 has been shown to promote migration and invasion (20-22).

The present study aimed to investigate the functions of the PCSK9 in human ESCC progression. The present study may provide novel theoretical perspectives regarding ESCC diagnosis and treatment.

Materials and methods

Patients and specimens. A total of 60 ESCC tissues and 20 normal esophageal tissues were collected from patients (41 men and 19 women; age range, 49-76 years; mean age, 68 years) who received esophagectomy between January and May 2020 at The Fourth Hospital of Hebei Medical University (Shijiazhuang, China). A total of 100 esophageal cancer tissue samples were also obtained between October 2017 and January 2018 from patients (48 men and 19 women; age range, 48-84 years; mean age, 65 years) who had undergone esopha-gectomy at The Fourth Hospital of Hebei Medical University (Shijiazhuang, China). 5 Paired carcinomatous and para-carci-nomatous tissues were from patients with ESCC (3 men and 2 women; age range, 63-77 years; mean age, 71 years) who had undergone esophagectomy at The Fourth Hospital of Hebei Medical University (Shijiazhuang, China). All patients did not undergo preoperative adjuvant chemotherapy and radio-therapy. All patients signed informed consent forms, and the study was approved (no. 2018MEC015) by the Medical Ethics Committee of Hebei Medical University.

Cell culture. *In vitro* experiments were conducted using TE1, KYSE30, KYSE150 and KYSE170 human ESCC cell lines grown in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cell lines were purchased from Procell Life Science & Technology Co., Ltd. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Transient transfection. To establish cell lines with PCSK9 over-expression, transfection was performed when the cell density reached ~90%. The PCSK9 plasmid (cat. no. RC220000; Origene Technologies, Inc.) was employed to induce overex-pression of PCSK9, whereas the empty pCMV6-Entry plasmid (cat. no. PS100001; Origene Technologies, Inc.) served as a control. A total of 2 μg plasmid was used for transfection of each well of a 6-well culture plate for 6 h at 37°C. According to the manufacturer's protocol, transient transfection was

conducted using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and subsequent functional experiments were conducted 24 h post-transfection.

Small interfering RNA (siRNA) transfection. KYSE-150 cells were subjected to transfection with a double-stranded siRNA obtained from Guangzhou RiboBio Co., Ltd. using Hi-perfect Transfection Reagent (Qiagen GmbH) according to the manufacturer's protocol. To establish cell lines with PCSK9 knockdown, transfection was performed when the cell density reached ~50% of a 6-well culture plate. Cells were transfected with 50 nM siRNAs for 6 h at 37°C and the knockdown gene effect was assessed 48 h post-transfection via western blot analysis and RT-qPCR. The sequences were as follows: si-PCSK9-1 sense, 5'-GCACCCUCAUAGGCC UGGAGUUUAU-3'; and antisense, 5'-AUAAACUCCAGG CCUAUGAGGGUGC-3'; si-PCSK9-2 sense, 5'-GACAUC AUUGGUGCCUCCAGCGACU-3'; and antisense, 5'-AGU CGCUGGAGGCACCAAUGAUGUC-3'; si-PCSK9-3 sense, 5'-CAUAGGCCUGGAGUUUAUUCGAAA-3'; and antisense, 5'-UUUCGAAUAAACUCCAGGCCUAUG-3'; si-negative control (NC) sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.

Cytokine treatment. CCL25 was purchased from PeproTech EC Ltd. In the rescue experiment, after transfection of si-PCSK9, ESCC cells were cultured with CCL25 (150 ng/ml) for 24 h at 37°C in a humidified atmosphere containing 5% CO_2.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from ESCC tissues and cells was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was prepared using GoScript Reverse Transcriptase according to the manufacturer's protocol (Promega Corporation). GoTaq qPCR Master Mix (Promega Corporation) was used to perform qPCR amplifi-cation. RT-qPCR was conducted by using an SYBR Green PCR Kit (Promega Corporation) with a real-time PCR System (ABI 7500). The thermocycling conditions of qPCR were as follows: Initial denaturation, 70°C for 5 min; annealing, 25°C for 5 min; extension, 42°C for 60 min; and denaturation, 70°C for 15 min (23). The primer sequences are as follows: PCSK9 forward, 5'-GCTGAGCTGCTCCAGTTTCT-3', reverse, 5'-AATGGCGTAGACACCCTCAC-3'; GAPDH forward, 5'-AAGGTGAAGGTCGGAGTCAAC-3' and reverse, 5'-GGG GTCATTGATGGCAACAATA-3'. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (24).

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 kit (Dojindo Laboratories, Inc.) was used to evaluate cell viability. First, $4x10^3$/cells well were inoculated into 96-well plates (100 μm/well) and incubated at 37°C overnight. Subsequently, 10 μl CCK-8 solution was added to each well and incubated for 1.5 h at 37°C. Finally, absorbance was measured at 450 nm using a microplate reader at 24, 48, 72 and 96 h.

Colony formation assay. ESCC cells (~1,000) were isolated and seeded in a 6-well plate. After 10-14 days, the colonies were fixed by 4% paraformaldehyde for 15 min at room temperature and stained with 0.1% crystalline violet-stained

for 10 min at room temperature. The colonies (>50 cells) were then counted using ImageJ (version 1.8.0_172).

Transwell migration and invasion assays. Transwell migration and invasion assays were conducted using empty (migration) or precoated (30 μl Matrigel at 37˚C for 1 h) (invasion) Transwell chambers (BD Biosciences). Briefly, $4x10^4$ cells were plated into the upper chamber in serum-free culture medium, whereas complete culture medium was added to the lower chamber. After 36-48 h incubation (dependent on cell type) in a 37˚C incubator, cells that were attached to the lower compartment of the filter were identified by crystal violet staining (0.1%; 37˚C for 15 min) and were counted under a light microscope.

Wound healing assay. A previously described method was used to conduct the wound healing assay (23). Briefly, $3x10^5$ transfected cells were evenly distributed onto a 6-well plate. Once the cells reached 80% confluence in serum-free medium, they were scratched using a 200-μl pipette tip. Subsequently, images were captured at 0 and 24 h to determine the wound healing rate. The wound healing rate was calculated as follows: (Original wound area-non-healing wound area)/original wound area x100.

Western blotting. Following PCSK9 overexpression or knockdown, total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) and was denatured by boiling. Protein concentrations were detected by using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (40 μg/lane) were then separated on 10% gels using SDS-PAGE and were transferred onto PVDF membranes (MilliporeSigma). After blocking the proteins with 5% non-fat dried milk at 37˚C for 1 h (Beijing Solarbio Science & Technology Co., Ltd.), incubation was performed at 4˚C overnight with primary antibodies at a 1:1,000 dilution. Rabbit polyclonal antibodies against E-cadherin, N-cadherin and vimentin (cat. nos. 20874-1-AP, 22018-1-AP and 10366-1-AP; 1:1,000; Proteintech Group, Inc.) were used to detect the corresponding proteins. Anti-PCSK9 antibody (cat. no. 27882-1-AP; 1:1,000; Proteintech Group, Inc.) was used to detect the transfection effect. The loading control was β-actin (cat. no. 20536-1-AP; 1:1,000; Proteintech Group, Inc.) Membranes were then washed with Tris-buffered saline plus Tween (1% Tween-20) and incubated with secondary HRP-conjugated antibodies (1:10,000) for 2 h at room temperature. The secondary antibodies were purchased from Proteintech Group, Inc. (cat. no. PR30012). Enhanced chemiluminescence SuperSignal™ West Atto reagent (cat. no. A38554; Thermo Fisher Scientific, Inc.) was used to visualize the proteins according the protocol described by Scherbakov *et al* (25). ImageJ software (version 1.8.0_172; National Institutes of Health) was used to analyze the gray value of the western blot.

Immunohistochemistry. ESCC samples and normal esophageal epithelial tissues were dehydrated in 4% paraformaldehyde in PBS (4˚C), made transparent, dipped in wax, embedded, and sliced into 5-μm thick serial sections using a microtome (RM2235; Leica Microsystems GmbH). Slides were deparaffinized in xylene, rehydrated using a decreasing alcohol gradient, and washed three times for 5 min in 1X PBS.

Following antigen retrieval, the sections were heat-treated for 5 min in 10 mmol/l Na-citrate buffer (pH 6.0) and washed again in 1X PBS. For antigen retrieval, the sections were heated with EDTA buffer (pH 9.0) in a microwave oven for 5 min. Subsequently, the sections were incubated for 45 min at 37˚C in 5% normal goat serum (Proteintech Group, Inc.), after which, the sections were incubated with an anti-PCSK9 antibody (cat. no. 27882-1-AP; 1:100; Proteintech Group, Inc.) at 4˚C overnight. A streptavidin-biotinylated HRP-based detection system was used to reveal specific binding after incubation with a secondary antibody (cat. no. PR30011; 1:100; Proteintech Group, Inc.) at 37˚C for 1 h. The sections were then incubated with 3,3'-diaminobenzidine chromogen for 1 h at 37˚C. Sections were observed at x200 magnification using a Leica DM4000 B LED microscope (Leica Microsystems GmbH). ImageJ (version 1.8.0_172; National Institutes of Health) was used to calculate the average optical density of positive expression (26). The expression was ranked on the sum of intensity and area from 0 to 7: 0-2, negative expression; 3-7, positive expression (3-4, weak positive expression; 5-7, strong positive expression). Staining intensity was graded as follows: 0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The staining area was scored as follows: 0, no staining; 1, 1-25% area; 2, 26-50% area; 3, 51-75% area; and 4, 76-100% area.

Enzyme-linked immunosorbent assay (ELISA). Cell supernatants were collected for ELISA. ELISA assays were performed in 96-well ELISA plates using a CCL25 ELISA kit (cat. no. ab256624; Abcam), according to the manufacturer's instructions.

Bioinformatics analysis. The Cancer Genome Atlas (TCGA; https://portal.gdc.com) was used to obtain RNA-sequencing expression profiles (level 3) for ESCC (tumor, n=163; adjacent paracancerous tissue, n=11). Statistical analysis was performed using R program v4.0.3 (URL http://www.R-project.org/), with ggplot2 (v3.3.2) used to detect the expression level of PCSK9 in normal tissues adjacent to ESCC and cancer tissues. Differential expression analysis was performed using DESeq2 (bioconductor.org/packages/DESeq2/) for PCSK9 high and low expression groups in ESCC. P<0.05 was considered to indicate a statistically significant difference.

Statistical analysis. For the *in vitro* studies, experiments were conducted with at least three biological repeats. SPSS 25.0 (IBM Corp.) was used for statistical analysis. Paired and unpaired Student's t-tests were used to compare continuous data between two groups. For comparisons between multiple groups, ANOVA (parametric) and the Kruskal-Wallis test (nonparametric) were used. Subsequently, if the obtained results were deemed significant, a post hoc test (LSD/SNK) was conducted for ANOVA, while Dunn's test was employed for the Kruskal-Wallis test. The Kaplan-Meier method and Cox regression analysis were used to evaluate cumulative survival, and a χ^2 test was used to examine the association between PCSK9 expression and clinicopathological findings. Kaplan-Meier survival curves were analyzed by log-rank test. P<0.05 was considered to indicate a statistically significant difference.
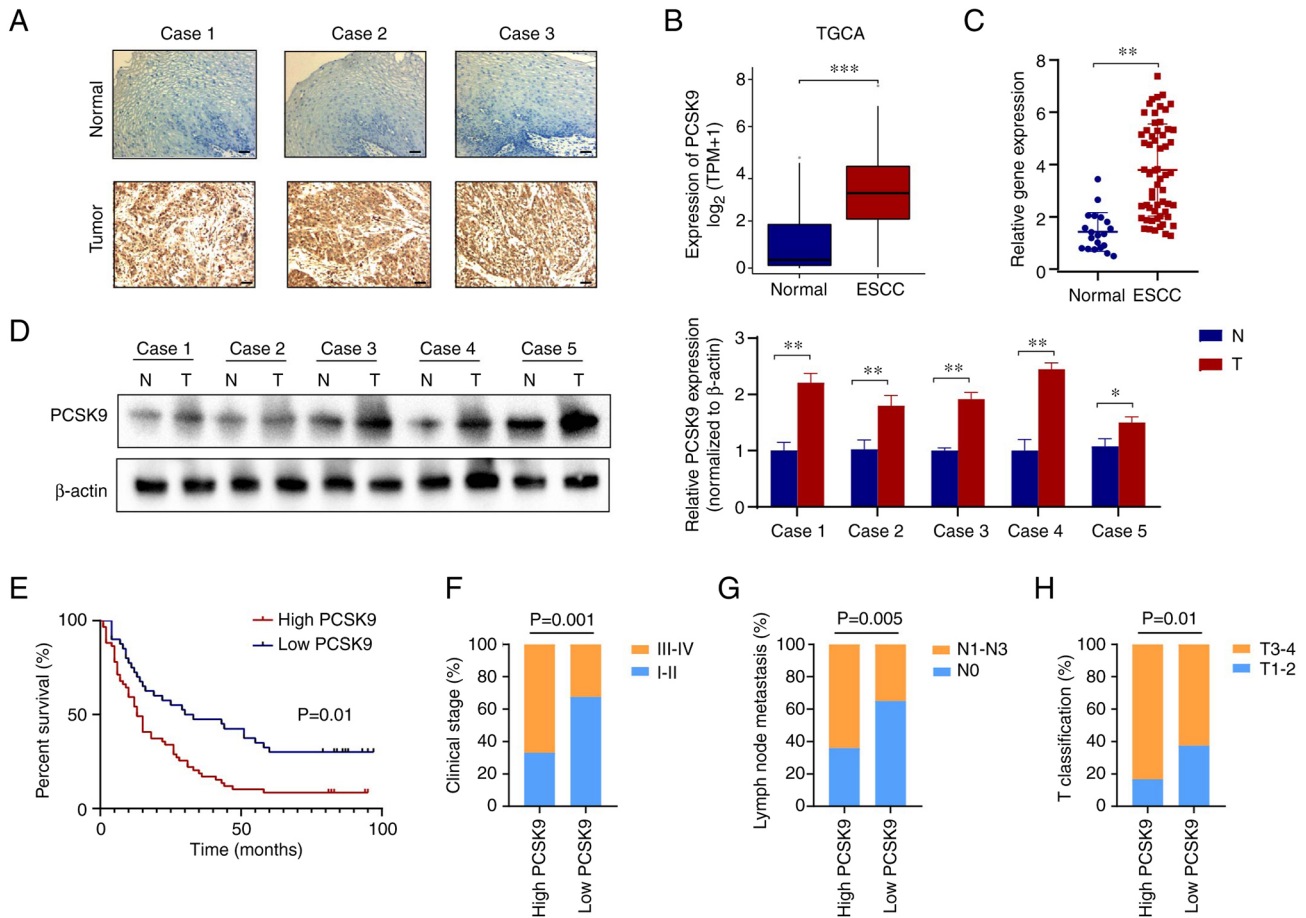
Figure 1. High PCSK9 expression is associated with poor prognosis in patients with ESCC. (A) Immunohistochemistry of normal esophageal epithelial and ESCC tissues from patients with ESCC. Typical cases with PCSK9 expression are shown. Scale bar, 100 μm. (B) PCSK9 expression levels in an ESCC validation set obtained from The Cancer Genome Atlas database. (C) Differential expression of PCSK9 in ESCC tissues and normal esophageal epithelial tissues from 60 patients. (D) PCSK9 expression levels in freshly obtained esophageal cancer tissues compared with adjacent paracancerous tissues in five patients. (E) Based on the expression of PCSK9, Kaplan-Meier analysis showed that high PCSK9 was associated with a shorter overall survival in 100 patients with ESCC. (F) In the PCSK9-high expression group, the clinical stage was higher than that in the PCSK9-low expression group. (G) PCSK9-high expression groups demonstrated a higher rate of lymph node metastasis. (H) A higher T classification was observed in the PCSK9-high expression group than that in the PCSK9-low expression group. Scale bars, 100 μm. *P<0.05, **P<0.01, ***P<0.001. PCSK9, proprotein convertase subtilisin/kexin type 9; ESCC, esophageal squamous cell carcinoma; N, normal; T, tumor.

Results

PCSK9 expression in ESCC tissues is associated with poor prognosis. To investigate the role of PCSK9 in ESCC, 20 normal esophageal and 60 ESCC tissues were assessed. At the protein level, immunohistochemical staining results suggested that PCSK9 expression was markedly higher in ESCC tissues compared with that in normal tissues (Fig. 1A). In addition, TCGA database revealed that PCSK9 was significantly elevated in ESCC tissues compared with that in normal tissues (Fig. 1B). The results of RT-qPCR analysis verified this finding in ESCC samples (Fig. 1C). Furthermore, analysis of PCSK9 expression levels was conducted in freshly obtained esophageal cancer tissues compared with adjacent paracancerous tissues in a cohort of 5 patients. The findings indicated a significant upregulation of PCSK9 expression in ESCC tissues when compared to para-carcinomatous tissues (Fig. 1D).

A total of 100 patients with ESCC were analyzed to explore the role of PCSK9 in ESCC. Based on PCSK9 expression levels, 60 patients were categorized as having

high PCSK9 expression and 40 as having low PCSK9 expression. The PCSK9 expression cut-off value was identified as the median IHC score. Based on the survival analysis, the PCSK9-high expression group had a shorter overall survival (OS) than the PCSK9-low expression group (Fig. 1E). In addition, it was observed that high levels of PCSK9 expression were associated with clinical stage, lymph node metastasis and T classification in patients with ESCC, but not with their age or sex (Fig. 1F-H; Table I). Based on univariate analysis, OS was associated with clinical stage, T classification, lymph node metastasis and PCSK9 expression. Multivariate analysis, however, showed that clinical stage, T classification, lymph node metastasis and PCSK9 expression were associated with OS (Table II). Overall, the expression of PCSK9 was revealed to be increased in ESCC tissue and was associated with poor prognosis.

PCSK9 promotes ESCC cell proliferation, invasion and migration in vitro. To explore the biological function of PCSK9 *in vitro*, its expression level was first measured in four ESCC cell lines using RT-qPCR. The expression levels

Table I. Relationship between the expression levels of PCSK9 and clinical pathological features in patients with esophageal squamous cell carcinoma.

Characteristic	n	PCSK9 expression		χ^2	P-value
		High	Low		
Age, years				3.038	0.053
≤60	34	18	16		
>60	66	42	24		
Sex				0.554	0.457
Male	74	46	28		
Female	26	14	12		
T stage				6.645	0.013
T1-2	25	10	15		
T3-4	75	50	25		
Lymph node metastasis				7.719	0.005
No	51	22	29		
Yes	49	38	11		
Clinical stage				10.635	0.001
I and II	47	20	27		
III	53	40	13		

PCSK9, proprotein convertase subtilisin/kexin type 9.

Table II. Univariate and multivariate analyses of prognostic factors in esophageal squamous cell carcinoma.

Variable	Univariate analysis			Multivariate analysis		
	HR	P-value	95% CI	HR	P-value	95% CI
Expression of PCSK9, high vs. low	2.113	0.002	1.328-3.364	1.755	0.030	1.057-2.915
Sex, male vs. female	1.555	0.126	0.884-2.732			
Age, <60 vs. ≥60 years	1.208	0.443	0.745-1.958			
T stage, T1-2 vs. T3-4	2.012	0.015	1.146-3.565	1.855	0.036	1.042-3.301
Lymph node metastasis, no vs. yes	1.545	0.001	1.216-1.962	1.332	0.032	1.025-1.729
Clinical stage, I and II vs. III	2.333	<0.001	1.482-3.671			

PCSK9, proprotein convertase subtilisin/kexin type 9.

of PCSK9 were lowest in TE1 cells and highest in KYSE150 cells (Fig. 2A). Therefore, biological function experiments were conducted in TE1 and KYSE150 cells. A PCSK9 overexpression vector was used for the overexpression experiment in TE1 cells, whereas PCSK9 siRNA was used for the knockdown experiment in KYSE150 cells. RT-qPCR was used to determine the efficiency of overexpression and knockdown (Fig. 2B and C). si-PCSK9-2 with the greatest efficiency was then selected for further experiments. Additionally, western blotting was conducted to analyze alterations in the protein expression levels of PCSK9 subsequent to its overexpression and knockdown. The overexpression of PCSK9 resulted in an upregulation of its protein expression level, while the knockdown of PCSK9 led to the inhibition of its expression (Fig. 2D and E).

Following PCSK9 overexpression, the proliferation of TE1 cells was increased, as determined by CCK-8 assay (Fig. 3A). Similar results were determined using the colony formation assay (Fig. 3B). In addition, PCSK9 affected the migration and invasion of ESCC cells. The Transwell assay showed that PCSK9 overexpression promoted the migration and invasion of TE1 cells (Fig. 3C). The wound healing assay also confirmed that PCSK9 overexpression could promote the migration of TE1 cells (Fig. 3D). Conversely, following PCSK9 knockdown, the proliferation of KYSE150 cells was inhibited, as determined by CCK-8 and colony formation assays (Fig. 3E and F). Furthermore, the migration and invasion of the cells were inhibited (Fig. 3G and H). These findings suggested that PCSK9 could facilitate the proliferation, migration and invasion of ESCC cells *in vitro*.
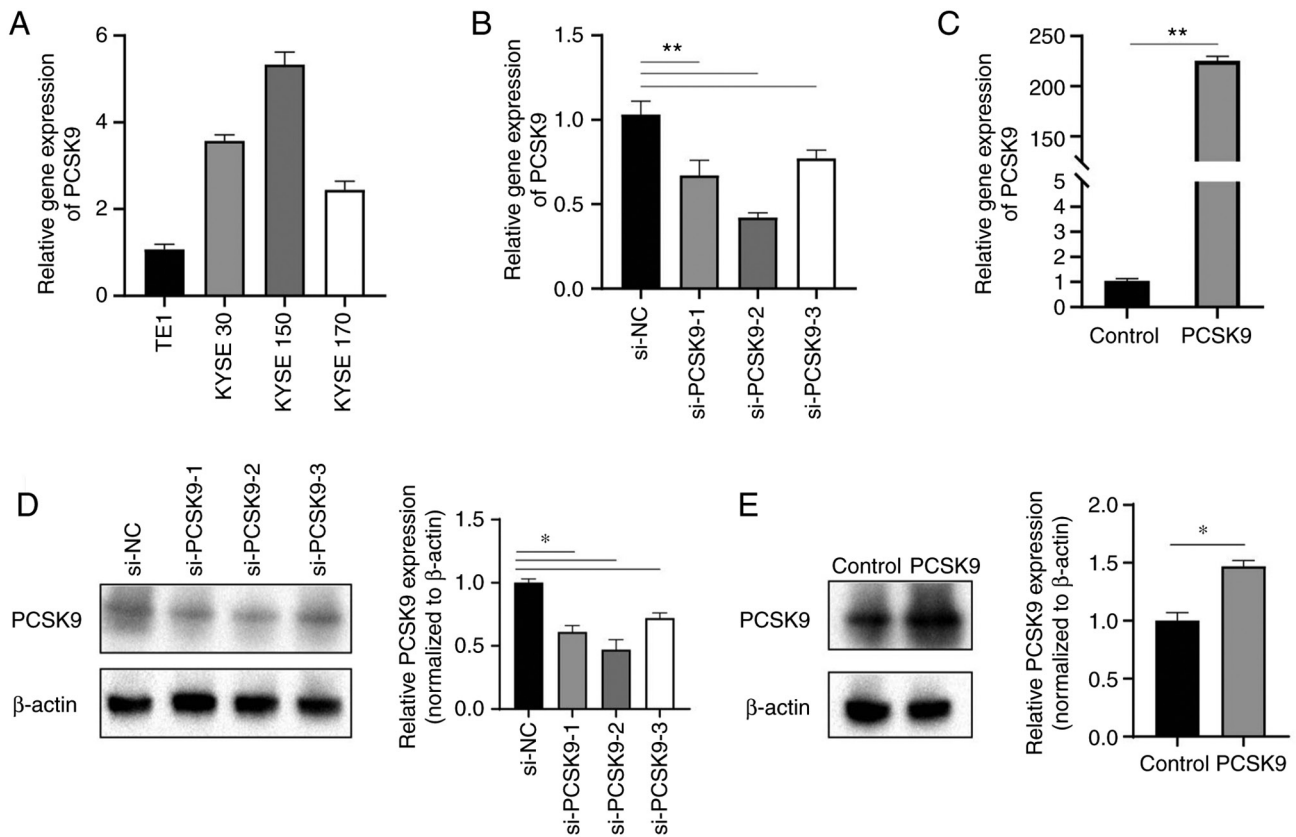
Figure 2. PCSK9 knockdown and overexpression in ESCC cell lines. (A) In ESCC cell lines, the mRNA expression levels of PCSK9 were lowest in TE1 cells and highest in KYSE150 cells. (B) Transfection efficiency of si-PCSK9 in the KYSE150 cell line; subsequent experiments were conducted using si-PCSK9-2, which had the highest knockdown efficiency. (C) Transfection efficiency of PCSK9 overexpression in the TE1 cell line. (D) PCSK9 protein expression following PCSK9 knockdown in KYSE150 cells. (E) PCSK9 protein expression following PCSK9 overexpression in TE1 cells. $^*P<0.05$, $^{**}P<0.01$. PCSK9, proprotein convertase subtilisin/kexin type 9; ESCC, esophageal squamous cell carcinoma; si, small interfering; NC, negative control.

PCSK9 modulates the EMT of ESCC cells. EMT is the process of epithelial cells acquiring mesenchymal characteristics. Tumor initiation, invasion, metastasis and resistance to cancer treatment are all affected by the EMT process (18). The key proteins involved in EMT were analyzed to better understand the effects of PCSK9 on ESCC. Following PCSK9 overexpression, the protein expression levels of E-cadherin were suppressed; however, the expression levels of N-cadherin and vimentin were increased (Fig. 4A). PCSK9 knockdown increased the protein expression levels of E-cadherin, while inhibiting those of vimentin and N-cadherin (Fig. 4B). These data indicated that PCSK9 was involved in EMT progression, and may regulate ESCC migration and invasion through EMT.

PCSK9 promotes ESCC progression by upregulating CCL25. To further investigate how PCSK9 promoted the progression of ESCC, ESCC data were extracted from the selected TCGA public database, and divided into high and low expression groups by PCSK9 expression The data were organized based on the levels of expression exhibited by the respective molecules, with the lower 50% being classified as the low expression group, and the upper 50-100% as the high expression group (high expression group, n=82; low expression group, n=81). Based on standard procedures, raw count matrices from the public data were compared using DESeq2. Among the differentially expressed genes, CCL25 was

highly associated with PCSK9 (Fig. 5A). Therefore, it was hypothesized that PCSK9 could promote ESCC progression by affecting the secretion of CCL25. To validate this hypothesis, ELISA experiments were performed to determine the effect of PCSK9 on CCL25 secretion. The results suggested that PCSK9 overexpression promoted CCL25 secretion, whereas PCSK9 knockdown had the opposite effect (Fig. 5B). Subsequently, rescue experiments were performed. As determined by the CCK-8 assay, PCSK9 knockdown inhibited cell proliferation, whereas the exogenous addition of CCL25 suppressed this effect (Fig. 5C). The colony formation assay also confirmed that the addition of CCL25 inhibited the diminished cell proliferation induced by PCSK9 knockdown (Fig. 5D). Furthermore, the migration and invasion of cells were inhibited following PCSK9 knockdown, whereas CCL25 reversed this effect (Fig. 5E and F).

Discussion

ESCC is a common malignant gastrointestinal tumor that seriously endangers human health. Although surgery, endoscopy and radiotherapy techniques have improved and complications have decreased, the overall treatment outcome of ESCC remains unsatisfactory (2). Therefore, it is essential to identify more effective tumor markers and discover new therapeutic targets for ESCC to improve the long-term survival rate of patients.
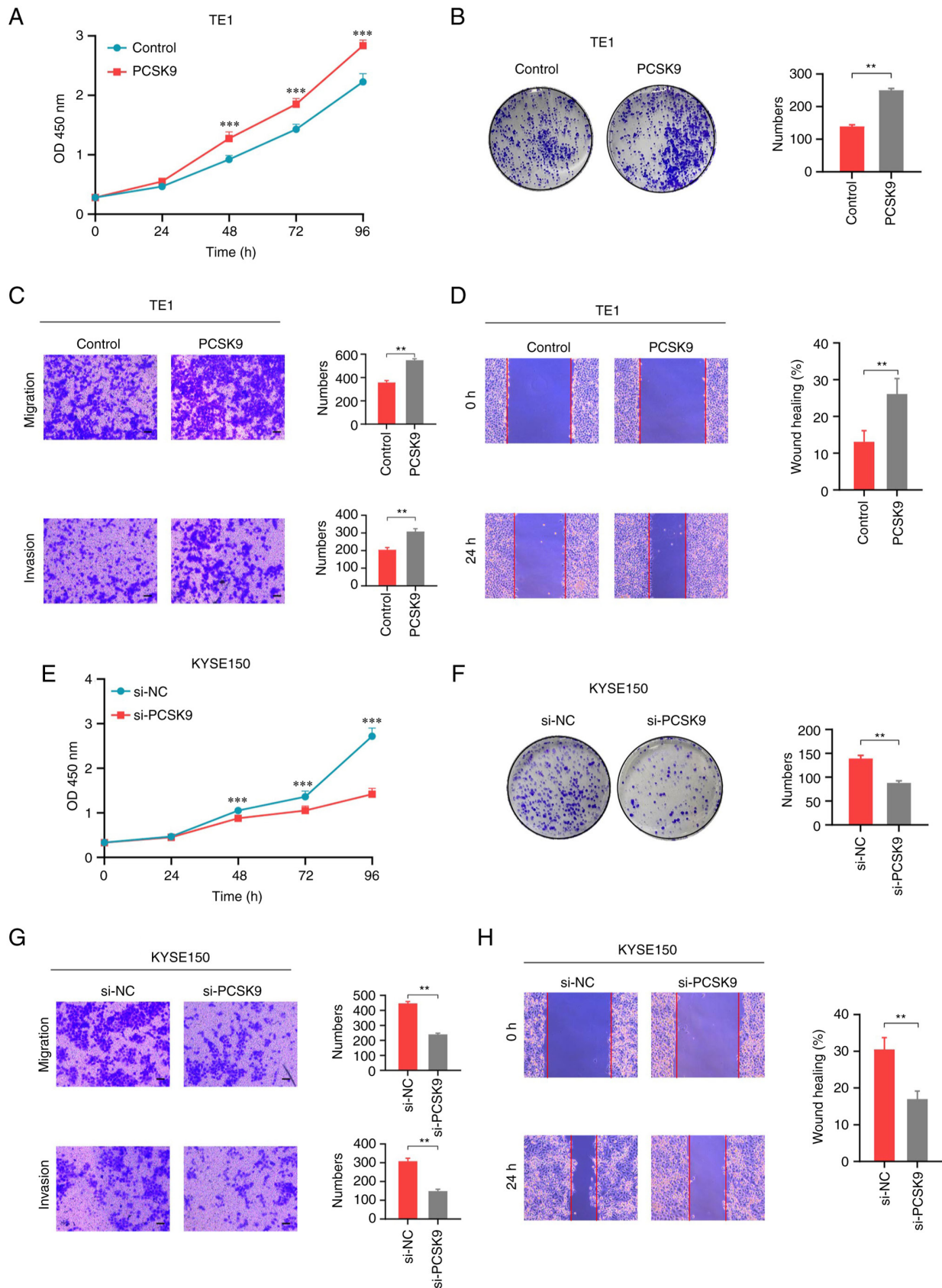
Figure 3. PCSK9 promotes cell proliferation, migration and invasion *in vitro*. (A) Proliferation of TE1 cells overexpressing PCSK9 and those transfected with a vector control, as determined using CCK-8 assay. PCSK9 promoted ESCC cell proliferation. $^{***}P<0.001$ vs. Control. (B) Colony formation assay of TE1 cells; PCSK9 overexpression promoted colony formation. Migration and invasion of TE1 cells with PCSK9 overexpression were detected using (C) Transwell migration and invasion assays, and (D) wound healing assays. PCSK9 promoted the invasion and migration of ESCC cells. (E) Proliferation of KYSE150 cells with PCSK9 knockdown and those transfected with si-NC, as determined using CCK-8 assay. (F) Colony formation assay of KYSE150 cells. (G) Transwell migration and invasion, and (H) wound healing assays detected migration and invasion of KYSE150 cells. Scale bars, 100 μm. $^{**}P<0.01$. $^{***}P<0.001$. PCSK9, proprotein convertase subtilisin/kexin type 9; CCK-8, Cell Counting Kit-8; ESCC, esophageal squamous cell carcinoma; si, small interfering; NC, negative control.
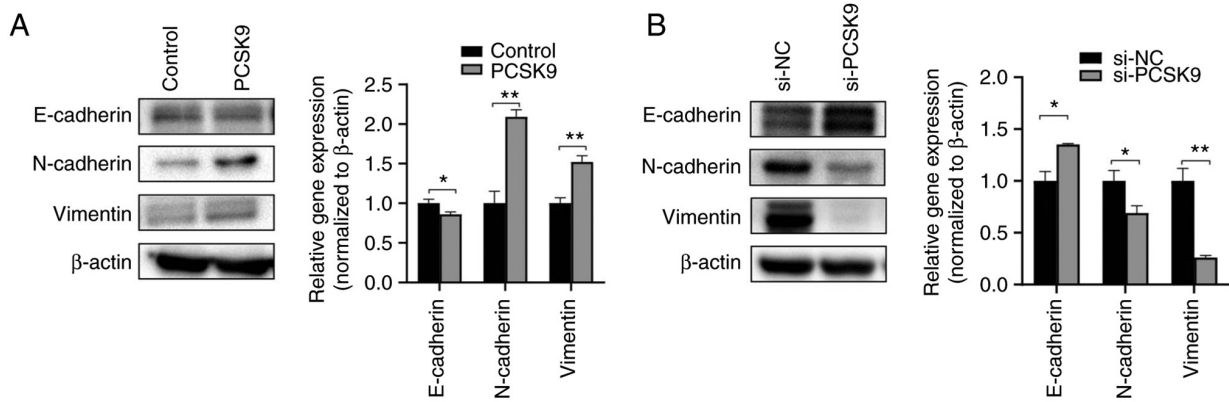
Figure 4. PCSK9 modulates the EMT of ESCC cells. (A) PCSK9 regulates esophageal squamous cell carcinoma progression and metastasis in TE1 cells through EMT. Overexpression of PCSK9 inhibits E-cadherin expression, and upregulates N-cadherin and vimentin protein expression. (B) Western blotting revealed that PCSK9 knockdown in the KYSE150 cells increased E-cadherin, and inhibited N-cadherin and vimentin expression. $^*P<0.05$, $^{**}P<0.01$. PCSK9, proprotein convertase subtilisin/kexin type 9; si, small interfering; NC, negative control; EMT, epithelial-mesenchymal transition.

Research has shown that lipid metabolism is critical to cancer progression (27-29). PCSK9 is a subtilisin-like proprotein convertase family member that serves a critical role in maintaining plasma cholesterol levels and fatty acid metabolism. Aside from regulating lipid transport, PCSK9 also affects cell functions, including viral infection and insulin resistance, and tumor progression and tumor apoptosis (30,31). Furthermore, PCSK9 has been implicated in cancer biology in several studies (9). In hepatocellular carcinoma, PCSK9 promotes tumor growth by inhibiting tumor cell apoptosis through the FASN/Bax/Bcl-2/caspase 9/caspase 3 pathway (10). PCSK9 also promotes colon cancer cell progression and metastasis by regulating EMT and PI3K/AKT signaling (11). In gastric cancer, PCSK9 promotes invasion and suppresses apoptosis by promoting the MAPK signaling pathway through the upregulation of heat shock protein 70 (12). Notably, it has been reported that serum PCSK9 antigen levels are not associated with esophageal cancer (32); this may be due to the fact that PCSK9 is mainly produced and metabolized by the liver. In the present study, ESCC tissues exhibited higher expression levels of PCSK9 than non-ESCC tissues, and PCSK9 expression was associated with T classification, clinical stage and lymph node metastasis. It was also identified as an independent risk factor for the OS of patients with ESCC. *In vitro* experiments revealed that PCSK9 stimulated the proliferation, invasion and migration of ESCC cells. The present findings on the role of PCSK9 in ESCC are consistent with findings in other types of cancer, suggesting that PCSK9 is an oncogenic gene that has a vital role in cancer development.

EMT refers to the transformation of epithelial cells into mesenchymal cells (33). The epithelial cells lose polarity and intercellular adhesion junctions during this process and become mesenchymal cells, with increased migratory and invasive abilities (18). Studies have shown that EMT serves a crucial role in the development of malignancies, cell invasion and tumor metastasis. During EMT, the cell phenotype is transformed from epithelial to mesenchymal, and cellular markers change. The expression of epithelial markers, such as E-cadherin and cytokeratin, is decreased; the expression of mesenchymal markers, such as N-cadherin, vimentin and smooth muscle actin proteins, are increased (14,16). E-cadherin is a vital adhesion molecule for maintaining the epithelial phenotype, and has a decisive role in intercellular adhesion and cell polarity (15,17,34). The tumor microenvironment also plays an important role in EMT. In the tumor microenvironment, mesenchymal stem cells release growth factors that promote EMT, such as IL-6 and TNF-α. In non-small cell lung cancer, IL-6 and TNF-α levels have been shown to be positively correlated with the protein levels of vimentin and N-cadherin, and negatively correlated with the protein levels of E-cadherin in tumor tissues. These results suggested that IL-6 and TNF-α may have an essential role in tumor EMT development (35). Decreased or absent E-cadherin expression in tumor cells is considered the gold standard for the loss of epithelial features in cancer cells in EMT and a critical manifestation of tumor metastasis (36). In the present study, the overexpression of PCSK9 inhibited the protein expression levels of E-cadherin, and promoted those of N-cadherin and vimentin. During PCSK9 knockdown, the opposite was observed. By knocking down PCSK9 in colorectal cancer, colon cancer cells have been shown to display reduced EMT and PI3K/AKT activation (11). Thus, PCSK9 may promote tumor cell metastasis and invasion by facilitating EMT.

Chemokines and their receptors are closely associated with tumor cell genesis, development and metastasis, and appear highly expressed in several tumors. Thymus-expressed chemokine 25, also known as CCL25, is the sole ligand of C-C chemokine receptor type 9. Various studies have shown that CCL25 is involved in tumor metastasis and tumorigenesis, and is overexpressed in malignant tumors (21,22,37). VEGF and MMPs play essential roles in tumor neovascularization and metastasis. Notably, tumor size and the number of metastasized lymph nodes are positively correlated with the serum levels of MMP-2 and MMP-9 in breast cancer (38), whereas in non-small cell lung cancer, CCL25 can promote migration and invasion by regulating VEGF and MMPs (21). Breast cancer cells may also express MMPs through CCL25 (20).

In addition, CCL25 plays a vital role in leukemia invasion and osteosarcoma metastasis (39,40). The present study showed that PCSK9 could promote the development of ESCC by promoting the secretion of CCL25. The rescue experiment showed that PCSK9 knockdown inhibited cell migration and invasion, whereas these effects could be reversed through the addition of CCL25.
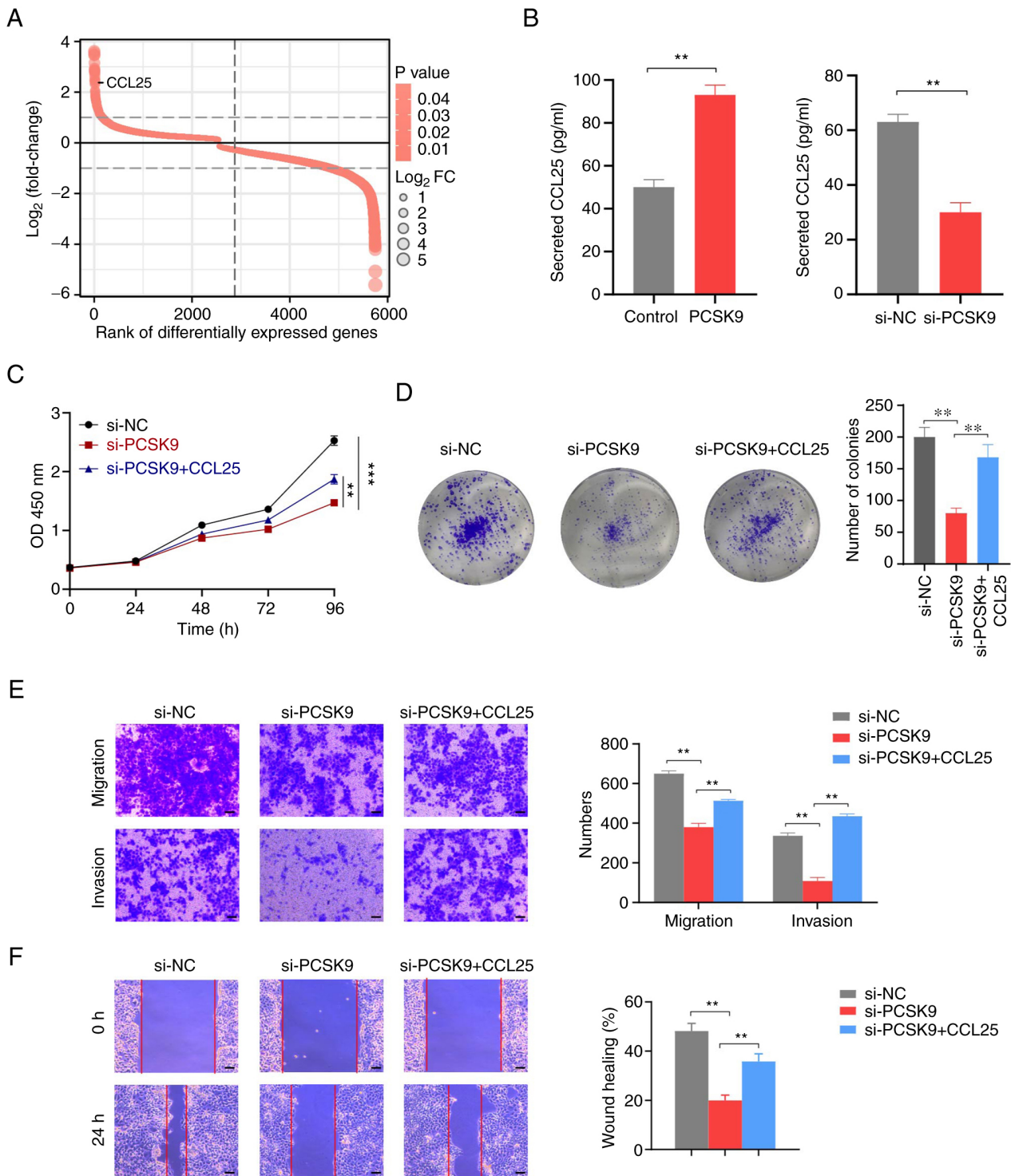
Figure 5. PCSK9 promotes ESCC progression through CCL25 upregulation. (A) PCSK9 data were extracted from The Cancer Genome Atlas public database and divided into high and low expression groups, according to the expression of the PCSK9. The original count matrix of the selected public data was analyzed for differences according to the standard process using the DESeq2 package. CCL25 expression was also low in the PCSK9-low expression group. (B) As determined by ELISA, PCSK9 overexpression increased CCL25 secretion, whereas PCSK9 knockdown inhibited CCL25 secretion. (C) Cell Counting Kit-8 assay showed that the exogenous addition of CCL25 inhibited PCSK9 knockdown-induced inhibition of cell proliferation. (D) Colony formation assay showed that the exogenous addition of CCL25 rescued PCSK9 knockdown-induced colony formation inhibition. (E) Transwell migration and invasion, and (F) wound healing assays were performed to examine whether the exogenous addition of CCL25 could reverse PCSK9 knockdown-induced inhibition of the migration and invasion of ESCC cells. Scale bars, 100 μm. $^{**}P<0.01$, $^{***}P<0.001$. PCSK9, proprotein convertase subtilisin/kexin type 9; ESCC, esophageal squamous cell carcinoma; CCL25, chemokine (C-C motif) ligand 25; si, small interfering; NC, negative control.

The present results demonstrated that PCSK9 may act as a tumor promoter in ESCC. PCSK9 could facilitate the proliferation, migration and invasion of ESCC *in vitro*.

Mechanistically, PCSK9 could promote EMT by secreting CCL25. Hence, the present study could provide novel theoretical perspectives regarding ESCC diagnosis and treatment.

In conclusion, a comprehensive *in vitro* cell experiment and tissue sample analysis was conducted in the present study to assess the involvement of PCSK9 in ESCC progression and metastasis. In ESCC tissues, PCSK9 protein was highly expressed and associated with poor prognosis. In addition, it was suggested that PCSK9 may promote tumor progression through EMT in ESCC, potentially by secreting CCL25. Overall, the present data demonstrated the oncogenic activity of PCSK9 in ESCC. Future studies should investigate PCSK9 inhibition as a potential treatment for ESCC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZT and HW were involved in study conception and design. ZT provided administrative support. HW provided study materials or patients. HW and QG performed the experiments. QG and MW collected and assembled data. HW and CL analyzed and interpreted data. ZT and HW confirm the authenticity of all the raw data. All authors wrote the manuscript, and read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of The Fourth Hospital of Hebei Medical University. Written informed consent was obtained from each patient included and this study was performed in accordance with The Declaration of Helsinki.

Patient consent for publication

The patients provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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