RUNX3 inhibits the invasion and migration of esophageal squamous cell carcinoma by reversing the epithelial-mesenchymal transition through TGF-β/Smad signaling

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Abstract. Runt-related transcription factor 3 (RUNX3) is a candidate tumor suppressor, and its inactivation may play a crucial role in the carcinogenesis process of numerous cancer types, including esophageal squamous cell carcinoma (ESCC). We previously revealed that RUNX3 inactivation was correlated with lymph node metastasis (LNM) and ESCC recurrence. However, the exact mechanisms of this process are still under investigation. The aim of the present study was to examine the potential roles and underlying molecular mechanisms of RUNX3 in ESCC metastasis and the epithelial-mesenchymal transition (EMT). According to the results, RUNX3 expression in ESCC tissue was significantly reduced compared with that in adjacent normal tissue (0.50±0.20 vs. 0.83±0.16; P<0.001). In addition, statistical analysis revealed a close association between decreased RUNX3 expression and T status (P=0.027) and LNM (P=0.017) in ESCC patients. Pearson's correlation coefficient analysis was then used to evaluate correlations between RUNX3 and EMT-related marker expression. The results revealed that RUNX3 expression in ESCC tissues was negatively correlated with the expression of N-cadherin (r=-0.429; P<0.01) and Snail (r=-0.364; P<0.01) and positively correlated with the expression of E-cadherin (r=0.580; P<0.01). Moreover, Eca109 and EC9706 cell invasion, migration, MMP-9 expression and EMT were significantly inhibited by RUNX3 overexpression. Notably, further analysis revealed that RUNX3 overexpression markedly inhibited the phosphorylation of Smad2/3; RUNX3-overexpressing cells also displayed less sensitivity to TGF-β1-induced EMT than control cells. Thus, RUNX3 may inhibit the invasion and migration of ESCC cells by reversing EMT through TGF-β/Smad signaling and may be useful as a therapeutic target.

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Abbreviations: RUNX3, runt-related transcription factor 3; ESCC, esophageal squamous cell carcinoma; LNM, lymph node metastasis; EMT, epithelial-mesenchymal transition

Key words: runt-related transcription factor 3, esophageal squamous cell carcinoma, lymph node metastasis, epithelial-mesenchymal transition, TGF-β/Smad signaling pathway

Introduction

With approximately 442,000 new cases diagnosed and 440,000 associated deaths worldwide in 2013, esophageal carcinoma is ranked as the eighth most common human malignancy and the sixth leading cause of cancer-related deaths worldwide (1). Esophageal squamous cell carcinoma (ESCC), as the predominant histopathological type, accounts for approximately 90% of esophageal cancer cases (2). Despite advances in diagnosis and multimodal therapy, the prognosis remains less than satisfactory, with 5 year overall survival rates ranging between 15 and 50% (3-5). Lymph node metastasis (LNM) is the single most important prognostic factor and remains the major cause of mortality after complete resection in ESCC patients (6,7). In fact, it has been revealed that the number of metastatic nodes is closely related to survival, and the 5 year survival rate of patients with 0, 1-2, ≥3 positive lymph nodes was 59.8%, 33.4% and 9.4%, respectively (3). Overall, there is an urgent need to elucidate the underlying mechanisms of LNM by identifying key molecules that may contribute to the development of reasonable treatment plans for different patients and improve the outcomes of ESCC patients.

Runt-related transcription factor 3 (RUNX3), located on human chromosome 1p36.1, is a member of the runt-domain family of transcription factors (8,9). RUNX3 has been

identified as a potential tumor suppressor gene in a variety of malignancies, especially in gastrointestinal cancers, such as gastric cancer, hepatocellular cancer, colorectal cancer, and ESCC (8,10-13). RUNX3 inactivation is involved in tumor development and metastasis through different processes, including the cell cycle, apoptosis, angiogenesis, EMT, invasion and migration (14). Although researchers have investigated the expression profile and roles of RUNX3 in ESCC progression (15), the molecular mechanisms of RUNX3 in EMT and ESCC metastasis have not yet been investigated and need to be elucidated.

Tumor metastasis is a multicellular process that involves cell-cell adherence, invasion, migration, angiogenesis, extracellular matrix (ECM) degradation and EMT (16,17). Among these processes, EMT is thought to have crucial functions. EMT is a dynamic process in which epithelial cells typically lose their epithelial characteristics, including cell polarity and cell-cell contact, and acquire a spindle shape migration phenotype (18,19). In addition, the expression of the epithelial markers E-cadherin and claudin-1 is reduced, whereas that of the mesenchymal markers N-cadherin and vimentin is increased (20).

The transforming growth factor β (TGF-β) pathway plays a complex dual role in tumor development. It acts as a tumor suppressor pathway during the early stages of epithelial neoplasia by inhibiting tumor cell proliferation and inducing apoptosis. At later stages of carcinogenesis, TGF-β signaling contributes to tumor invasion and metastasis by inducing EMT (18,21,22). TGF-β/Smad signaling, the main pathway in EMT, is initiated by transforming growth factor β1 (TGF-β1) binding to its type II receptor (TβRII). TβRII forms a heterodimer with the TGF-β type I receptor (TβRI); activated TβRI phosphorylates R-Smad (Smad2 and Smad3) at the distal C-terminal SXS motif, after which pSmad2/3 and Co-Smad (Smad4) form a transcription complex that transduces the signal to the nucleus. In the nucleus, the Smad complex directly or indirectly binds to various transcription factors, thus providing additional regulation of target genes that mediate EMT (23-25).

On the basis of our previous studies, the aim of the present study was to investigate the potential roles and molecular mechanisms of RUNX3 in ESCC metastasis and EMT.

Materials and methods

Ethics statement. The present study was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated with Shandong University. Written informed consent for use of the tissues and data analysis was obtained from every patient or their relatives.

Patients and specimens. In total, 102 ESCC tissues and 30 adjacent normal tissues (>5 cm from the margin of the tumor) were harvested from patients who underwent Ivor-Lewis esophagectomy with two-field lymphadenectomy at the Department of Thoracic Surgery, Provincial Hospital Affiliated to Shandong University from May 2017 to December 2018. All patients underwent esophagectomy with complete resection, and none received neoadjuvant radio-/chemotherapy. Postoperative staging was based on the eighth edition of the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification criteria published in 2017. Among the 102 ESCC patients, 45 had LNM, and 57 did not have LNM. The detailed clinical data for these patients are presented in Table I.

Cell culture and transfection. Five human ESCC cell lines (TE-1, Eca109, KYSE150, KYSE450, and EC9706) were purchased from the Cell Bank of the Shanghai Institute in China. All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). For activation of the TGF-β pathway, cells were treated with 10 ng/ml recombinant TGF-β1 (cat. no. AF-100-21C-10; PeproTech, Inc.). Cell culture plates were maintained at 37˚C in a humidified 5% CO_2 incubator. Human RUNX3 overexpression plasmids (NM-004350) were chemically synthesized and packaged into lentiviruses (Shanghai GeneChem Co., Ltd.). Puromycin was used at 5 μg/ml for 1 week to select stably transfected cells. Cells were labeled BC (without lentivirus transfection), vector (transfected with the control vector), and RUNX3 (transfected with the RUNX3 overexpression plasmid).

Immunohistochemical (IHC) analysis. IHC analysis was performed to detect the expression level of RUNX3 as well as the correlation between the expression of RUNX3 and EMT-related markers using the streptavidin-peroxidase (SP) method. Sections were stained with anti-RUNX3 (dilution 1:100; cat. no. ab40278), anti-N-cadherin (dilution 1:100; cat. no. ab76011), and anti-Snail (dilution 1:100; cat. no. ab180714; all from Abcam), and anti-E-cadherin (dilution 1:100; cat. no. 20874-1-AP; ProteinTech Group, Inc.) antibodies at 4˚C overnight. The primary antibodies were replaced with phosphate-buffered saline (PBS) as a negative control to rule out nonspecific binding. The secondary antibody and avidin-biotin peroxidase complex methods were performed according to the standard protocols provided by the manufacturer (ZSGB Biotech Beijing; OriGene technologies, Inc.). All sections were evaluated by two pathologists who were blinded to the clinical data.

Western blot analysis. Protein was extracted from tissue samples and tumor cells using radioimmunoprecipitation (RIPA) lysis buffer (Beyotime Institute of Biotechnology), and the concentration was determined using a bicinchoninic acid (BCA) kit. Equal amounts of protein (30 μg) were separated by 8% or 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Briefly, 5% nonfat dry milk was used to block nonspecific binding. The membranes were incubated overnight at 4˚C with primary antibodies. Following three washes, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:5,000; goat anti-mouse cat. no. ZB-2305 and goat anti-rabbit cat. no. ZB-2301; both from ZSGB Biotech) for 1 h at room temperature. Finally, the protein levels were quantified using an enhanced chemiluminescence (ECL) detection system (Amersham imager 600; General Electric). The antibodies used in the present study were as follows: anti-RUNX3 (dilution 1:500; cat. no. ab40278), anti-N-cadherin (dilution 1:1,000; cat. no. ab76011), and anti-Snail (dilution 1:1,000;

Table I. Correlations between RUNX3 expression and clinicopathological features of 102 ESCC patients.

Parameters	Cases (n=102)	RUNX3 expression (%) Positive (n=23)	Negative (n=79)	P-value
Sex				0.475
Male	45	12 (26.7)	33 (73.3)	
Female	57	11 (19.3)	46 (80.7)	
Age (years)				0.810
≤50	41	10 (24.4)	31 (75.6)	
>50	61	13 (21.3)	48 (78.7)	
Tumor size (cm)				0.347
<3	44	12 (27.3)	32 (72.7)	
≥3	58	11 (19.0)	47 (81.0)	
T status				**0.027**
T1-2	63	19 (30.2)	44 (69.8)	
T3-4	39	4 (10.3)	35 (89.7)	
Differentiation degree				0.158
Low	54	9 (16.7)	45 (83.3)	
Mid-high	48	14 (29.2)	34 (70.8)	
LNM				**0.017**
No	57	18 (31.6)	39 (68.4)	
Yes	45	5 (11.1)	40 (88.9)	

Bold values indicate P<0.05. ESCC, esophageal squamous cell carcinoma; RUNX3, runt-related transcription factor 3; LNM, lymph node metastasis.

cat. no. ab180714; all from Abcam), anti-matrix metallopeptidase 9 (MMP-9) (dilution 1:1,000; cat. no. 10375-2-AP), anti-GAPDH, (dilution 1:1,000; cat. no. 10494-1-AP), and anti-E-cadherin (dilution 1:1,000; cat. no. 20874-1-AP; ProteinTech Group, Inc.), anti-Smad2 (dilution 1:1,000; cat. no. ab40855) and anti-Smad3 (dilution 1:1,000; cat. no. ab40854; both from Abcam), anti-pSmad2$^{ser465/467}$ (dilution 1:1,000; cat. no. 3108T; Cell Signaling Technology, Inc.), and anti-pSmad3$^{s423+s425}$ antibodies (dilution 1:1,000; cat. no. ab52903; Abcam).

RNA extraction and real-time PCR. Total RNA was isolated from cultured cells using RNAiso Plus (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. All RNA samples were treated with RNase-free DNase (Takara Biotechnology Co., Ltd.) and stored at -80˚C. One microgram of RNA was subjected to reverse transcription using a reverse transcriptase reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.) to obtain cDNA. GAPDH was used for normalization. Relative transcript quantities were calculated using the $2^{-\Delta\Delta Cq}$ method (26). The thermocycling conditions were as follows: 95˚C for 10 min and then 40 cycles of 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. The experiments were performed in triplicate. The primer sequences used were as follows: RUNX3, 5'-TCAACGACC TTCGCTTCGTG-3' (forward primer) and 5'-ACCTTGATG GCTCGGTGGTA-3' (reverse primer); GAPDH, 5'-GCACCG TCAAGGCTGAGAAC-3' (forward primer) and 5'-TGGTGA AGACGCCAGTGGA-3' (reverse primer).

Transwell assay. For both the migration and invasion assays, cells were pre-incubated in FBS-free medium for 24 h. Uncoated Transwells were used for the migration assay, whereas 40 μl of Matrigel (1:4; BD Biosciences) was used to pre-coat the upper surface of the Transwells in the invasion assay. Next, $1.5x10^5$ serum-starved cells were seeded in the upper Transwells chamber in a 24-well plate (8 μm pore size polycarbonate membrane; EMD Millipore) with 200 μl of FBS-free medium; 600 μl of medium with 15% FBS was added to the lower chamber. The cells were incubated for 24 h for the migration assay and for 48 h for the invasion assay. Finally, the cells migrating to the lower surface of the membrane were fixed with 4% paraformaldehyde for 20 min and stained with hematoxylin for 5 min at room temperature; cells from three randomly selected fields were counted. The data are presented as the mean ± SD.

Wound-healing assay. Cells were plated into 6-well plates and cultured in RPMI-1640 with 10% FBS until they reached 90% confluence. The confluent cell monolayers were scratched using a 10 μl pipette tip and incubated in culture medium with 1% FBS. Images were captured using a LEICA DMi8 inverted microscope every 12 h (magnification x100).

Immunofluorescence analysis. Following fixation in 4% formaldehyde for 20 min, permeabilization with 0.5% Triton X-100 for 20 min and blocking with 10% normal goat serum for 1 h, cells were incubated with rabbit anti-pSmad2 (dilution 1:200) and rabbit anti-pSmad3 (dilution 1:100) antibodies at 4˚C overnight. The cells were then incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (dilution 1:100) for 1 h at 37˚C, and the nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Images were captured using an Olympus BX43 fluorescence microscope (magnification x200).

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used for all statistical analyses. Quantitative data was expressed as the mean ± standard deviation (SD). The χ^2 test was used to analyze the association between RUNX3 expression and clinicopathological variables. A paired Student's t-test was used to compare RUNX3 in paired ESCC tumor tissues and adjacent normal tissues, and for all other comparisons between two groups, unpaired Student's t-tests were performed. The significance of differences among more than two groups was calculated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Pearson's correlation analysis was used to analyze correlations between RUNX3 and EMT-related marker expression. A statistically significant difference was defined as a P-value <0.05.

Results

RUNX3 expression is decreased in ESCC. The expression profile of RUNX3 in 30 pairs of ESCC tumor tissues and adjacent normal tissues by was first assessed with IHC analysis. Significant staining was readily observed in the nuclei of the

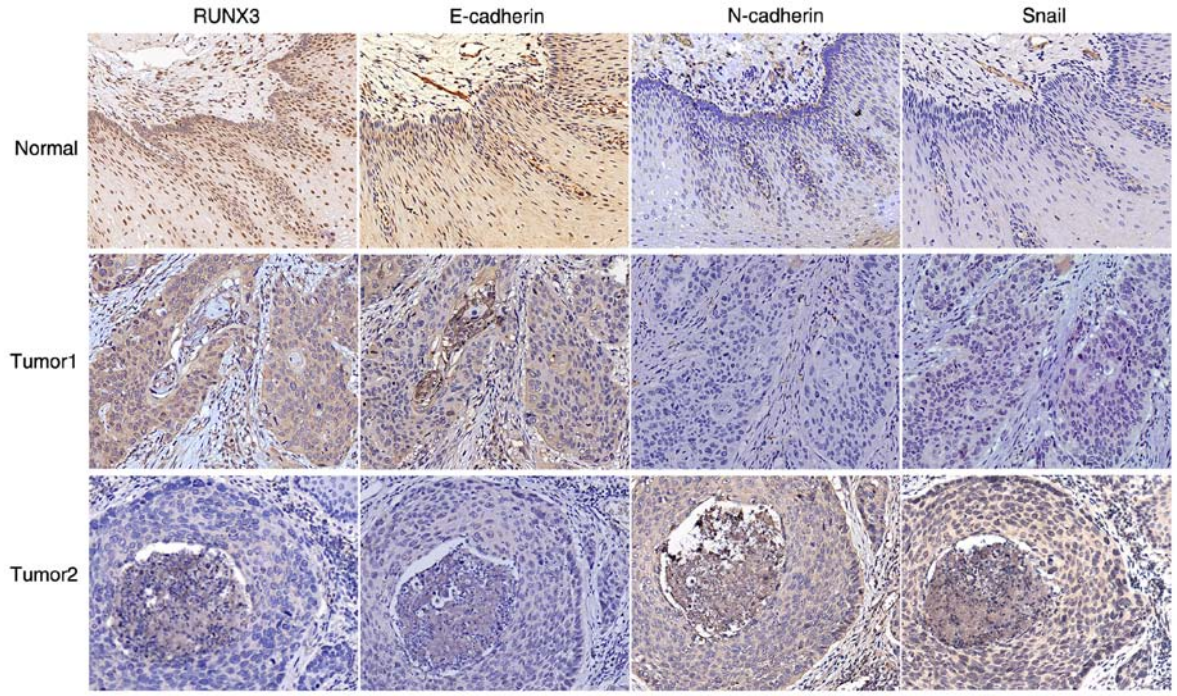

Figure 1. IHC staining of RUNX3, E-cadherin, N-cadherin, and Snail in esophageal ESCC and adjacent normal tissues (original magnification x400). Tumor 1, a tumor tissue without LNM; Tumor 2, a tumor with LNM. IHC, immunohistochemical; ESCC, esophageal squamous cell carcinoma; LNM, lymph node metastasis.

noncancerous tissue; weak immunostaining was observed in tumor cells (Fig. 1). Furthermore, RUNX3 expression levels were verified through western blot analysis and it was revealed that RUNX3 expression in tumor tissues was significantly lower than that in adjacent normal tissues (Fig. 2A and B; RUNX3/GAPDH: 0.50±0.20 vs. 0.83±0.16; P<0.001).

RUNX3 expression is associated with T status and LNM. Tissues were considered RUNX3-negative when nuclear staining was present in <5% of the cells, with or without cytoplasmic staining. According to these criteria, all 102 ESCC patient samples were divided into two groups: 23 cases (22.55%) were categorized as the positive expression group, and 79 cases (77.45%) were categorized as the negative expression group. The chi-square test was next used to investigate the relationship between RUNX3 expression and clinicopathological parameters (including age, sex, tumor size, T status, differentiation degree, and LNM). As revealed in Table I, negative RUNX3 expression was more common in T3-4 than in T1-2 cases (89.7% vs. 69.8%, respectively; P=0.027) and more prevalent in node-positive than in node-negative cases (88.9% vs. 68.4%, respectively; P=0.017). Thus, it was surmised that decreased RUNX3 expression may play a vital role in ESCC metastasis. No significant differences were revealed between RUNX3 expression and other clinicopathological parameters.

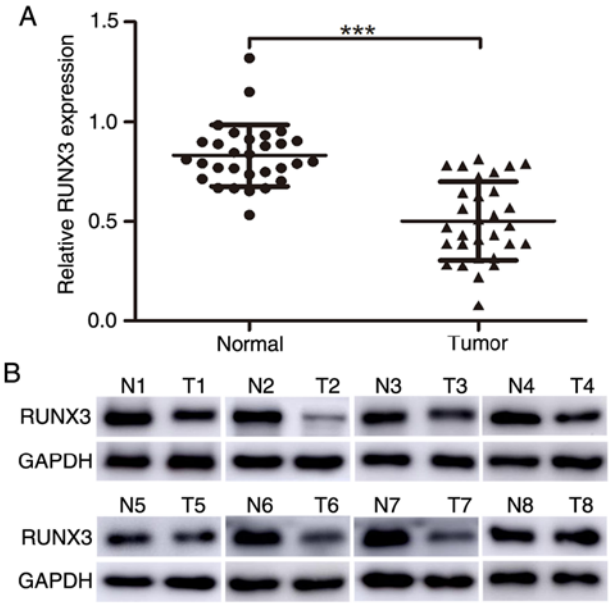

Figure 2. Western blot analysis of RUNX3 expression in ESCC tumors and adjacent normal tissues. (A) Quantitative analysis of RUNX3 protein expression in 30 pairs of tissue specimens normalized to GAPDH. The results are expressed as the mean ± standard deviation. ***P<0.001. (B) RUNX3 and GAPDH levels in 8 pairs of tissue specimens. N, adjacent normal tissues; T, tumor tissues. RUNX3, runt-related transcription factor 3; ESCC, esophageal squamous cell carcinoma.

RUNX3 expression is negatively correlated to Snail and N-cadherin expression and positively correlated to E-cadherin expression in ESCC tissues. EMT has been reported to contribute to tumor invasion, migration and metastasis in various cancers (27). To elucidate the mechanisms of RUNX3 in ESCC LNM, the correlation between RUNX3 and EMT-related marker expression in LNM tissues and non-LNM tissues (Fig. 1) was evaluated. IHC results revealed RUNX3 expression to be negatively correlated to the expression of the mesenchymal marker N-cadherin (r=-0.429; P<0.01) and transcription factor Snail (r=-0.364; P<0.01) and

Table II. Correlations between expressions of RUNX3 and EMT related markers in 102 ESCC patients.

	E-cadherin		N-cadherin		Snail	
Parameters	Positive	Negative	Positive	Negative	Positive	Negative
RUNX3						
Positive	17	6	7	16	11	12
Negative	10	69	62	17	67	12
r	0.580		-0.429		-0.364	
P-value	<0.01		<0.01		<0.01	

RUNX3, runt-related transcription factor 3; EMT, epithelial-mesenchymal transition; ESCC, esophageal squamous cell carcinoma.

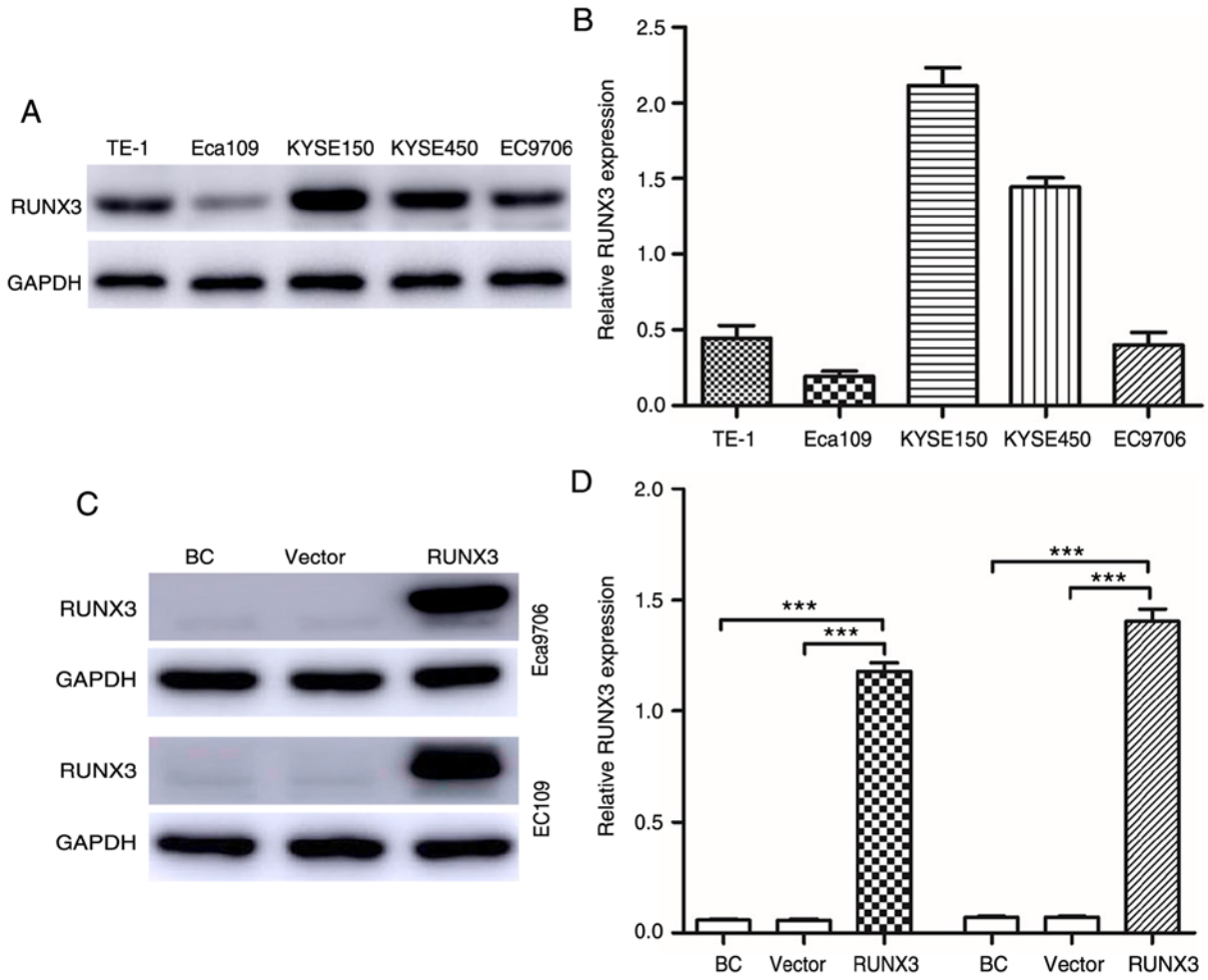

Figure 3. Cell selection and lentivirus transfection of ESCC cells. Relative expression of the RUNX3 (A) protein and (B) mRNA in five ESCC cell lines. Establishment and selection of notable and stably RUNX3-overexpressing Eca109 and EC9706 cells. (C) Western blot and (D) RT-PCR analyses were performed to detect RUNX3 protein and mRNA levels in Eca109 and EC9706 cells. The results are expressed as the mean ± standard deviation. ***P<0.001. BC, blank control group; Vector, vector group; RUNX3, RUNX3 group. ESCC, esophageal squamous cell carcinoma; RUNX3, runt-related transcription factor 3.

positively correlated to the expression of the epithelial marker E-cadherin (r=0.580; P<0.01) in ESCC tissues (Table II). These results indicated that RUNX3 expression was associated with ESCC EMT.

Cell transfection. The expression levels of RUNX3 in five ESCC cell lines were detected via qRT-PCR and western blot analyses. According to the results, Eca109 and EC9706 cells exhibited the lowest expression of RUNX3 at both the protein (Fig. 3A) and mRNA (Fig. 3B) levels. Thus, Eca109 and EC9706 cells were used to analyze the upregulation of RUNX3 expression. Compared with the blank control and vector groups, the RUNX3 group exhibited significantly upregulated RUNX3 expression, although there was no difference in RUNX3 expression between the blank control and vector groups (Fig. 3C and D).

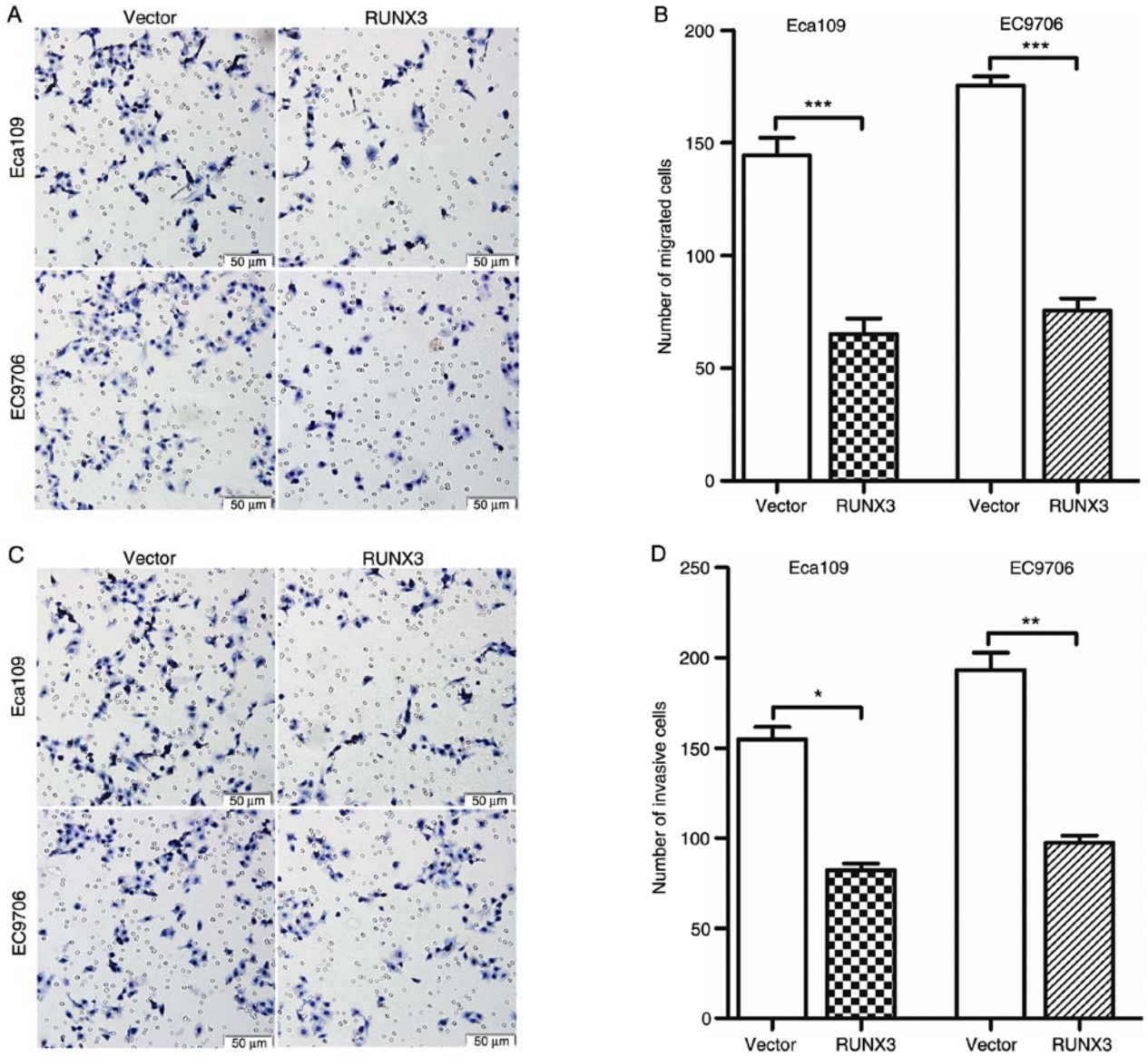

Figure 4. Upregulation of RUNX3 decreases the invasion and migration abilities of ESCC cells and decreases MMP-9 expression. (A) Representative images of cell migration (original magnification x200). (B) Representative percentages of cell migration. (C) Representative images of cell invasion (original magnification x200). (D) Representative percentages of cell invasion. Vector, vector group; RUNX3, RUNX3 group. *P<0.05, **P<0.01 and ***P<0.001.

Upregulation of RUNX3 significantly decreases the invasion and migration abilities of ESCC cells as well as MMP-9 expression. Since RUNX3 expression was negatively correlated with T status and LNM in ESCC patients, the effect of RUNX3 on cell migration and invasion in ESCC cells was further investigated using wound-healing and Transwell assays. In the Transwell assays, the number of cells in the RUNX3 group that traversed the membrane was significantly decreased compared with that in the vector group (Fig. 4A and B; P<0.01). Moreover, the number of cells that invaded Matrigel was also significantly attenuated in the RUNX3 group (Fig. 4C and D; P<0.01). The data from the wound-healing assay further supported this finding, as upregulating expression of RUNX3 suppressed cell migration compared with the vector group (Fig. 4E). Furthermore, restoration of RUNX3 expression led to a significant decrease in the expression of MMP-9 (Fig. 4F and G; P<0.01), which can promote invasion and LNM through ECM degradation (28).

Upregulation of RUNX3 may reverse EMT and decrease Smad2/3 phosphorylation in ESCC cells. To evaluate the mechanism of RUNX3 in ESCC metastasis, the levels of EMT markers (E-cadherin, N-cadherin and Snail) were detected by western blotting and it was revealed that N-cadherin and Snail expression levels were downregulated in RUNX3-overexpressing cells but that E-cadherin expression was significantly upregulated (Fig. 5A). TGF-β/Smad-induced EMT has been suggested to be associated with the development and progression of ESCC. To further assess whether RUNX3 reverses ESCC EMT through the TGF-β/Smad pathway, the levels of Smad2, Smad3 and their phosphorylated versions (pSmad2, pSmad3) were evaluated. The results of the western blot analysis revealed that levels of both pSmad2 and pSmad3 were decreased in the RUNX3 group compared to the vector group; in contrast, the levels of Smad2 and Smad3 were unaltered (Fig. 5B). Immunofluorescence assays were also used to validate the effect of RUNX3 overexpression on pSmad2 and pSmad3 staining, and as revealed in

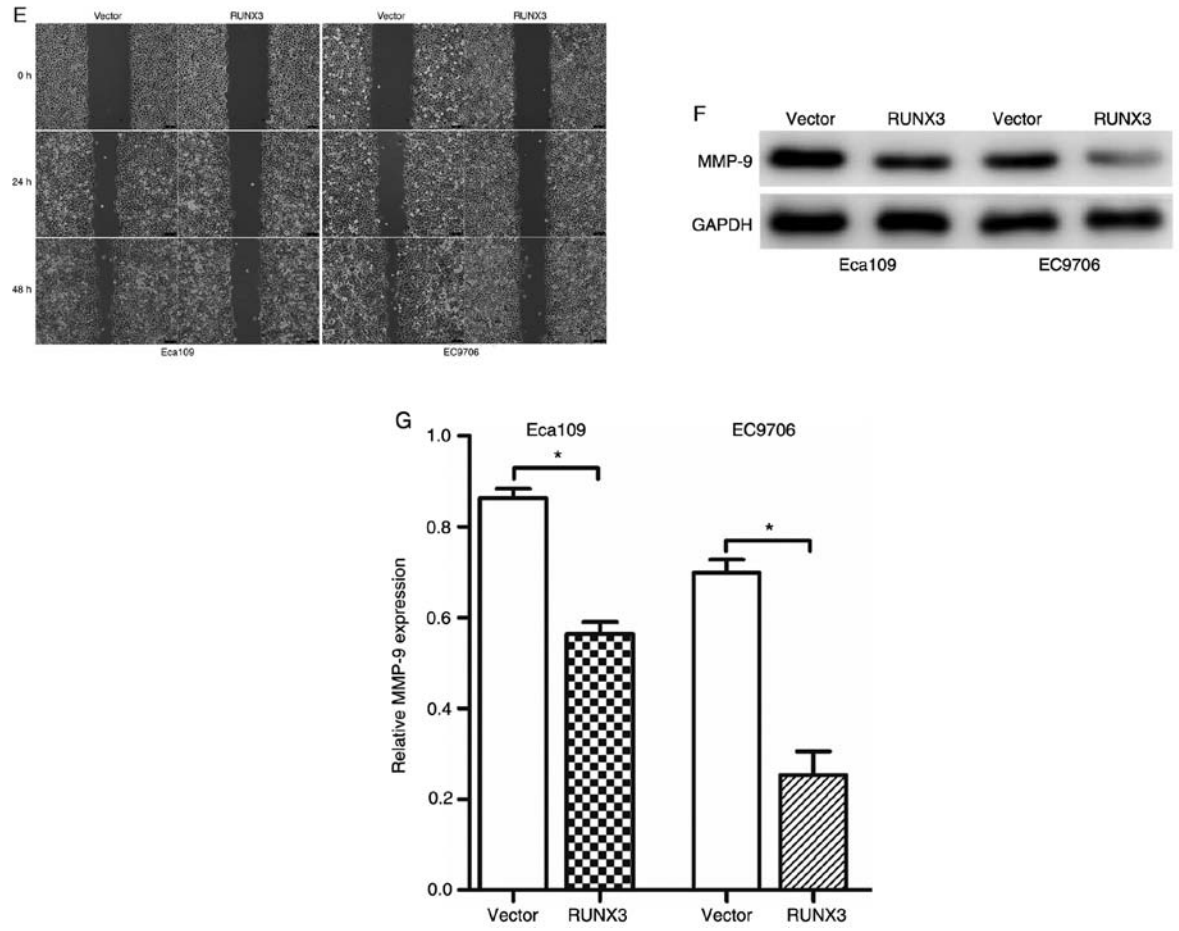

Figure 4. Continued. Upregulation of RUNX3 decreases the invasion and migration abilities of ESCC cells and decreases MMP-9 expression. (E) Representative images of the wound-healing assay performed with Eca109 and EC9706 cells; the scale bars represent 100 μm. (F and G) Effect of RUNX3 on MMP-9 expression in Eca109 and EC9706 cells. Vector, vector group; RUNX3, RUNX3 group. *P<0.05, **P<0.01 and ***P<0.001. RUNX3, runt-related transcription factor 3; ESCC, esophageal squamous cell carcinoma; matrix metallopeptidase 9.

Fig. 5C, pSmad2 and pSmad3 staining was markedly decreased in the RUNX3 group.

RUNX3-overexpressing cells display diminished responsiveness to TGF-β1-induced EMT. A previous study indicated that Runx3-null gastric epithelial lines are unexpectedly sensitive to TGF-β1-induced EMT (29). The sensitivity of RUNX3 to TGF-β1-induced EMT was next investigated and it was revealed that the levels of pSmad2/3 peaked at 1 h after TGF-β treatment (Fig. 6A and B). Thus, the temporal pattern of Smad2/3 phosphorylation after treatment with 10 ng/ml TGF-β1 for 1 h was investigated. Based on the results, the levels of pSmad2/3 significantly increased in the vector group, whereas no significant changes were evident in RUNX3-overexpressing cells (Fig. 7A). The expression of EMT-related markers after 72 h of stimulation with TGF-β1 was also assessed. TGF-β1-induced increases in N-cadherin and Snail and decreases in E-cadherin levels were significantly greater in the vector control cells than in the RUNX3-overexpressing cells (Fig. 7B). One hallmark of EMT is phenotypic change in epithelial cell morphology. Eca109 and EC9706 cells displayed an elongated fibroblast-like morphology and reduced cell-cell contact after 72 h of TGF-β1 stimulation, whereas noticeable morphological changes and cell-cell contact were not detected in RUNX3-overexpressing cells (Fig. 7C and D).

Discussion

It is widely accepted that surgery is the standard treatment for patients with localized ESCC (30). Although surgical treatment can cure 90% of patients with early ESCC, the prognosis is suboptimal for advanced-stage ESCC due to a lack of early diagnosis methods, local relapse, distant metastasis and resistance to traditional treatments such as chemotherapy and radiotherapy. The esophagus has a unique histological structure involving a rich lymphatic-capillary network in the submucosa, which contributes to variable lymphatic spread and skip metastasis (3,31). In fact, Li *et al* reported that approximately 33.1% of ESCC patients experience LNM when the submucosa (T1b) is invaded and that 4.3% of ESCC patients still experience LNM even when the tumor is confined to the mucosa (T1a) (31). We previously demonstrated that RUNX3 expression was significantly related to LNM and that RUNX3 inactivation was predictive of poor survival (32). In the present study, we focused on exploring the underlying mechanisms of RUNX3-mediated EMT and metastasis.

According to the present results, RUNX3 expression was markedly lower in tumor tissues than in adjacent normal tissues, and this decreased expression of RUNX3 was correlated with T status and LNM in ESCC patients. In an attempt to validate the biological function of RUNX3 in ESCC

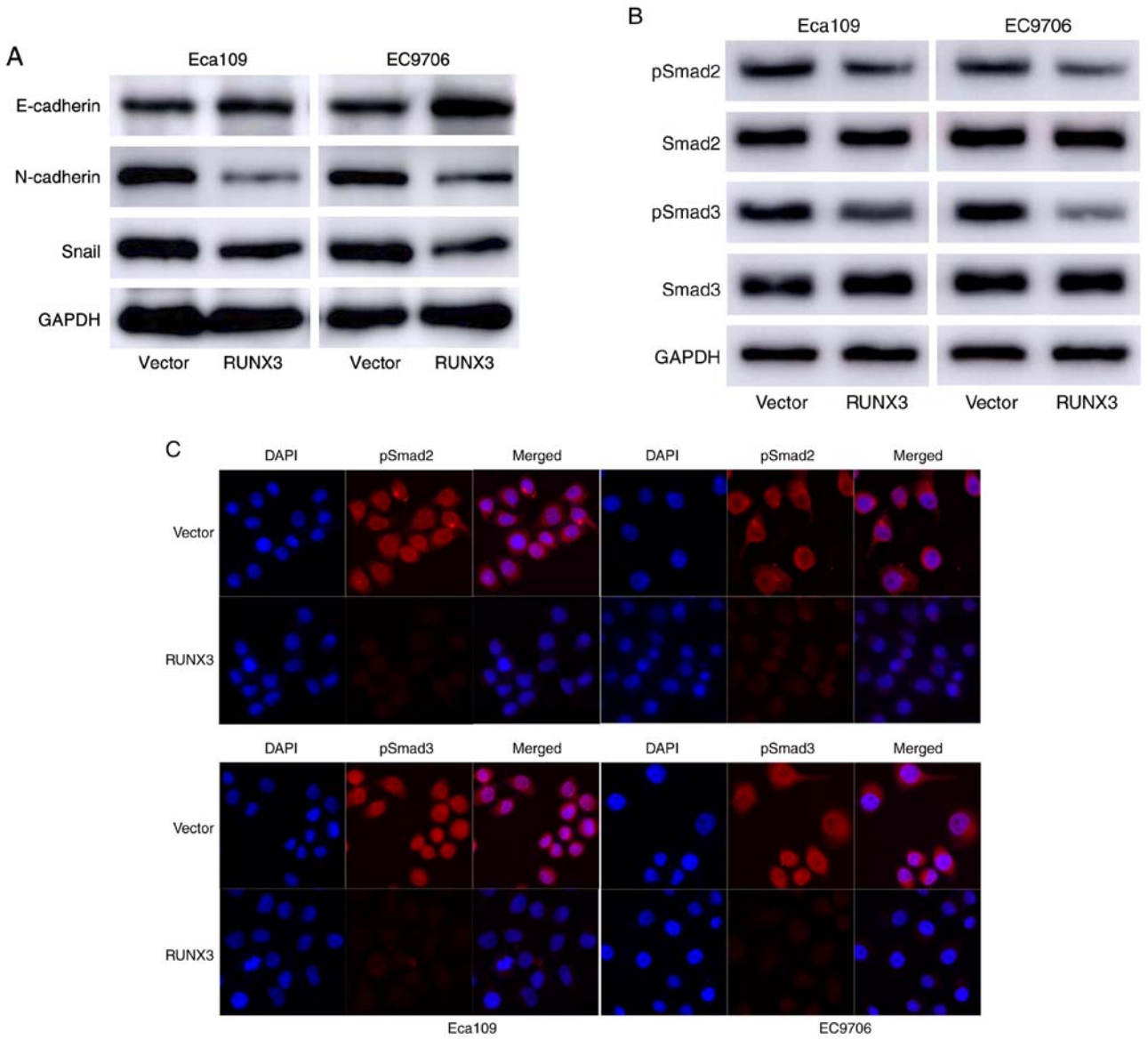

Figure 5. Upregulation of RUNX3 reverses EMT and decreases Smad2/3 phosphorylation in ESCC cells. (A) Western blot results revealed that expression of E-cadherin was increased and that expression of N-cadherin and Snail was decreased in the RUNX3 group compared with the vector group. (B) The expression levels of pSmad2/3 were decreased with RUNX3 overexpression. (C) Eca109 and EC9706 cells were subjected to immunofluorescence staining with an anti-pSmad2 or -pSmad3 antibody. Vector, vector group; RUNX3, RUNX3 group. RUNX3, runt-related transcription factor 3; EMT, epithelial-mesenchymal transition; ESCC, esophageal squamous cell carcinoma.

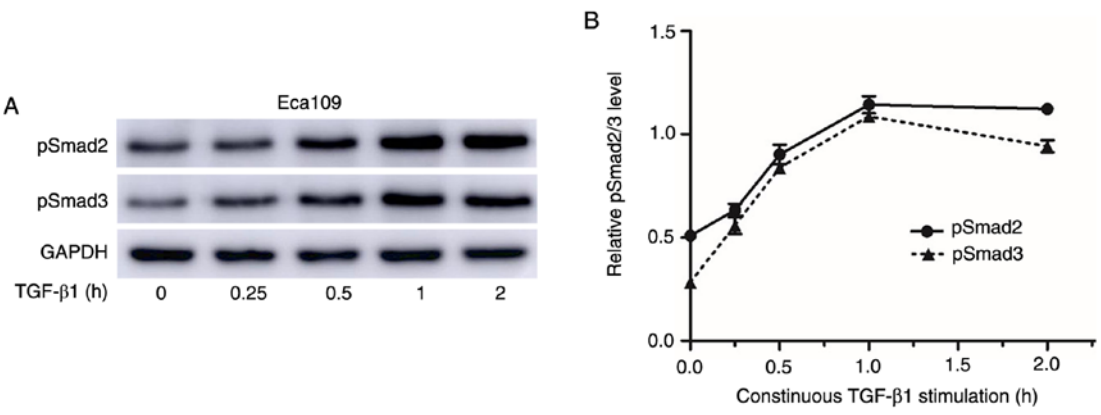

Figure 6. TGF-β1 activates Smad2/3 phosphorylation. (A) ESCC cells were treated with 10 ng/ml TGFβ1 for 0, 0.25, 0.5, 1, and 2 h, and western blotting was performed to analyze Smad2/3 phosphorylation. (B) Relative pSmad2/3 levels were quantified using Graph Prism6 software. TGF-β1 causes time-dependent phosphorylation of Smad2/3, and the levels of pSmad2/3 peaked at 1 h after TGF-β1 treatment. TGF-β1, transforming growth factor β1; ESCC, esophageal squamous cell carcinoma; pSmad2/3, phosphorylated Smad2/3.

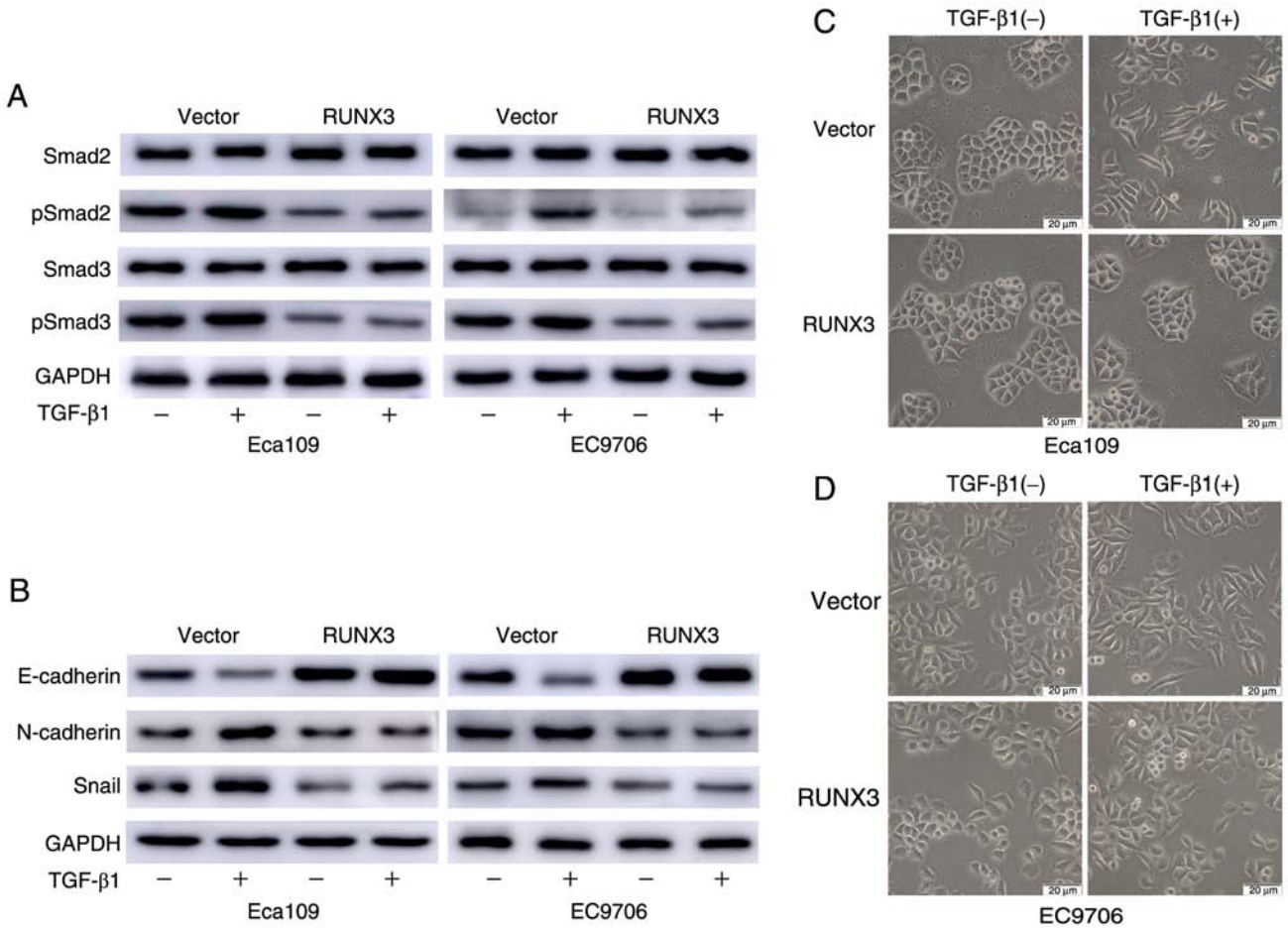

Figure 7. RUNX3-overexpressing cells exhibit reduced responsiveness to TGF-β1-induced EMT. (A) Western blotting results revealed that phosphorylated Smad2/3 (pSmad2/3) was significantly increased in the vector group compared with the RUNX3-overexpressing group after 1 h of TGF-β1 stimulation. (B) N-cadherin and Snail were significantly increased and E-cadherin was decreased in the vector group compared with the RUNX3-overexpressing group after 72 h of TGF-β1 stimulation. (C and D) Representative images of morphological changes in RUNX3-overexpressing and control cells with or without 72 h of TGF-β1 stimulation. Vector, vector group; RUNX3, RUNX3 group. RUNX3, runt-related transcription factor 3; TGF-β1, transforming growth factor β1; EMT, epithelial-mesenchymal transition.

metastasis, Eca109 and EC9706 cells were transfected with a RUNX3-expressing lentivirus and it was revealed that restoration of RUNX3 expression attenuated invasion and migration abilities. Moreover, RUNX3 overexpression led to a significant decrease MMP-9 expression. All of these results indicated that RUNX3 markedly inhibited ESCC cell metastasis. Since EMT plays an important role in tumor invasion and metastasis, it was surmised that RUNX3 may prevent ESCC cell metastasis by modulating EMT. The expression levels of E-cadherin were determined to be increased and those of Snail and N-cadherin to be decreased in the RUNX3-overexpressing group compared with the vector control group. IHC results also revealed that RUNX3 expression was positively correlated with E-cadherin expression and inversely with Snail and N-cadherin expression in ESCC tissues, which strongly supports our hypothesis.

Recently, an accumulating number of studies have demonstrated that EMT can be induced by numerous molecules, such as inflammatory cytokines, growth factors and numerous transcription factors (20). Among these factors, TGF-β is a key driver of EMT (19). Cumulative research has revealed that TGF-β signaling is tightly controlled by the phosphorylation of R-Smads and that dephosphorylation of R-Smads disrupts signal relay (33). Lin *et al* reported that PPM1A dephosphorylates TGF-β-activated Smad2/3, dissociates the Smad complex, and promotes the nuclear export of Smad2/3 (34). RUNX3, as a critical downstream effector in the TGF-β signaling pathway, physically interacts with R-Smad through its C-terminal region (35-38). In this study, it was revealed that RUNX3 overexpression resulted in significant dephosphorylation of pSmad2/3, indicating that the effect of RUNX3 on reversing EMT may be attributable, at least in part, to TGF-β/Smad signaling.

A previous study has indicated that the gastric mucosa of Runx3-null mice is resistant to TGF-β1-induced growth suppression (8). However, in another study, Voon *et al* reported that Runx3-null gastric epithelial lines were unexpectedly sensitive to TGF-β1-induced EMT (29). Therefore, the sensitivity of RUNX3 to TGF-β1-induced EMT was further assessed. Upon TGF-β1 stimulation, significantly decreased levels of E-cadherin and increased levels of N-cadherin, Snail and pSmad2/3 were observed in the vector group. However, no effect on these proteins was observed in the RUNX3 group. Morphological studies also revealed that TGF-β1 treatment induced phenotypic changes, resulting in the cells in the vector group adopting a more spindle-like morphology than the cells in the RUNX3 group, which indi-

cates that RUNX3-overexpressing ESCC cells were resistant to TGF-β1-induced EMT.

RUNX3 is thought to exist in a basal, inactive state in the cytoplasm and cannot act as a transcription factor (9). Increasing evidence indicates that RUNX3 can be reactivated and is therefore considered to be a good drug target (39). Our previous studies demonstrated that reactivation of RUNX3 by 5-azacytidine (an anticancer drug) inhibited the malignant behavior of ESCC cells (40). Moreover, heterologous RUNX3 expression was able to reverse cisplatin resistance in ESCC cell lines (41). In the present study, evidence is provided that restoration of RUNX3 expression decreased the invasion and migration of ESCC cells by inhibiting EMT. Collectively, the present results revealed that novel drugs targeting RUNX3 are a promising treatment strategy for ESCC.

There are several limitations to the present study. Firstly, the number of samples was relatively small, which may affect the reliability of our findings. Therefore, larger sample sizes and multicenter randomized studies are required. Secondly, the cells were not serum-starved in the wound-healing assay. In our future studies, we will attempt to knock down RUNX3 and develop a lung metastasis model to further validate our results *in vivo*. Immunoprecipitation assays will also be performed to verify the binding of RUNX3 to R-Smad.

In summary, in the present study it was revealed that RUNX3 inhibited EMT by abrogating TGF-β1-mediated Smad2/3 phosphorylation and decreasing the expression of the transcription factor Snail, thereby inhibiting the invasion and migration abilities of ESCC cells. This study is the first to elucidate the detailed mechanisms of RUNX3-mediated regulation of EMT and metastasis in ESCC. The findings herein support the hypothesis that targeted therapies for RUNX3 may serve as complementary treatment approaches to control postoperative LNM and improve the survival of ESCC patients.

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Availability of data and materials

The data used during the study are available from the corresponding author upon reasonable request.

Authors' contributions

YT and QS collected the samples and acquired the experimental data. WJ, GC and MS performed the analysis of the data. ZX wrote the manuscript. ZX, YT, YJ and BS performed the experiments. ZW and XGZ designed, supervised and funded the experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University (protocol no. 2017550). Written informed consent was obtained from each patient or their relatives for use of the tissues and data analysis.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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