International Journal of Biochemistry and Cell Biology 114 (2019) 105557

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Homeobox C8 is a transcriptional repressor of E-cadherin gene expression in non-small cell lung cancer

Jie Zhang, Mengqi Yang, Dongjia Li, Siqi Zhu, Jin Zou, Shanshan Xu, Yun Wang, Jialu Shi, Yong Li*

School of Life Sciences, Anhui University, Hefei, Anhui Province, PR China

ARTICLE INFO

Keywords:
E-cadherin
HOXC8
Epithelial-mesenchymal transition (EMT)
Non-small cell lung cancer (NSCLC)

ABSTRACT

Loss of E-cadherin expression is a hallmark of epithelial-mesenchymal transition (EMT) in tumor progression. Because previous findings suggested that homeobox C8 (HOXC8) promotes EMT in non-small-cell lung cancer (NSCLC), we investigated whether E-cadherin is a target of HOXC8 protein. In this study, we report that HOXC8 binds to the E-cadherin promoter and acts as a transcriptional repressor to regulate E-cadherin transcription in NSCLC. We further show that loss of E-cadherin leads to an increase in anchorage-independent growth and migration of NSCLC cells, and the inhibitory effects mediated by HOXC8 knockdown can be largely rescued by reduction of E-cadherin expression, suggesting that the HOXC8-E-cadherin pathway is involved in lung cancer progression. Moreover, analysis of E-cadherin and HOXC8 expression indicates that expression of HOXC8 is strongly correlated with loss of E-cadherin expression, and high HOXC8 / low E-cadherin expression is significantly correlated with poor survival for lung cancer patients. Taken together, these data indicate that E-cadherin is a target gene of HOXC8 and that the loss of E-cadherin promotes the growth and migration of NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide and has two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Siegel et al., 2014). NSCLC, which includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma, is the predominant histological type accounting for more than 80% of cases(Travis, 2002). Due to the tumor migration and invasion, the survival rates for NSCLC patients remain poor(Chambers et al., 2002). Therefore, it is important to elucidate the molecular mechanism governing the regulation of NSCLC aggressiveness.

E-cadherin (CDH1) is a transmembrane protein mediating calcium-dependent cell-cell adhesion, and downregulation of E-cadherin, which can be caused by loss of heterozygosity (LOH) or transcriptional silencing, is a hallmark of epithelial-mesenchymal transition (EMT) (van Roy, 2014). EMT is a key step in the progression of tumors toward migration and metastasis, in which cells lose epithelial characteristics and acquire a migratory, mesenchymal phenotype(Thiery, 2002). E-cadherin has been shown to be transcriptionally repressed through the action of EMT transcription factors (TFs) including Snail, Twist, and Zeb (Thiery, 2002). It has been well documented that repression of E-cadherin is involved in EMT which leads to migration and metastasis of cancer cells including NSCLC (Schmalhofer et al., 2009; Tsoukalas et al., 2017; Wang et al., 2017).

Homeobox C8 (HOXC8) belongs to HOX family which contains 39 members in mammals, and HOX proteins participate in a number of physiological and pathological processes including embryogenesis and tumorigenesis (Krumlauf, 1994; Shah and Sukumar, 2010). It has been reported that HOXC8 is deregulated in various types of cancer, including breast, cervical, prostate and ovarian cancers (Alami et al., 1999; Axlund et al., 2010; Li et al., 2010; Lu et al., 2016), and functions as a transcription factor to regulate a number of gene transcription. Recently we reported that HOXC8 was significantly upregulated in clinical specimens of NSCLC compared to normal tissues, and upregulation of HOXC8 played an important role in tumorigenicity of NSCLC cell lines A549 and NCI-H460 (Liu et al., 2018).

In this study, we further explore the underlying mechanism of HOXC8 on tumorigenicity of NSCLC cells. Using chromatin

Abbreviations: EMT, epithelial-mesenchymal transition; HOXC8, homeobox C8; CDH1, E-cadherin; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinoma; LOH, loss of heterozygosity; TGFβ1, transforming growth factor beta 1; ChIP, chromatin immonuprecipitation; TSS, transcription start site; qRT-PCR, quantificational real-time; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

* Corresponding author at: School of Life Sciences, Anhui University, Hefei, Anhui Province, 230601, PR China.
E-mail address: liyongahu@163.com (Y. Li).

https://doi.org/10.1016/j.biocel.2019.06.005
Received 8 March 2019; Received in revised form 11 June 2019; Accepted 13 June 2019
Available online 13 June 2019

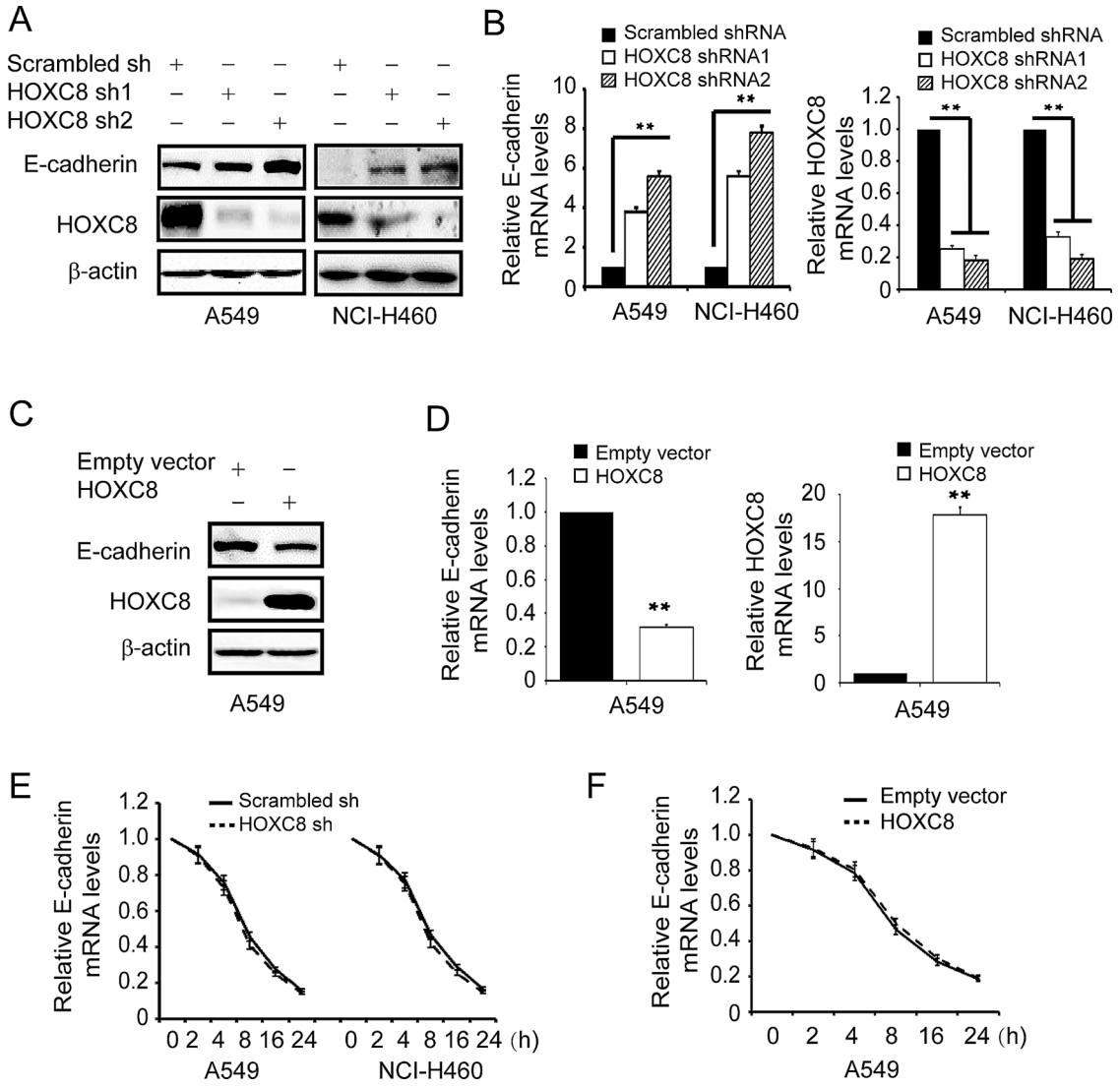

Fig. 1. HOXC8 negatively regulates E-cadherin transcription in NSCLC cells. (A) A549 or NCI-H460 cells were lentivirally transduced with scrambled or HOXC8 shRNAs, and cell lysates were subjected to Western blot to detect E-cadherin, HOXC8, and β-actin. (B) A549 or NCI-H460 cells were lentivirally transduced with scrambled or HOXC8 shRNAs, and total RNAs were subjected to qRT-PCR to measure the levels of E-cadherin mRNA; 18S rRNA was used for standardization. Columns, means; bars, SEM; n = 3; ** $P < 0.01$. (C) A549 cells were lentivirally transduced with empty or HOXC8 expression vectors, cell lysates were subjected to Western blot to detect E-cadherin, HOXC8, and β-actin. (D) A549 cells were lentivirally transduced with empty or HOXC8 expression vectors, and then total RNAs were subjected to qRT-PCR to measure the levels of E-cadherin mRNA; 18S rRNA was used for standardization. Columns, means; bars, SEM; n = 3; ** $P < 0.01$. (E) A549 or NCI-H460 cells were lentivirally transduced with scrambled or HOXC8 shRNA, and then treated with 2μg/ml actinomycin for different time. E-cadherin mRNA levels were analyzed by qRT-PCR, 18S rRNA was used for standardization. Experiments were done in triplicates. (F)A549 cells were lentivirally transduced with empty or HOXC8 expression vectors, and then treated with 2μg/ml actinomycin for different time. E-cadherin mRNA levels were analyzed by qRT-PCR. Experiments were done in triplicates using 18S rRNA for normalization.

immunoprecipitation (ChIP) and luciferase assays, we showed that HOXC8 bound directly to the promoter of E-cadherin and functioned as a transcription repressor to inhibit the transcription of E-cadherin in A549 or NCI-H460 cells. We found that HOXC8 promoted cell viability, anchorage-independent growth and migration of NSCLC via inhibiting E-cadherin expression. Moreover, Kaplan-Meier analysis indicated that high expression of HOXC8 was statistically associated with poor progression free survival for lung cancer patients, whereas low expression of E-cadherin correlated with shorter survival of NSCLC. These data indicated that HOXC8 promoted cell growth and migration of NSCLC at least partly by transcriptionally inhibiting the expression of E-cadherin.

2. Materials and methods

2.1. Cells and materials

The human non-small-cell lung cancer A549 and NCI-H460 cell lines and normal lung epithelial BEAS-2B cell line were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). Cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Thermo Scientific Shanghai, China) at 37 ˚C in a 5% CO_2 atmosphere. The cell lines have been authenticated and tested by the Cell Bank. For verification, we performed mycoplasma tests in our laboratory, and the cell behavior and morphology were consistent with the descriptions in the Cell Bank. Anti-HOXC8 antibody (15448-1-AP; titer, 1:1000) was purchased from ProteinTech Group (Wuhan, China), anti-E-cadherin antibody (24E10; titer, 1:1000) was obtained from Cell Signaling

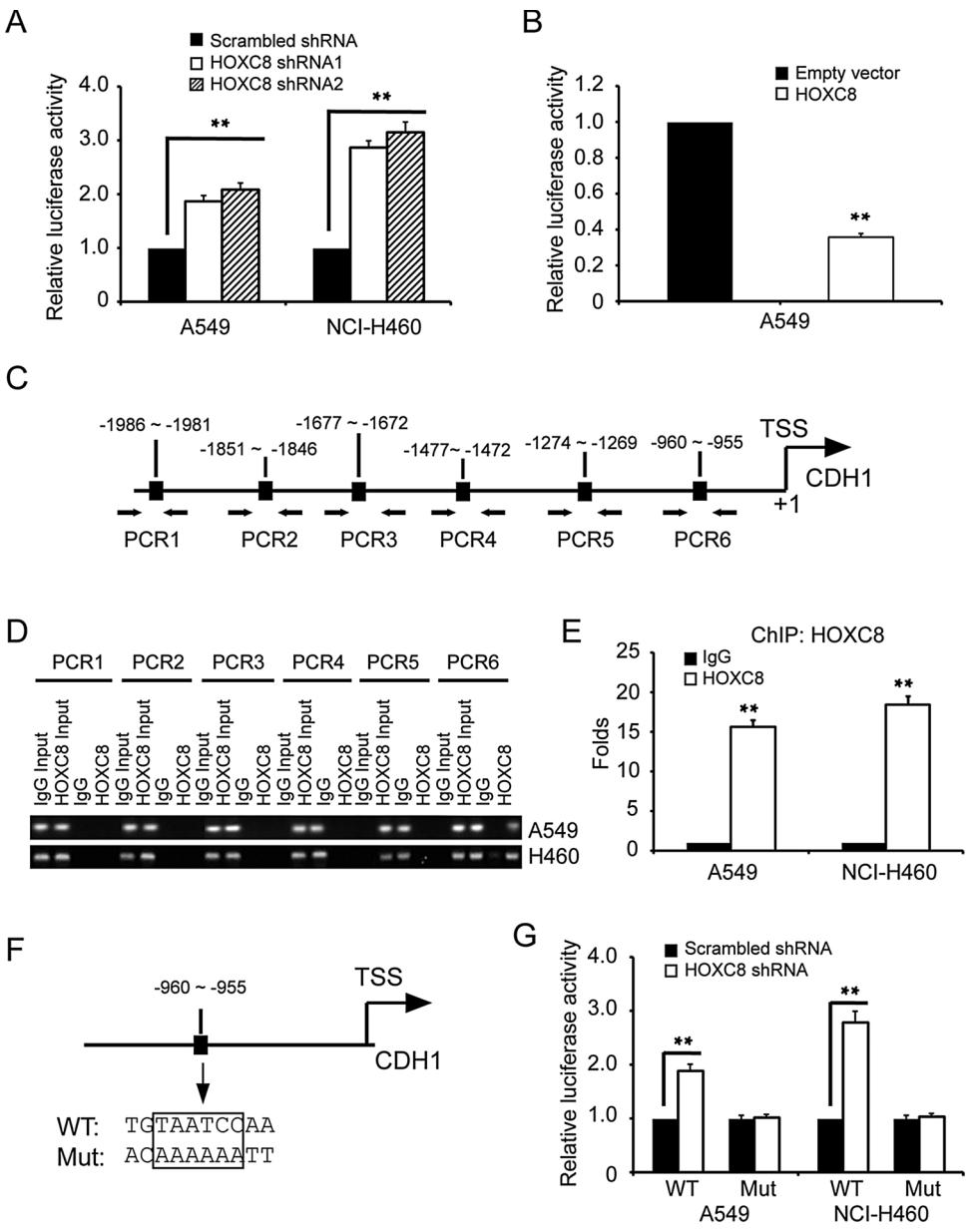

Fig. 2. HOXC8 binds directly to E-cadherin promoter to suppress the promoter activities. (A) The 2-kb E-cadherin promoter was subcloned into luciferase reporter vector by PCR and then was transfected into A549 or NCI-H460 cells that were lentivirally transduced with scrambled or HOXC8 shRNA. After 24 h, luciferase activities were measured with Renilla for normalization. Columns, mean; bars, SEM; ** $P < 0.01$. (B) E-cadherin promoter luciferase reporter vector was transfected into A549 cells that were lentivirally transduced with empty vector or HOXC8 expression vector. After 24 h, luciferase activities were measured with Renilla for normalization. Columns, mean; bars, SEM; ** $P < 0.01$. (C) Positions of PCR primers designed to amplify the regions containing putative HOX binding sites (TAATXX) in E-cadherin promoter. CDH1: E-cadherin (D) Chromatin was immunoprecipitated from A549 or NCI-H460 cells using anti−HOXC8 antibody or control IgG, and precipitated chromatins were amplified by PCR. (E) Chromatin was immunoprecipitated from A549 or NCI-H460 cells using anti−HOXC8 antibody or control IgG, and subjected to real-time PCR. Columns, mean; bars, SEM; ** $P <$ 0.01. (F) Mutagenesis of the HOXC8 binding sites in E-cadherin promoter was performed using overlap extension PCR and subcloned into luciferase reporter vector. CDH1: E-cadherin (G) A549 or NCI-H460 cells that were transduced with HOXC8 shRNA vectors were transiently transfected with wildtype or mutant MGP promoter luciferase vectors. Luciferase activities were measured 24h posttransfection and normalized by Renilla. Columns, mean; bars, SEM; ** $P < 0.01$.

Technology (Beijing, China), anti-tubulin (sc-8035; titer, 1:1000) and anti-β-actin antibody (sc-1616; titer, 1:1000) was from Santa Cruz Biotechnology (Shanghai, China). TRIzol RNA extraction reagent was purchased from Sangon Biotech (Shanghai, China). Lipofectamine 2000 and 3000 were purchased from Life Technologies (Shanghai, China). ECL SuperSignal West Femto Maximum Sensitivity Substrate was purchased from Thermo Scientific (Shanghai, China). Chemical reagents were purchased from Sigma (Shanghai, China).

2.2. Construction of shRNA and expression vectors

E-cadherin and HOXC8 shRNA sequences were designed by web-based Invitrogen Block-It program and were subcloned into pLV-shRNA vector (BioSettia, San Diego, CA). E-cadherin and HOXC8 lentiviral expression vectors were subcloned into pCDH-CMV-MCS-EF1-Puro (System Biosciences, Mountain View, CA). E-cadherin promoter sequence was amplified using genomic DNA isolated from A549 cells and then was subcloned into the pGL4.23 vector (Promega, Beijing, China) that contains the firefly luciferase reporter gene. The mutagenesis of HOXC8 binding site was generated through end-prolongation PCR (all primer sequences are provided in Supplemental Table S1).

2.3. Western blotting and quantitative reverse transcriptase-PCR (qRT-PCR)

Western blotting and qRT-PCR were performed as described previously(Liu et al., 2018). In brief, protein samples were separated by SDS–PAGE and electroblotted onto Immobilon-P membranes (Millipore, Beijing, China). 1 μg of total RNA was reverse-transcribed and then subjected to real-time PCR with gene-specific primer sets using the ABI7900 system (ThermoFisher, Shanghai, China). 18S rRNA was used as an internal control for normalization (Saviozzi et al., 2006), and all primers used for the qRT–PCR analysis are described in Supplementary Table S1.

2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out as previously described(Liu et al., 2018). Briefly, A549 cells were grown to ˜70% confluence in 15-cm dishes and fixed in 1% formaldehyde. Sheared chromatin was

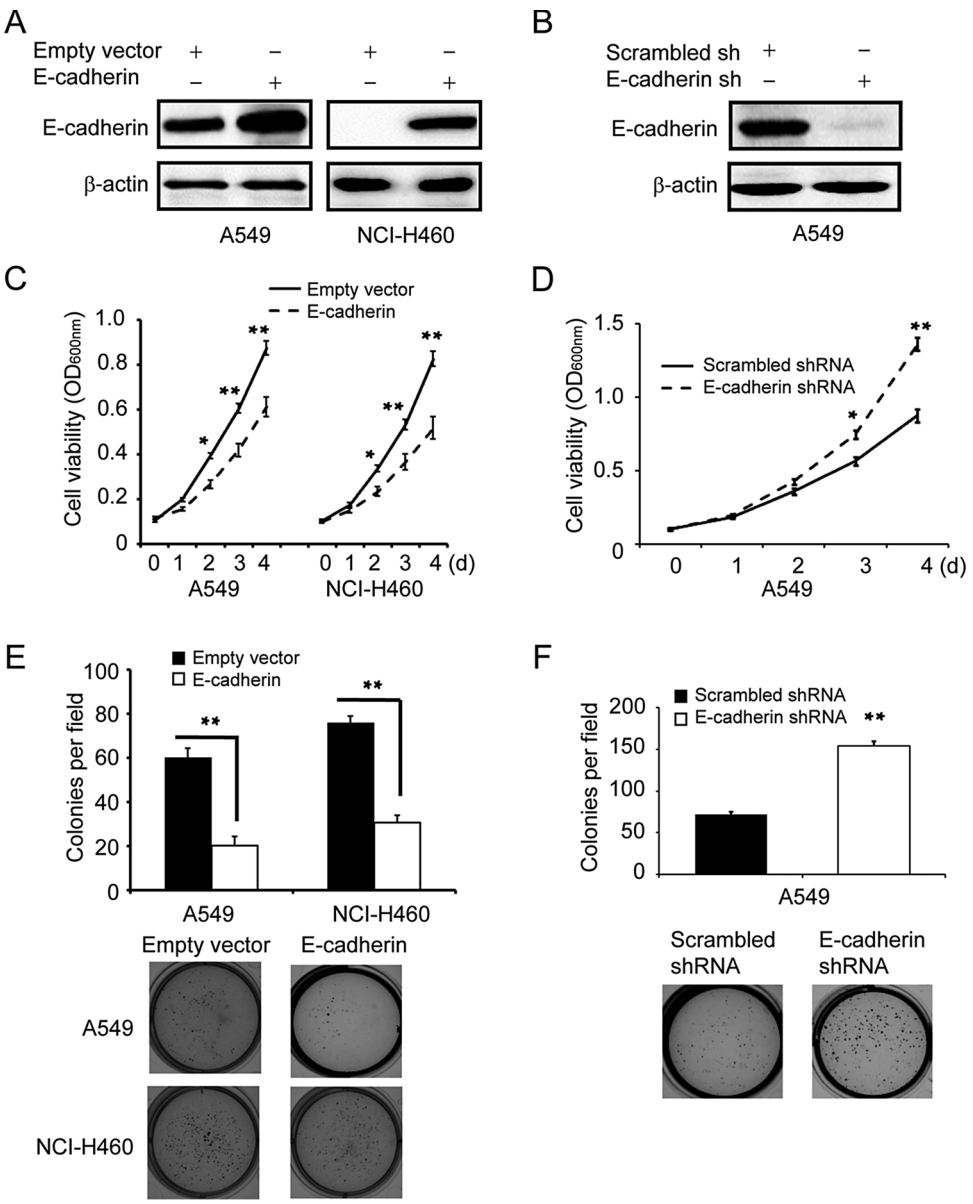

Fig. 3. E-cadherin inhibits the viability and anchorage-independent growth of NSCLC cells. (A) A549 or NCI-H460 cells were lentivirally transduced with empty or E-cadherin expression vectors, and cell lysates were subjected to Western blot to detect E-cadherin and β-actin. (B) A549 cells were lentivirally transduced with scrambled or E-cadherin shRNA vectors, and cell lysates were subjected to Western blot to detect E-cadherin and β-actin. (C) MTT assays were carried out in A549 or NCI-H460 cells that were lentivirally transduced with empty or E-cadherin expression vectors. Data are the mean ± SEM; n = 3; * $P < 0.05$; ** $P < 0.01$. (D) MTT assays were carried out in A549 cells that were lentivirally transduced with scrambled or E-cadherin shRNA vectors. Data are the mean ± SEM; n = 3; * $P < 0.05$; ** $P < 0.01$. (E) A549 or NCI-H460 cells transduced with empty or E-cadherin expression vectors were subjected to soft agarose colony formation. Columns, mean; bars, SEM; n = 3; ** $P < 0.01$. (F) A549 cells that were lentivirally transduced with scrambled or E-cadherin shRNA vectors were subjected to soft agarose colony formation. Columns, mean; bars, SEM; n = 3; ** $P < 0.01$.

immunoprecipitated with anti−HOXC8 antibody and the precipitated DNA was analyzed by PCR or real-time PCR.

2.5. Luciferase activity assay

Luciferase assays were carried out as previously described(Liu et al., 2018). Briefly, A549 or NCI-H460 cells were grown to 70% confluence, 1 μg of wild-type or mutant E-cadherin promoter luciferase vectors were transiently transfected into cells using lipofectamine 3000, and 10 ng of Renilla luciferase vectors were included in the transfection for standardization. After 24 h, the luciferase assays were conducted using the dual luciferase system (Promega, Beijing, China), and luminescence was measured with a PHERAstar FS (BMG Labtech) according to the protocols of the manufacturer.

2.6. MTT cell viability assay

MTT assays were carried out as previously described(Liu et al., 2018). Briefly, 5×10^3 cells per well were seeded into 24-well plates and cultured for indicated times. Cell viability was tested by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 50 μl, 5 mg/ml) solution for 4 h at 37 °C. After removing the medium, dimethylsulfoxide (100 μL/well) was added to dissolve the formazan precipitate. The color intensity in each plate was measured spectrophotometrically using a microplate reader at 560 nm.

2.7. Soft agar colony formation assay

For colony formation assays, 2×10^4 cells per well were added into 24-well plate which consisted of a bottom base layer (0.6% agarose diluted in DMEM) and top layer (0.3% agarose diluted in DMEM). After 3–4 weeks, colonies were stained with iodonitrotetrazolium chloride (INT) and counted under a phase-contrast microscope.

2.8. Transwell migration assay

Briefly, 5×10^4 cells were added into each Transwell upper chamber. After a 6 h migration period, the remaining cells in the upper chamber were removed with cotton swabs, while the cells on the undersurface of the chamber were stained with a crystal violet solution. The number of migratory cells was determined by counting the stained cells in three different fields under a phase-contrast microscope with

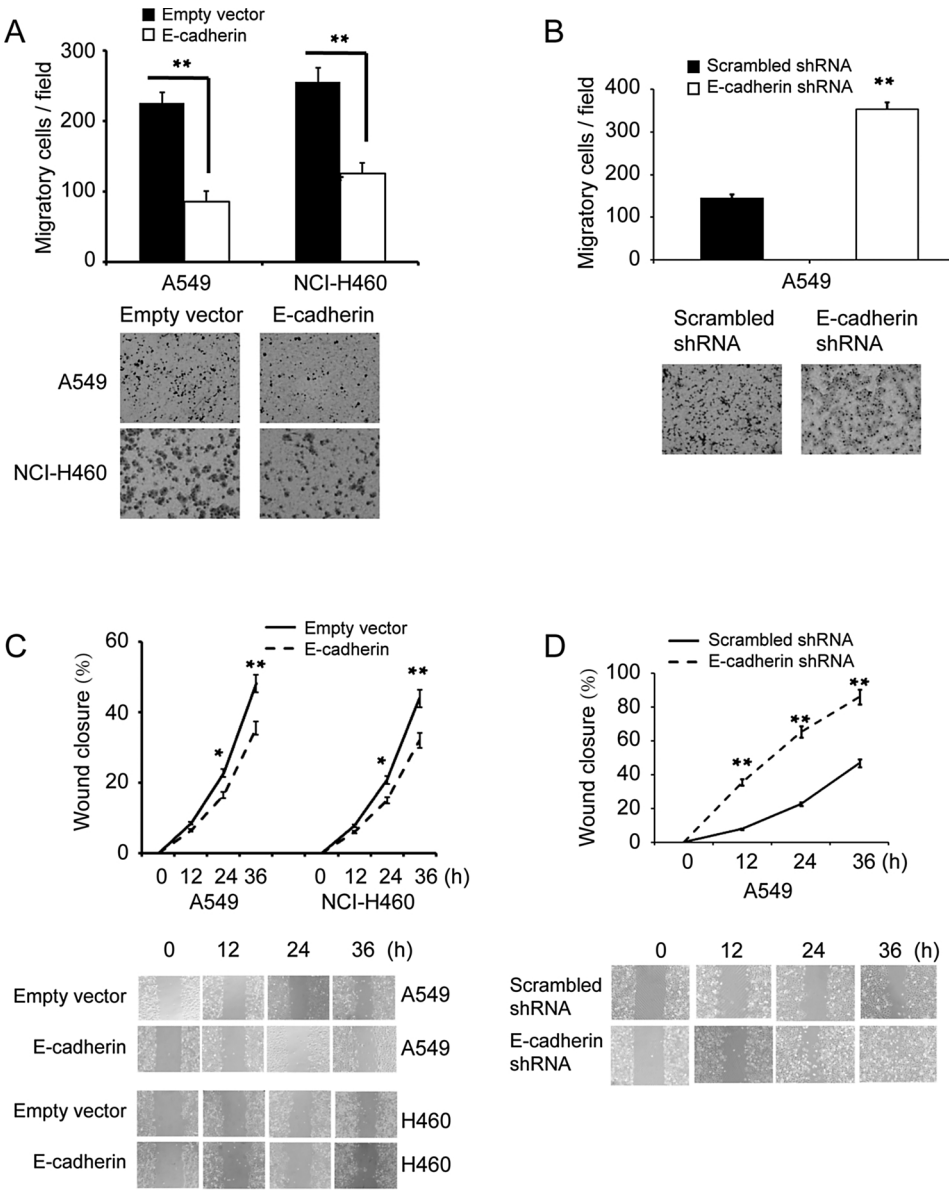

Fig. 4. E-cadherin decreases the migratory abilities of NSCLC cells. (A) Transwell assays to analyze migration of A549 or NCI-H460 cells lentivirally transduced with empty vectors or E-cadherin expression vectors. Columns, mean; bars, n = 3; SEM; ** $P < 0.01$. (B) Transwell assays to analyze migration of A549 cells that were lentivirally transduced with scrambled or E-cadherin shRNA. Columns, mean; bars, SEM; n = 3; ** $P < 0.01$. (C) Wound healing assays to measure the motility of A549 or NCI-H460 cells that were lentivirally transduced with empty vectors or E-cadherin expression vectors. Data are the mean ± SEM; n = 3;; SEM; * $P < 0.05$; ** $P < 0.01$. (D) Wound healing assays to measure the motility of A549 cells transduced with scrambled shRNA or E-cadherin shRNA. Data are the mean ± SEM; n = 3;; SEM; * $P < 0.05$; ** $P < 0.01$.

200 × magnification.

2.9. Wound healing assay

A549 or NCI-H460 cells were planted on 6-well plates and cultured as confluent monolayers. The cells were carefully scraped using a 20 μl pipette tip and debris was removed by washing with 1 × PBS. The denuded areas were quantified using Image *J* software. The wound closure was calculated as followed: (Original width–width of actual wound at different time)/Original width × 100%. The experiment was repeated 3 times.

2.10. Bioinformatics analysis

The Kaplan-Meier analysis was performed using the online Kaplan-Meier Plotter (http://www.kmplot.com) to estimate progression-free survival curves of lung cancer patients (Gyorffy et al., 2013), and the patients samples were split into two groups (high and low groups of HOXC8 or E-cadherin expression) according to median expression levels.

2.11. Statistical analysis

The data are presented as the means ± SEM. Statistical analyses were performed on data collected from at least three independent experiments. Student's *t*-test (two-tailed) was used to compare two groups, and differences were considered to be statistically significant when $P < 0.05$. Statistical analyses were performed with GraphPad Prism with significance levels set at * $P < 0.05$ and ** $P < 0.01$.

3. Results

3.1. HOXC8 represses E-cadherin transcription in NSCLC cells

We previously reported that ectopic expression of HOXC8 promoted EMT by activating the expression of transforming growth factor beta 1 (TGFβ1) in NSCLC cell lines A549 and NCI-H460 (Liu et al., 2018). Given the significant role of E-cadherin in the EMT process, we investigated whether HOXC8 is involved in the regulation of E-cadherin expression in A549 and NCI-H460 cells. Both A549 and NCI-H460 cells are non-small lung cancer cell lines and frequently used for exploring the mechanisms of EMT process (Lv et al., 2016; Thomson et al., 2005).

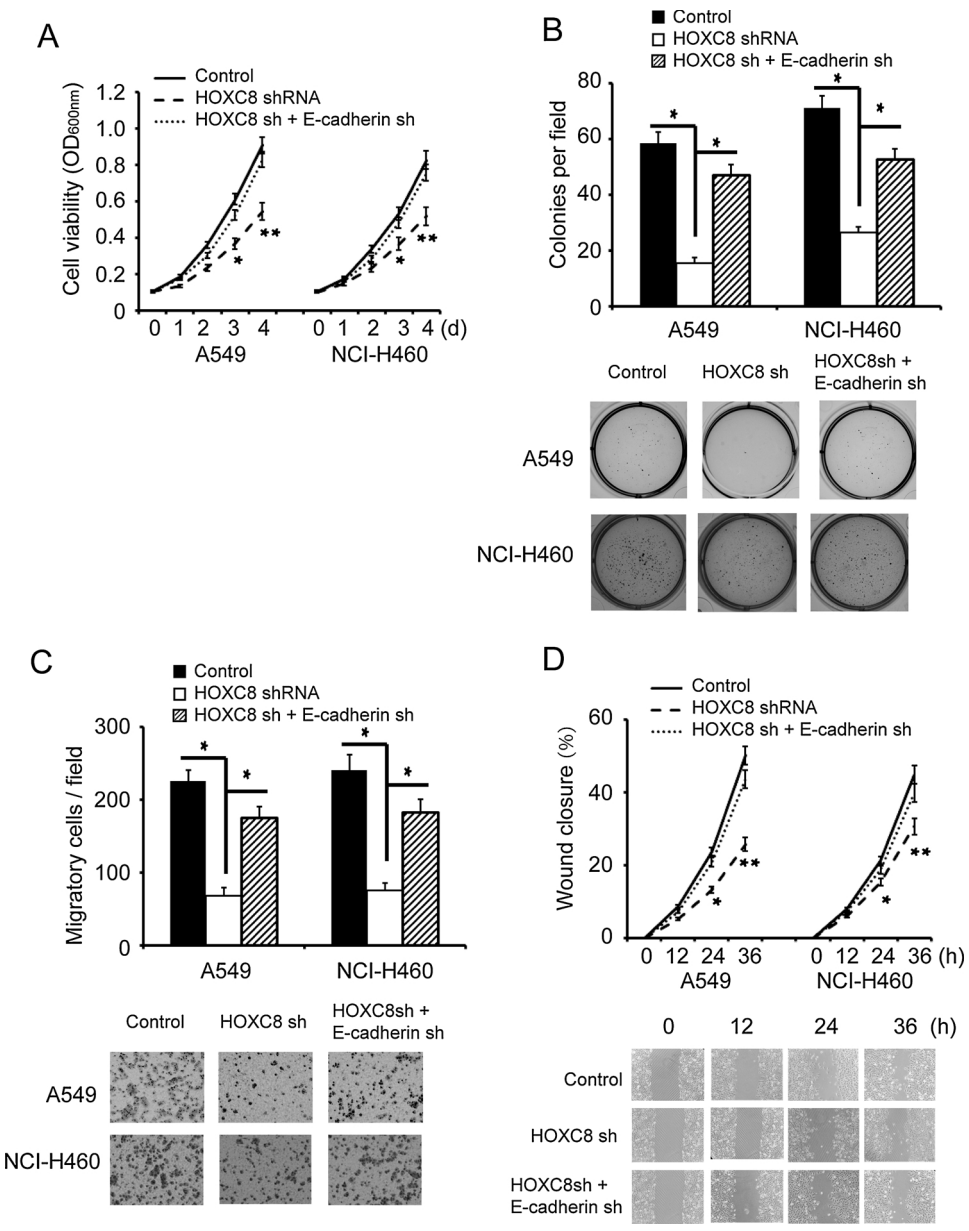

Fig. 5. HOXC8-E-cadherin pathway is involved in cell growth and migration of NSCLC. A549 or NCI-H460 cells were lentivirally transduced with scrambled or HOXC8 shRNA vectors, and then transduced with E-cadherin shRNA vectors. (A) MTT assays were performed to examine cell growth. Data are the mean ± SEM; n = 3; * $P <$ 0.05; ** $P < 0.01$. (B) Soft agar assays were carried out to examine the anchorage-independent growth. Columns, mean; bars, SEM; n = 3; * $P <$ 0.05. (C) Transwell assays were performed to analyze cell migration. Columns, means; bars, SEM; n = 3; *, $P <$ 0.05. (D) Wound healing assays were carried out to measure cell motility. Data are the mean ± SEM; n = 3; * $P <$ 0.05; ** $P < 0.01$.

First, we depleted HOXC8 expression by lentivirally delivering HOXC8 shRNA into A549 or NCI-460 cells, and examined the expression of E-cadherin by Western blotting or qRT-PCR analysis. We found that depletion of HOXC8 resulted in an increase in both the protein and mRNA levels of E-cadherin in A549 cells (Fig. 1A & 1B). To determine whether HOXC8 expression will decrease the expression of E-cadherin gene, we ectopically expressed HOXC8 protein in A549 cells, but not in NCI-H460 cells due to the absence of E-cadherin expression in the cells (Liu et al., 2018; Thomson et al., 2005). We found that forced expression of HOXC8 decreased both protein and mRNA levels of E-cadherin in A549 cells (Fig. 1C & 1D). To assess whether HOXC8 affects E-cadherin mRNA stability, we treated cells with the transcription inhibitor actinomycin D to block further transcription to assess mRNA half-life. As shown in Fig. 1E & 1 F, actinomycin D chase experiments showed that silencing HOXC8 or ectopic expression of HOXC8 had no significant effects on the stability of E-cadherin mRNA. Taken together, these data suggest that HOXC8 is likely involved in regulating E-cadherin transcription in NSCLC cells.

3.2. HOXC8 binds directly to the E-cadherin promoter and inhibits the activities of the E-cadherin promoter

Since all HOX proteins function as transcription factors to regulate their target gene expression, we hypothesized that HOXC8 could serve as a transcription factor for E-cadherin gene. To test this hypothesis, we generated an E-cadherin promoter reporter gene plasmid by cloning the 2,000-nucleotide region of E-cadherin promoter upstream of the transcription start site (TSS) into firefly luciferase vector. Luciferase analyses with this plasmid showed that silencing HOXC8 greatly enhanced the luciferase activities (Fig. 2A) and that forced expression of HOXC8 resulted in a significant decrease in luciferase activities compared to the negative control (Fig. 2B). These data indicated that HOXC8 inhibited the activities of E-cadherin promoter in NSCLC cells.

Based on the reported HOX protein-binding consensus sequences (Svingen and Tonissen, 2006), we identified 6 such sequences in the 2-kb E-cadherin promoter (Fig. 2C). We designed several sets of PCR primers that specifically amplified each region containing the putative HOX binding sequence in E-cadherin promoter (Fig. 2C) and performed ChIP using anti−HOXC8 antibodies and IgG as negative control. ChIP

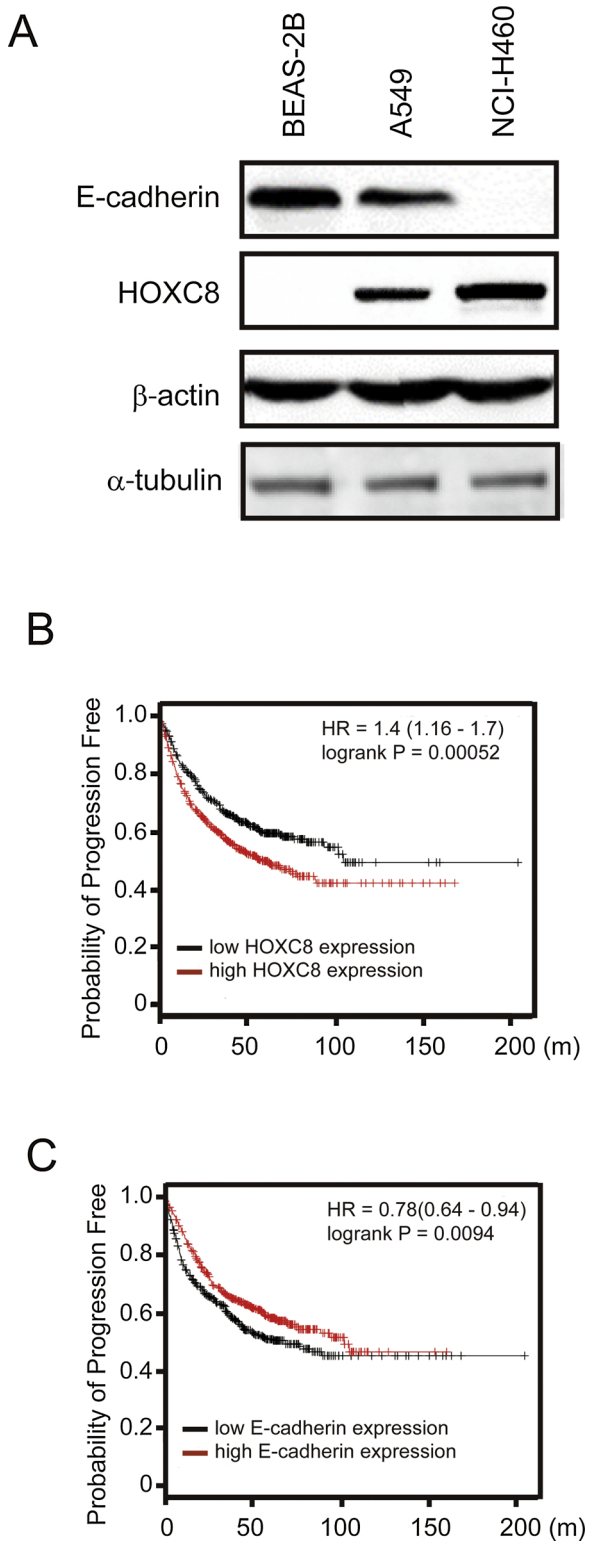

Fig. 6. HOXC8 and E-cadherin expression is inversely correlated in lung cells and patients survival. (A) Western blot was performed to examine the protein levels of HOXC8 and E-cadherin in lung cancer cell lines (A549 and NCI-H460) and normal lung cell line (BEAS-2B). β-actin and α-tubulin were used as the loading control. (B) Kaplan-Meier survival plots show progression-free survival for lung cancer patients with high and low expression of HOXC8 (www.kmplot.com, probe ID: 202291_s_at, logrank $P = 0.00052$). Patients sample were split into two group according to median expression (high HOXC8 expression group: 496 samples; low HOXC8 expression group: 486 samples) (C) Kaplan-Meier survival plots show progression-free survival for lung cancer patients with high and low expression of E-cadherin (www.kmplot.com, probe ID: 201131_s_at, logrank $P = 0.0094$). Patients sample were split into two groups according to median expression (high E-cadherin expression group: 492 samples; low E-cadherin expression group: 490 samples).

analyses showed that HOXC8 bound to E-cadherin promoter on nucleotides −960 to −955 (Fig. 2D), and quantitative PCR further showed that the region was enriched more than 16 fold in the HOXC8 immunoprecipitates compared to the IgG negative control in A549 or NCI-H460 cells (Fig. 2E). Furthermore, mutagenesis of the HOXC8 binding site completely abolished the effects of HOXC8 on the promoter activities of E-cadherin (Fig. 2F & G). These data suggest that HOXC8 binds directly to the promoter of E-cadherin and functions as a transcription suppressor to regulate E-cadherin transcription in NSCLC cells.

3.3. E-cadherin suppresses growth and migration of NSCLC cells

Given that HOXC8 regulates E-cadherin transcription, we analyzed the effects of E-cadherin on cell growth and migration in NSCLC. We first carried out experiments to ectopically express E-cadherin in A549 and NCI-H460 cells, or knockdown E-cadherin expression in A549 cells. Forced expression of E-cadherin greatly increased both E-cadherin protein and mRNA levels in A549 or NCI-H460 cells (Fig. 3A & Supplementary Fig. S1A), and E-cadherin shRNA knockdown led to a clear reduction in E-cadherin expression at both protein levels and mRNA levels of E-cadherin in A549 cells (Fig. 3B & Supplementary Fig. S1B). Next, we performed MTT and colony formation to examine the effects of E-cadherin on cell viability and anchorage-independent growth. MTT assays showed that ectopic expression resulted in decreased cell viability (Fig. 3C), whereas depletion of E-cadherin by shRNA knockdown increased the viability of cells (Fig. 3D). Consistent with the results, soft agar colony formation assays showed that overexpressing E-cadherin significantly inhibited anchorage-independent cell growth (Fig. 3E), and silencing E-cadherin expression markedly enhanced anchorage-independent cell growth (Fig. 3F).

We then analyzed the effects of E-cadherin on cell migratory capability. In Transwell migration assays, we found that ectopic expression of E-cadherin resulted in significant reduction of migratory cells (Fig. 4A), and silencing of E-cadherin by shRNA knockdown significantly increased cell migration (Fig. 4B). Wound healing assays further showed that overexpressing E-cadherin reduced the migratory rate of A549 or NCI-H460 cells (Fig. 4C), and knockdown of E-cadherin accelerated the cell migratory rate compared to control cells (Fig. 4D). Collectively, the above data indicated that E-cadherin exerted repressive effects on cell viability, anchorage-independent growth and migration in NSCLC.

3.4. HOXC8-E-cadherin axis is involved in the growth and migration of NSCLC

We previously reported that silencing of HOXC8 significantly impaired the abilities of cell growth and migration (Liu et al., 2018); therefore, it is necessary to explore whether HOXC8 affects these behaviors by regulating E-cadherin transcription in NSCLC cells. To explore whether the effects of HOXC8 were functionally linked to its regulation of E-cadherin transcription, HOXC8 knockdown cells were lentivirally transduced with E-cadherin shRNA, and then examined with MTT, colony formation, migration and wound healing assays. MTT and colony formation assays showed that HOXC8 depletion greatly decreased cell viability or anchorage-independent cell growth, which was largely recovered by inhibition of E-cadherin expression (Fig. 5A & 5B). Moreover, Transwell and wound healing assays indicated that the impaired migratory capabilities by HOXC8 knockdown were almost

restored by the depletion of E-cadherin in NSCLC cells (Fig.5C, D & Supplementary Fig. S2). These results suggest that the HOXC8-E-cadherin pathway is involved in the growth and migration of NSCLC.

3.5. HOXC8 expression is correlated with a loss of E-cadherin

Because HOXC8 was capable of repressing E-cadherin expression, we sought to characterize the expression levels of HOXC8 and E-cadherin in NSCLC cell lines A549 and NCI-H460 or the normal human bronchial epithelial cell line BEAS-2B. By Western blot analysis, we found that the normal human bronchial epithelial cell BEAS-2B exhibited considerably higher E-cadherin protein levels and no detectable HOXC8 expression compared to A549 or NCI-H460 cells. Consistent with this observation, we found that HOXC8 expression is inversely correlated with the expression levels of E-cadherin in both NSCLC cell lines A549 and NCI-H460 (Fig. 6A). These data indicated that HOXC8 expression is tightly correlated with a loss of endogenous E-cadherin expression in normal bronchial epithelial cells and NSCLC cells. Furthermore, online Kaplan-Meier survival analysis showed that patients with high expression of HOXC8 had a significantly lower probability of progression free survival for lung cancer patients (logrank $P = 0.00052$) (Fig. 6B), while patients with low expression of E-cadherin were significantly associated with poor survival rate of lung cancer (logrank $P = 0.0094$) (Fig. 6C). These results further supported the experimental finding that HOXC8 acted as a transcription repressor of the E-cadherin and HOXC8-E-cadherin axis played an important role in the tumorigenesis of NSCLC.

4. Discussion

HOX (homeobox) genes encode homeodomain-containing transcription factors critical to development, differentiation and homeostasis. The dysregulation of these genes has been implicated in a variety of cancers, in which altered expression of HOX genes are regarded as indicators of tumor progression or suppression (Shah and Sukumar, 2010; Svingen and Tonissen, 2006). As a member of the HOX family, HOXC8 is reported to be involved in the development of different types of human cancers, such as breast, ovarian, prostate, cervical and pancreatic cancers, demonstrating the importance of HOXC8 in tumor development (Alami et al., 1999; Axlund et al., 2010; Li et al., 2010; Lu et al., 2016). In our previous study, we reported that HOXC8 promoted tumorigenesis of NSCLC by upregulating TGFβ1 expression and downregulating E-cadherin expression (Liu et al., 2018). In the current study, we observed that HOXC8 functions as a transcriptional repressor to inhibit E-cadherin transcription in NSCLC cells.

Transforming growth factor beta 1(TGFβ1) is an important cytokine in cancer progression, and plays an important role in the EMT process (Colak and Ten Dijke, 2017). Several reports have shown that TGFβ1 promotes EMT by downregulating E-cadherin expression in NSCLC (Chang et al., 2016; Wang et al., 2017). We found that HOXC8 expression resulted in loss of E-cadherin mRNA and upregulation of TGFβ1, which prompted us to examine whether HOXC8 regulates the expression of E-cadherin directly or indirectly. Using luciferase reporter and ChIP assays, we found that HOXC8 acted as a transcriptional repressor by binding to and inhibiting the E-cadherin promoter directly. In combination with our previous study (Liu et al., 2018), these results show that HOXC8 is involved in the bidirectional regulation of E-cadherin and TGFβ1, indicating that HOXC8 may regulate a battery of genes to promote EMT in NSCLC.

As a member of the HOX family, it has been reported that HOXC8 is able to function as a transcriptional activator or repressor to regulate transcription of different genes, including osteopontin (OPN), proliferating cell nuclear antigen (PCNA), CDH11, Mgl1 and Smad6 (Lei et al., 2005; Li et al., 2014; Min et al., 2010; Ruthala et al., 2011). Moreover, studies showed that HOXC8 forms protein complexes to regulate its target gene expression (Shi et al., 1999; Zhang et al., 2017). Although our results show that HOXC8 expression inhibits E-cadherin transcription, these results do not rule out the possibility that HOXC8 regulates E-cadherin transcription through interactions with other transcription factors. It has been reported that several transcription factors participate in the downregulation of E-cadherin, including Snail, Slug, ZEB1 and ZEB2 (Bolos et al., 2003; Cano et al., 2000; Comijn et al., 2001; Schmalhofer et al., 2009). It is still unclear how these transcription factors cooperate in specific cellular contexts or in different types of tumors. We believe that it is plausible that HOXC8 may cooperate with these factors to promote EMT process of NSCLC in a coordinated fashion. It should also be noted that downregulation of E-cadherin is a transient and dynamic event in tumor progression (Erami et al., 2015; Graff et al., 2000). Therefore, further studies are needed to determine the relationship between HOXC8 and other factors in the regulation of E-cadherin transcription and to explore whether the relationship between HOXC8 and E-cadherin is a common phenomenon in other tumors.

The loss of E-cadherin is a crucial event for EMT and has been found to increase tumor cell migration and invasion in various types of cancer (Brabletz et al., 2018; Frixen et al., 1991; Tsoukalas et al., 2017); however, this loss is not a necessary prerequisite for migration and invasion in some types of cancer (Hollestelle et al., 2013; Liu et al., 2014). We found that depletion of E-cadherin expression enhanced the capabilities for cell growth and migration. Moreover, knockdown of HOXC8 significantly inhibited growth and migration of lung cancer cells, which can be rescued by silencing E-cadherin, indicating that downregulation of E-cadherin by HOXC8 is important to cell growth and migration in NSCLC. Several studies revealed that loss of E-cadherin promotes cell invasion by activating epidermal growth factor receptor (EGFR) signaling, and enhanced E-cadherin expression increases the chemosensitivity of NSCLC cells to EGFR inhibitor treatment (Bae et al., 2013; Witta et al., 2006). Consistent with the findings of these studies, our previous work showed that depletion of HOXC8 significantly enhanced cisplatin-induced apoptosis in A549 and NCI-H460 cells. Although we did not explore the roles of HOXC8 in EGFR signaling, the loss of E-cadherin by HOXC8 suggested that depletion of HOXC8 in combination with EGFR inhibitors could be a plausible strategy to enhance therapeutic efficacy for NSCLC.

In summary, we demonstrated that HOXC8 functioned as a transcriptional repressor to regulate the expression of E-cadherin in NSCSL cells. We further showed that downregulation of E-cadherin by HOXC8 significantly enhanced cell growth and migration, and high HOXC8 / low E-cadherin expression was statistically linked to poor survival for lung cancer patients.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by grants from National Natural Science Foundation of China (81572864), and Anhui University startup Foundation (J01006039).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.06.005.

References

Alami, Y., Castronovo, V., Belotti, D., Flagiello, D., Clausse, N., 1999. HOXC5 and HOXC8 expression are selectively turned on in human cervical cancer cells compared to normal keratinocytes. Biochem. Biophys. Res. Commun. 257, 738–745.

Axlund, S.D., Lambert, J.R., Nordeen, S.K., 2010. HOXC8 inhibits androgen receptor signaling in human prostate cancer cells by inhibiting SRC-3 recruitment to direct androgen target genes. Mol. Cancer Res. 8, 1643–1655.

Bae, G.Y., Choi, S.J., Lee, J.S., Jo, J., Lee, J., Kim, J., et al., 2013. Loss of E-cadherin

activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer. Oncotarget 4, 2512–2522.
Bolos, V., Peinado, H., Perez-Moreno, M.A., Fraga, M.F., Esteller, M., Cano, A., 2003. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J. Cell. Sci. 116, 499–511.
Brabletz, T., Kalluri, R., Nieto, M.A., Weinberg, R.A., 2018. EMT in cancer. Nat. Rev. Cancer 18, 128–134.
Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., et al., 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. 2, 76–83.
Chambers, A.F., Groom, A.C., MacDonald, I.C., 2002. Dissemination and growth of cancer cells in metastatic sites. Nat. Rev. Cancer 2, 563–572.
Chang, H., Liu, Y., Xue, M., Liu, H., Du, S., Zhang, L., et al., 2016. Synergistic action of master transcription factors controls epithelial-to-mesenchymal transition. Nucleic Acids Res. 44, 2514–2527.
Colak, S., Ten Dijke, P., 2017. Targeting TGF-beta signaling in cancer. Trends Cancer 3, 56–71.
Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., et al., 2001. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol. Cell 7, 1267–1278.
Erami, Z., Timpson, P., Yao, W., Zaidel-Bar, R., Anderson, K.I., 2015. There are four dynamically and functionally distinct populations of E-cadherin in cell junctions. Biol. Open 4, 1481–1489.
Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., et al., 1991. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol. 113, 173–185.
Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B., Herman, J.G., 2000. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J. Biol. Chem. 275, 2727–2732.
Gyorffy, B., Surowiak, P., Budczies, J., Lanczky, A., 2013. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PLoS One 8, e82241.
Hollestelle, A., Peeters, J.K., Smid, M., Timmermans, M., Verhoog, L.C., Westenend, P.J., et al., 2013. Loss of E-cadherin is not a necessity for epithelial to mesenchymal transition in human breast cancer. Breast Cancer Res. Treat. 138, 47–57.
Krumlauf, R., 1994. Hox genes in vertebrate development. Cell 78, 191–201.
Lei, H., Wang, H., Juan, A.H., Ruddle, F.H., 2005. The identification of Hoxc8 target genes. Proc. Natl. Acad. Sci. U. S. A. 102, 2420–2424.
Li, Y., Chao, F., Huang, B., Liu, D., Kim, J., Huang, S., 2014. HOXC8 promotes breast tumorigenesis by transcriptionally facilitating cadherin-11 expression. Oncotarget 5, 2596–2607.
Li, Y., Zhang, M., Chen, H., Dong, Z., Ganapathy, V., Thangaraju, M., et al., 2010. Ratio of miR-196s to HOXC8 messenger RNA correlates with breast cancer cell migration and metastasis. Cancer Res. 70, 7894–7904.
Liu, H., Zhang, M., Xu, S., Zhang, J., Zou, J., Yang, C., et al., 2018. HOXC8 promotes proliferation and migration through transcriptional up-regulation of TGFbeta1 in non-small cell lung cancer. Oncogenesis 7, 1.
Liu, X., Huang, H., Remmers, N., Hollingsworth, M.A., 2014. Loss of E-cadherin and epithelial to mesenchymal transition is not required for cell motility in tissues or for metastasis. Tissue Barriers 2, e969112.
Lu, S., Liu, R., Su, M., Wei, Y., Yang, S., He, S., et al., 2016. Overexpression of HOXC8 is associated with poor prognosis in epithelial ovarian cancer. Reprod. Sci. 23, 944–954.
Lv, X.Q., Qiao, X.R., Su, L., Chen, S.Z., 2016. Honokiol inhibits EMT-mediated motility and migration of human non-small cell lung cancer cells in vitro by targeting c-FLIP. Acta Pharmacol. Sin. 37, 1574–1586.
Min, H., Lee, J.Y., Bok, J., Chung, H.J., Kim, M.H., 2010. Proliferating cell nuclear antigen (Pcna) as a direct downstream target gene of Hoxc8. Biochem. Biophys. Res. Commun. 392, 543–547.
Ruthala, K., Gadi, J., Lee, J.Y., Yoon, H., Chung, H.J., Kim, M.H., 2011. Hoxc8 down-regulates Mgl1 tumor suppressor gene expression and reduces its concomitant function on cell adhesion. Mol. Cells 32, 273–279.
Saviozzi, S., Cordero, F., Lo Iacono, M., Novello, S., Scagliotti, G.V., Calogero, R.A., 2006. Selection of suitable reference genes for accurate normalization of gene expression profile studies in non-small cell lung cancer. BMC Cancer 6, 200.
Schmalhofer, O., Brabletz, S., Brabletz, T., 2009. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev. 28, 151–166.
Shah, N., Sukumar, S., 2010. The Hox genes and their roles in oncogenesis. Nat. Rev. Cancer 10, 361–371.
Shi, X., Yang, X., Chen, D., Chang, Z., Cao, X., 1999. Smad1 interacts with homeobox DNA-binding proteins in bone morphogenetic protein signaling. J. Biol. Chem. 274, 13711–13717.
Siegel, R., Ma, J., Zou, Z., Jemal, A., 2014. Cancer statistics, 2014. CA Cancer J. Clin. 64, 9–29.
Svingen, T., Tonissen, K.F., 2006. Hox transcription factors and their elusive mammalian gene targets. Heredity. 97, 88–96.
Thiery, J.P., 2002. Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cancer 2, 442–454.
Thomson, S., Buck, E., Petti, F., Griffin, G., Brown, E., Ramnarine, N., et al., 2005. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. Cancer Res. 65, 9455–9462.
Travis, W.D., 2002. Pathology of lung cancer. Clin. Chest Med. 23, 65–81 viii.
Tsoukalas, N., Aravantinou-Fatorou, E., Tolia, M., Giaginis, C., Galanopoulos, M., Kiakou, M., et al., 2017. Epithelial-mesenchymal transition in non small-cell lung Cancer. Anticancer Res. 37, 1773–1778.
van Roy, F., 2014. Beyond E-cadherin: roles of other cadherin superfamily members in cancer. Nat. Rev. Cancer 14, 121–134.
Wang, H., Wu, Q., Zhang, Y., Zhang, H.N., Wang, Y.B., Wang, W., 2017. TGF-beta1-induced epithelial-mesenchymal transition in lung cancer cells involves upregulation of miR-9 and downregulation of its target, E-cadherin. Cell. Mol. Biol. Lett. 22, 22.
Witta, S.E., Gemmill, R.M., Hirsch, F.R., Coldren, C.D., Hedman, K., Ravdel, L., et al., 2006. Restoring E-cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. Cancer Res. 66, 944–950.
Zhang, Y., Yang, C., Zhang, M., Liu, H., Gong, C., Zhang, J., et al., 2017. Interleukin enhancer-binding factor 3 and HOXC8 co-activate cadherin 11 transcription to promote breast cancer cells proliferation and migration. Oncotarget. 8, 107477–107491.