

Induction of CXCL8 by endoplasmic reticulum stress promotes migration and invasion of esophageal squamous cell carcinoma through activation of SMAD2/3

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Abstract. Endoplasmic reticulum stress (ERS) is a protective stress response aimed at mitigating its own abnormal proteins, which is closely associated with tumors. However, the molecular mechanism of ERS in the pathogenesis of esophageal squamous cell carcinoma (ESCC) remains unclear. In the present study, RNA sequencing was carried out in the ESCC ERS cell model *in vitro*, and differentially expressed genes were screened, among which CXCL8 with exhibited differential expression which was studied. CXCL8 was significantly upregulated after thapsigargin (TG; an ERS inducer) treatment in ESCC cells. A marked elevated expression of CXCL8 and its receptors were observed in ESCC cells. CXCL8 was induced by the IRE1 α and PERK pathways of ERS, transcription of which was activated by the downstream transcription factors XBP1 and ATF4. TG and rh-CXCL8 facilitated migration and invasion of ESCC cells, and the migration and invasion effect of TG on ESCC cells could be partially prevented by knockdown of CXCR1. Furthermore, CXCL8-CXCR1 could activate SMAD2/3 and the activation of SMAD2/3 directly or indirectly regulated the transcription of SNAI2 and ZEB1 to promote the progression of epithelial-mesenchymal transition (EMT) in ESCC. Both *in vivo* experiments and immunohistochemical analyses further demonstrated the oncogenic effects of CXCL8. In conclusion, the data obtained

in the present study indicated that CXCL8 may be induced via the IRE1 α /XBP1 and PERK/ATF4 pathways, and that the CXCL8-CXCR1/2-SMAD2/3-SNAI2/ZEB1 axis is involved in the EMT process of ER-stressed ESCC cells. Thus, blocking the CXCL8-CXCR1/2 axis may disrupt ERS-induced migration and invasion of ESCC cells, thereby improving the prognosis of patients with ESCC.

Introduction

Esophageal carcinoma is a prevalent malignant tumor originating from the esophageal mucosa, with varying global incidence in different regions. Increased incidences of esophageal carcinoma were reported in developing countries, particularly in the low-middle Social Demographic Index (SDI) region, where incident cases surged by 105.73% (1), which may be attributed to regional cultures, dietary patterns and other factors (2,3). In China, the main histologic type of esophageal carcinoma is esophageal squamous cell carcinoma (ESCC). Contributors to ESCC include smoking, alcohol intake (4,5), poor diet, chronic esophagitis, genetic mutations (6) and viral infections (7). Treatment strategies for ESCC include surgical resection, endoscopic therapy, radiotherapy, chemotherapy, targeted therapy and immunotherapy (8-10). Despite improvements in treatment strategies, the 5-year survival rate of patients with ESCC is 18% (11), warranting exploration of the molecular pathogenesis of ESCC to find new therapeutic strategies.

Endoplasmic reticulum stress (ERS) is a state of cellular stress caused by ER dysfunction and abnormal protein accumulation, which triggers an adaptive program response known as the unfolded protein response (UPR). In response to UPR, three transmembrane sensors, inositol-requiring enzyme 1 α [IRE1 α ; also known as endoplasmic reticulum to nucleus signaling 1 (ERN1)], protein kinase R (PKR)-like endoplasmic reticulum kinase [PERK; also known as eukaryotic translation initiation factor 2 α kinase 3 (EIF2AK3)] and activating transcription factor (ATF) 6 are activated by dissociating with the molecular chaperone glucose-regulated protein 78. Among them, IRE1 α catalyzes the shearing of X-box binding

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protein 1 (XBP1) mRNA, thus resulting in translation of active transcription factors called XBP1s. PERK phosphorylates its catalytic substrate, eIF2 α through autophosphorylation, and then stimulates the production of ATF4, which subsequently activates CHOP to initiate cell death, including apoptosis. ATF6 translocates to the golgi apparatus, where it is cleaved by sphingosine-1-phosphate and site-2 protease to release amino acid fragments with transcriptional activity called ATF6f (12). ATF4, XBP1s and ATF6f regulate transcription of a large range of target genes that participate in the UPR. In tumors, the biological effects of ERS are two-fold, the intensity of stress and different cell types will induce mRNA transcription and promote tumor cell proliferation (13), migration (14), autophagy (15-17) and apoptosis (18-20). However, the mechanism of ERS in ESCC is still not well clarified.

To explore the occurrence and mechanism of ERS in ESCC, RNA sequencing was carried out on the *in vitro* ESCC cell model of ERS and focused on the role of C-X-C motif chemokine ligand (CXCL) 8, also known as IL-8. CXCL8, is a cytokine and chemokine that belongs to the CXC chemokine family and is a small molecule protein produced by a variety of cells. CXCL8 carries out an important role in inflammation, tumor and immune response (21). CXCL8 exerts biological effects by binding to its chemokine receptors (CXCRs) 1 and 2, among which CXCR1 mainly binds to CXCL8, while CXCR2 also binds to CXCL1, 2, 3, 5 and 7 (22). Increased CXCL8 expression is associated with poor prognosis in a variety of tumors, including breast, colon and lung cancer (23-25). Furthermore, elevated CXCL8 expression has been associated with resistance to chemotherapy and immunotherapy in breast cancer, gastric cancer, lung adenocarcinoma and hepatocellular carcinoma (26-29). The CXCL8-CXCR1/2 axis promotes migration, proliferation, invasion and survival of tumor cells (30). CXCL8 and CXCR1/2 are reported to be highly expressed in ESCC tissues and are associated with factors and prognosis (31,32). In particular, CXCL8 promotes migration and invasion of ESCC cells through phosphorylation of AKT and ERK1/2 (32). Nevertheless, the role of CXCL8 in ERS and the potential downstream mechanisms promoting ESCC progression need to be further investigated.

The present study investigated the mechanisms underlying CXCL8 upregulation during ERS and its tumor-promoting effects in ESCC, in order to provide a new strategy for ESCC treatment.

Materials and methods

Cell culture and treatment. Human esophageal cancer cell lines (KYSE-150, KYSE-170 and TE-1) and the human normal esophageal epithelial cell line (HEEC) were purchased from the China Center for Type Culture Collection and cultured in RPMI1640 medium (cat. no. 11879020; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; cat. no. A5670201; Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained under standard culture conditions (37°C and 5% CO₂) in culture medium. For thapsigargin (TG; cat. no. T9033; MilliporeSigma) treatment, KYSE-150 and TE-1 cells were cultured at 37°C and treated with 100 nM TG for 0 to 24 h. For recombinant human (rh-)CXCL8 (cat. no. HY-P7379; MedChemExpress) treatment, KYSE-150 and

TE-1 cells were cultured at 37°C and treated with rh-CXCL8 at varying concentrations (0, 10, 20, 40, 80 or 160 ng/ml) for 0 to 48 h. For TGF- β 1 (cat. no. HY-P7118; MedChemExpress) treatment, KYSE-150 and TE-1 cells were cultured at 37°C and treated with 10 ng/ml TGF- β 1 for 48 h.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) assays. Total RNA was extracted from KYSE-150, KYSE-170, TE-1 and HEEC cells (4x10⁶ cells per sample) using Triquick reagent (cat. no. R1100; Beijing Solarbio Science & Technology Co., Ltd.) and reverse transcribed into cDNA with the Transcriptor First Strand cDNA Synthesis Kit (cat. no. 04896866001; Roche Diagnostics) according to the manufacturer's protocol. SYBR Green real-time fluorescent quantitative PCR premix (cat. no. B110031; Sangon Biotech Co., Ltd.) was carried out in StepOnePlus real-time fluorescent quantitative PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following cycling conditions: Initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 sec, 56°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 7 min. The expression levels of genes were analyzed with the 2^{- $\Delta\Delta C_q$} method (33) and normalized to endogenous GAPDH. Primer sequences are listed in Table SI.

RNA sequencing analysis. TE-1 cells were treated with 100 nM TG for 12 h under standard culture conditions (37°C; 5% CO₂), after which total RNA was extracted using TRIzol™ Reagent (cat. no. 15596026CN; Thermo Fisher Scientific, Inc.). The quality and integrity of the RNA samples were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Library construction, sequencing and data analysis were carried out by Sangon Biotech Co., Ltd. Sequencing was carried out on an Illumina platform using paired-end sequencing with a read length of 150 bp. The sequencing was performed using the Illumina NovaSeq 6000 system with the NovaSeq 6000 S4 Reagent Kit (300 cycles; cat. no. 20027466; Illumina, Inc.). The final library was quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Inc.) and the concentration was adjusted to 10-20 pM prior to sequencing, based on molar concentration calculations. Bioinformatics analysis was performed using the programs FastQC (v0.11.9; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), Cutadapt (v2.10; <https://cutadapt.readthedocs.io/>), HISAT2 (v2.2.1; <https://daehwankimlab.github.io/hisat2/>) and featureCounts (v2.0.1; <http://subread.sourceforge.net/featureCounts.html>). Statistical analysis and visualization were conducted in R (v4.3.2; <https://www.r-project.org/>; The R Foundation). Differential expression analysis was conducted in R (v4.3.2; <https://www.r-project.org/>) using DESeq2 (v1.30.1; <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>). Differentially expressed genes were screened based on the following criteria: $|\log_2\text{foldchange}| > 1$, adjusted $P < 0.05$.

Cell transfection. ERN1, EIF2AK3 and ATF6 small interfering (si)RNA, alongside si-negative control were purchased from Shanghai GenePharma Co., Ltd., sequences are listed in Table SII. pcDNA3.1-XBP1 and pcDNA3.1-ATF6 were purchased from GenScript Biotech Corporation. The cDNA encoding ATF4 was amplified and inserted into the pcDNA3.1 vector (cat. no. V79520; Invitrogen; Thermo

Fisher Scientific Inc.), named pcDNA3.1-ATF4, sequences are listed in Table SIII. KYSE-150 and TE-1 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and cultured for 24 h to allow adherence. Upon reaching 70–80% confluency, transfection was carried out according to the manufacturer's protocol for Lipofectamine® 2000 (cat. no. 11668500; Invitrogen; Thermo Fisher Scientific, Inc.). Specifically, 3 μ g of target overexpression plasmid and empty vector control plasmid or 100 pmol of siRNA and negative control siRNA was mixed with transfection reagent at room temperature for 20 min and added to the cell culture system. Post-transfection, cells were maintained in a 37°C, 5% CO₂ incubator. The culture medium was replaced with fresh medium at 6 h. After 48 h of total incubation, cells were harvested for transfection efficiency analysis by RT-qPCR and subsequent experiments.

Lentiviral infection. Full-length human CXCL8 cDNA was obtained via RT-qPCR and inserted into the lentiviral expression vector pCDH-CMV-MCS-EF1-Puro. The recombinant plasmid (10 μ g), along with the packaging plasmid psPAX2 (7.5 μ g) and the envelope plasmid pMD2.G (5 μ g), was mixed and incubated at room temperature for 20 min to form the transfection complex. The complex was then co-transfected into 293T cells using Lipofectamine 2000® to produce lentiviral particles at 37°C for 6 h, after which the medium was replaced with fresh medium. After 48 h, viral supernatants were harvested and subsequently used to transduce KYSE-150 cells. Infected cells were cultured in medium containing 1.0 μ g/ml puromycin for 14 days to generate a stable KYSE-150 cell line with CXCL8 overexpression.

Western blot analysis. KYSE-150 and TE-1 cells were washed in ice-cold PBS and RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.). PMSF (cat. no. P0100; Beijing Solarbio Science & Technology Co., Ltd.) was used to extract proteins from cells. Protein concentrations were measured using the BCA Protein Assay Kit (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.) per the manufacturer's instructions. Briefly, samples were mixed with working reagent (1:50 ratio) and incubated at 37°C for 30 min before absorbance measurement at 562 nm. Protein (50 μ g/lane) was then separated by 10% SDS-PAGE and transferred to PVDF membranes (cat. no. 1.15546; MilliporeSigma). Membranes were blocked with 5% non-fat milk in TBS with 0.05% Tween-20 for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4°C and corresponding HRP-conjugated secondary antibodies for 1 h at room temperature. The enhanced chemiluminescence detection reagent (cat. no. WBULS0100; MilliporeSigma) was used to detect the protein bands by Chemi XT 4 (Syngene). The antibodies are listed in Table SIV.

Gene expression profiling interactive analysis (GEPIA) database analysis. Expression profile analysis of CXCL8 across various types of cancer and normal tissues was carried out using the GEPIA web server (<http://gepia.cancer-pku.cn/>), which integrates RNA sequencing data from The Cancer Genome Atlas and Genotype-Tissue Expression projects. The

'Expression DIY' module was used to assess CXCL8 mRNA expression in pan-cancer types, and to compare its expression levels between ESCC and normal esophageal tissues. The thresholds were set at $\log_2$ fold change (FC) > 1 and $P < 0.01$ to define significant differential expression. Box plots were generated directly through GEPIA for visualization.

PROMO database analysis. Prediction of transcription factor binding sites was performed using the PROMO database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), which is based on the TRANSFAC transcription factor binding site database. The promoter region (–2,000 bp upstream of the transcription start site) of the CXCL8 gene was analyzed, with the species set to *Homo sapiens* and the maximum matrix dissimilarity rate set to 15%. Candidate transcription factors were selected based on their known relevance to ERS for further validation.

Cell proliferation assay. Cellular proliferation capability was detected using an MTS assay. Briefly, 1×10^3 cells pretreated with rh-CXCL8 for 24 h under standard culture conditions (37°C, 5% CO₂) were seeded per well in 96-well plates. At 0, 24, 48, 72 and 96 h post-seeding, CellTiter 96® Aqueous One Solution Reagent (cat. no. G3582; Promega Corporation) was added, followed by a 2-h incubation at 37°C before measuring absorbance at 490 nm.

Cell migration and invasion assays. Wound healing and Transwell assays were carried out to detect the migration and invasion ability of cells. For the wound healing assay, treated cells (KYSE-150 and TE-1 cells subjected to 24 h treatment with NC or 10 ng/ml rh-CXCL8, 12 h treatment with 100 nM TG, 48 h transfection with 100 pmol siCXCR1 and combined treatment with 100 nM TG and 100 pmol siCXCR1) were inoculated in 6-well plates and cultured in medium containing 10% FBS. A straight scratch was made in each well when the cell monolayer reached near complete confluence, and cells were then maintained in serum-free medium. Images were taken at the same position at 0 and 24 h using an inverted optical microscope (Nikon Eclipse 50i; Nikon Corporation). Subsequently, ImageJ (National Institutes of Health) software was employed to analyze the acquired images. The calculation of scratch area percentage was defined as the ratio of scratch area at 24 h to the scratch area at 0 h for each group.

For the Transwell invasion assay, 1×10^5 indicated cells in 200 μ l serum-free medium were placed in the upper chamber (Corning, Inc.) and cultured at 37°C for 24 h. The chamber had been previously coated with 200 μ l Matrigel (incubated at 37°C for 6 h), and 0.7 ml medium containing 10% FBS was placed in the lower chamber. Cells on the upper surface were wiped off after 24 h of incubation and 4% paraformaldehyde was used to fix the invasive cells on the lower surface of the chamber which was then stained with 0.1% crystal violet solution for 20 min at room temperature. Subsequently, the number of invading cells was counted in five random fields selected by an inverted microscope, and the average was calculated. The migration assay was carried out following the same protocol as the invasion assay, except that Transwell membranes were not coated with Matrigel.

Dual-luciferase reporter assay. In KYSE-150 cells, the promoter-reporter gene plasmids (containing promoter regions derived from CXCL8, SNAI2 and ZEB1) were separately co-transfected with pcDNA3.1-XBP1, pcDNA3.1-ATF4, pcDNA3.1 empty plasmid or treated with TGF- β 1. Luciferase activity was measured 48 h after transfection and treatment under standard culture conditions (37°C; 5% CO₂), using the Dual-Luciferase® Reporter Assay System (cat. no. E1910; Promega Corporation) according to the manufacturer's instructions and normalized to Renilla luciferase activity. The primer sequences for the dual-luciferase reporter plasmids are detailed in Table SIV.

Animal experiments. A total of 10 BALB/c-nude mice (6-week-old male and body weight of 18-22 g) were purchased from the Beijing Huafukang Biotechnology Co., Ltd. Mice were housed under standard conditions and had unlimited access to sterilized food and distilled water. Room temperature was maintained at 24±2°C, relative humidity was maintained at 60±10% and a 12-h light/dark cycle was used. After 7 days of acclimation, the mice were randomly divided into two experimental groups (n=5 per group): i) Control group, ii) CXCL8-overexpressed group. Group i) mice served as the normal control group and were subcutaneously injected with 5×10⁶ control (KYSE-150) cells into the dorsal flanks. Group ii) was the CXCL8-overexpressed group and the mice were injected with a total of 5×10⁶ CXCL8-overexpressing KYSE-150 cells into the dorsal flanks. The total duration of the animal study was 30 days. The mice were monitored daily for food and water intake, weight, body posture, behavior, distress and response to external stimuli.

Humane endpoints of the present study were loss of >20% body weight, severe dehydration, refusal of food, severe pain or distress or a moribund state, no animals reached these endpoints or were found dead during the present study. All mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium and 0.5-1 ml blood was collected by cardiac puncture followed by sacrifice by exsanguination. Indexes such as breathing, heartbeat, pupils and nerve reflexes were assessed to confirm death. Mice were euthanized after 30 days, and terminal volume and weight of tumor tissues were measured. The present study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Fourth Hospital of Hebei Medical University (approval no. IACUC-4th Hos Hebm-2023001).

Immunohistochemical staining assay. In the CXCL8-overexpressing mouse model, both esophageal carcinoma and adjacent normal tissues were collected and fixed in 4% formaldehyde at 4°C for 24-48 h. After washing with PBS, the specimens were dehydrated through a graded ethanol series, embedded in paraffin and sectioned at a thickness of 4- μ m. The sections were then blocked with 3% hydrogen peroxide (15 min at room temperature) to inhibit endogenous peroxidase activity and subjected to antigen retrieval in citrate buffer (pH 6.0) at >95°C for 5 min. Primary antibodies against CXCR1/2, p-SMAD2/3, SNAI2 and ZEB1 (all at 1:100 dilution) were applied and incubated overnight at 4°C, followed by a 2 h room temperature incubation with secondary antibodies. The stained sections were visualized using an inverted optical

microscope. The antibody information is identical to that used for western blotting (Table SIV).

Prediction of SMAD3-regulated miRNAs targeting ZEB1. To identify candidate miRNAs that may be transcriptionally regulated by SMAD3 and simultaneously target ZEB1, bioinformatics screening was conducted using the TransmiR v3.0 (<http://www.cuilab.cn/transmir>) and TargetScan Release 7.1 (http://www.targetscan.org/vert_71/). First, TransmiR v3.0 was queried to identify miRNAs either known or predicted to be regulated by SMAD3. Subsequently, TargetScan was used to predict miRNAs with conserved binding sites in the 3'-untranslated region (3'-UTR) of ZEB1. The predicted miRNAs from both TransmiR v3.0 and TargetScan databases were compared, and the overlapping miRNAs were identified as potential candidates linking SMAD3 and ZEB1.

Statistical analysis. GraphPad Prism 8.0 (Dotmatics) was used to carry out data analysis and graphing. Statistical significance was determined using an unpaired Student's t-test or one-way ANOVA with Tukey's post hoc test. All *in vitro* experiment data came from three independent experiments carried out in triplicate and presented as mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

CXCL8 is induced by ERS in ESCC. ESCC cells were treated with 100 nM TG (an ERS inducer) for 0-24 h (34), after which expression of effectors of the UPR were assessed. The mRNA and protein expression levels of XBP1, ATF4 and ATF6 peaked at 12 h after TG treatment (Fig. 1A and B). To explore the potential mechanism of ERS in ESCC progression, RNA sequencing analysis was employed to compare the gene expression profiles between TE-1 cells treated with either TG or DMSO. CXCL8 was one of the top ranked differentially altered genes in response to ERS (Fig. 1C). The elevated mRNA and protein expression levels of CXCL8 were further verified at 12 h in TG-treated KYSE-150 and TE-1 cells by RT-qPCR and western blot analysis (Fig. 1D and E). CXCL8 was significantly upregulated in a variety of types of cancer, including esophageal cancer by searching the GEPIA database (Fig. 1F and G). The mRNA expression level of CXCL8, as well as its receptors, CXCR1 and CXCR2, was increased in ESCC cells compared with that in HEEC cells (Fig. 1H).

Both the IRE1 α and PERK pathways regulate the transcription of CXCL8. To explore the signaling pathways which may be responsible for induction of CXCL8, three transmembrane sensors (IRE1 α , PERK and ATF6) were respectively knocked down in KYSE-150 and TE-1 cells using siRNAs, and the knockdown efficiency was validated by RT-qPCR assay (Fig. S1A). As shown in Fig. 2A and B, under the DMSO-treated condition, knockdown of the three sensors resulted in reduced expression of their downstream transcription factors (XBP1, ATF4, and ATF6). A decrease in CXCL8 expression was observed only in the siIRE1 α + DMSO and siPERK + DMSO groups under the DMSO-treated condition. Under the TG-treated condition, the siNS + TG group exhibited significantly increased mRNA and protein levels of the three

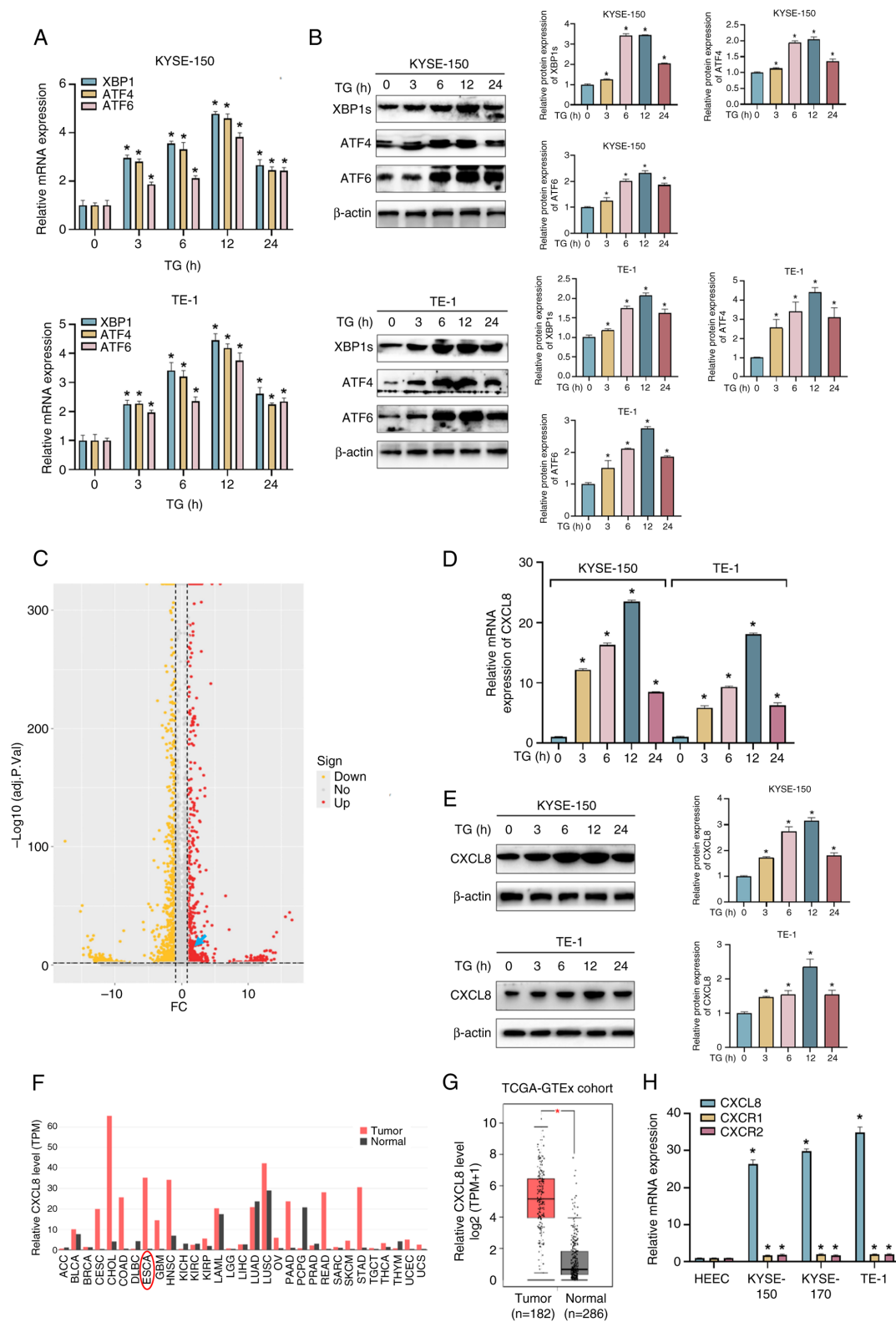


Figure 1. Endoplasmic reticulum stress induces CXCL8 expression in ESCC cells. KYSE-150 and TE-1 cells were treated with 100 nM TG for 0, 3, 6, 12 and 24 h, and expression levels of effectors in the UPR were examined by (A) RT-qPCR and (B) western blot analysis. (C) The volcano plot shows differentially expressed genes in TE-1 cells treated with 100 nM TG for 12 h. The x-axis represents the log2-transformed of FC ratios and the y-axis is the log10-transformed adjusted P-value. Red/yellow dots represent significantly up/downregulated genes ($|\log_2\text{FC}| \geq 1$, adj. $P < 0.05$). The blue arrow indicates the possible location of CXCL8. The (D) mRNA and (E) protein expression levels of CXCL8 in ESCC cells treated with 100 nM TG for 24 h. (F) The CXCL8 expression profile across all tumor samples and corresponding normal tissues was obtained from the GEPIA. (G) The mRNA expression levels of CXCL8 in ESCC tissues (n=182) and normal tissues (n=286) were obtained from the GEPIA database. (H) Expression levels of CXCL8, the receptors CXCR1 and CXCR2 in the normal esophageal epithelial cell line and ESCC cell lines were determined by RT-qPCR method. All data are expressed as mean $\pm$ SD, n=3/group. * $P < 0.05$ vs. TG-treated 0 h group. CXCL8, C-X-C motif chemokine ligand; ESCC, esophageal squamous cell carcinoma; TG, thapsigargin; FC, fold change; GEPIA, Gene Expression Profiling Interactive Analysis; RT-qPCR, reverse transcription-quantitative PCR; HEEC, human normal esophageal epithelial cell; XBP1, X-box binding protein 1; ATF, activating transcription factor; TCGA-GTEx, The Cancer Genome Atlas-Genotype-Tissue Expression; sign, significance; up, upregulated; no, no significance; down, downregulated; TPM, transcript per million.

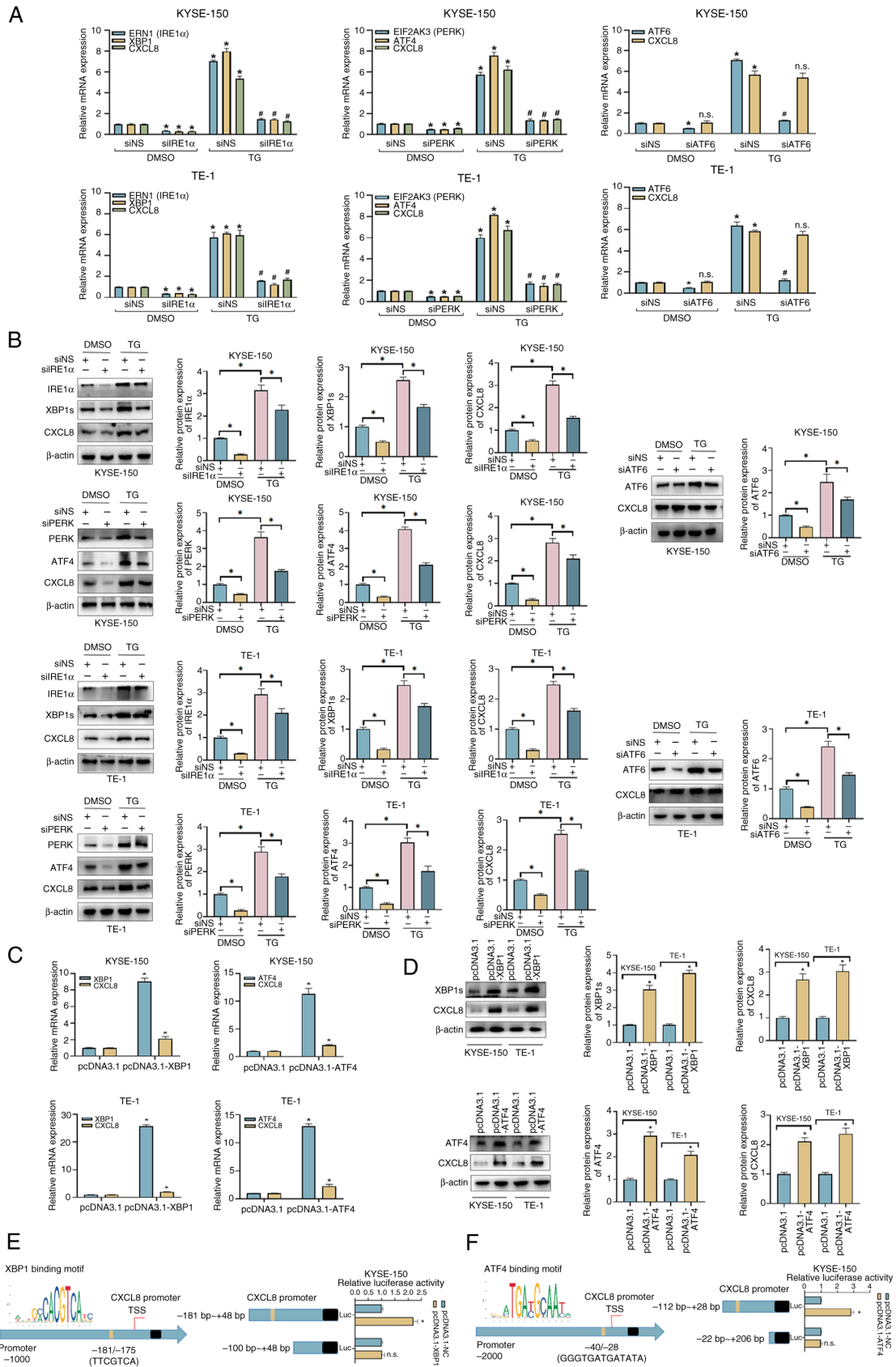


Figure 2. CXCL8 is induced by the PERK and IRE1 α pathways of the unfolded protein response in esophageal squamous cell carcinoma cells. Evaluation of the indicated (A) mRNA and (B) protein expression levels in siRNA-transfected KYSE-150 and TE-1 cells, which were treated with 100 nM TG or DMSO for 24 h. Evaluation of (C) mRNA and (D) protein expression levels of CXCL8 in transfected KYSE-150 and TE-1 cells by reverse transcription-quantitative PCR and western blot analysis. Dual-luciferase reporter assays were conducted in KYSE-150 cells to verify the direct binding effects of (E) XBP1 and (F) ATF4 on the CXCL8 promoter. All data are expressed as mean $\pm$ SD, $n=3$ /group. * $P<0.05$, vs. siNS + DMSO group, siNS + TG group or pcDNA3.1 group; # $P<0.05$ vs. siNS + TG group. n.s., not significant; IRE1 α , inositol-requiring enzyme 1 α ; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; si, small interfering; ERN, endoplasmic reticulum to nucleus signaling; EIF2AK3, ATF, eukaryotic translation initiation factor 2 alpha kinase 3; TG, thapsigargin; NS, non-silencing; CXCL, C-X-C motif chemokine ligand.

sensors, their downstream transcription factors, and CXCL8, compared with the siNS + DMSO group. In the siRNA + TG groups, the mRNA and protein levels of the three sensors, their downstream transcription factors, were decreased compared with those in the siNS + TG group; however, a reduction in CXCL8 mRNA and protein expression was observed only in the siIRE1 α + TG and siPERK + TG groups. Elevated levels of CXCL8 mRNA and protein in KYSE-150 and TE-1 cells overexpressing XBP1 and ATF4, the downstream transcription factors of IRE1 α and PERK, respectively were detected (Fig. 2C and D). In contrast, the expression level of CXCL8 was not influenced by the overexpression of ATF6 (Fig. S1B and C), indicating that upregulation of CXCL8 in ERS may be regulated by the IRE1 α /XBP1 and PERK/ATF4 signaling pathways. As transcription factors, XBP1 and ATF4 carry out key roles by regulating the transcription of various genes which are involved in ERS. A possible binding site of XBP1 on the promoter region of CXCL8 (-181 bp/-175 bp) was predicted by the PROMO database, and the binding effect was further verified by dual-luciferase reporter assay in KYSE-150 cells (Fig. 2E). A possible binding site of ATF4 on the promoter region of CXCL8 (-40 bp/-28 bp) was also predicted and the transcriptional regulatory effect of ATF4 on CXCL8 was also verified (Fig. 2F).

CXCL8 promotes ESCC cells migration and invasion in vitro. In order to study the biological function of CXCL8 in ESCC, KYSE-150 and TE-1 cells were treated with rh-CXCL8, and the expression levels of CXCR1 and CXCR2 were assessed. As shown in Fig. 3A-D, the mRNA and protein expression levels of both receptors peaked at 24 h with 10 ng/ml rh-CXCL8 treatment. ESCC cells were then treated with 10 ng/ml rh-CXCL8 for 24 h to detect the variation of cell function. CXCL8 had no effect on the viability of ESCC cells (Fig. 3E) but significantly promoted their migration and invasion (Fig. 3F and G).

CXCL8/CXCR1 promotes migration and invasion of ESCC cells under an ERS state. Roles of CXCL8/CXCR1 in the progression of ESCC cells under ERS state were further examined. The knockdown efficiency of CXCR1 was validated by an RT-qPCR assay (Fig. S1D). When ESCC cells were treated with 100 nM TG for 24 h, cell migration and invasion were significantly enhanced, while knockdown of CXCR1 partially prevented this effect (Fig. 4A and B).

CXCL8 increases the expression of EMT-related genes, SNAI2 and ZEB1. Considering that both ERS and CXCL8 promoted the migration and invasion of ESCC cells, the mRNA expression levels of a set of EMT-related genes were screened in rh-CXCL8-treated ESCC cells, including SNAI2, ZEB1, TWIST1, SNAIL, FN1, TWIST2, CDH2, VIMENTIN and ZEB2. Among these genes, SNAI2 and ZEB1 demonstrated the most significant trend of increased expression in KYSE-150 (Fig. 5A) and TE-1 cells (Fig. S2) when treated with 10 ng/ml rh-CXCL8. Nevertheless, further increases in the rh-CXCL8 concentration led to a progressive decline in their expression levels. The protein expression levels of SNAI2 and ZEB1, which were similar to their mRNA expression levels, were further verified in rh-CXCL8-treated ESCC cells by western blot analysis (Fig. 5B). Given that SNAI2

and ZEB1 are important genes involved in EMT process, we hypothesized whether CXCL8 promoted the expression of SNAI2 and ZEB1 by regulating the activation of EMT-related pathways. The PI3K/AKT, JAK2/STAT3, Wnt/ β -catenin and TGF- β /SMAD pathways are important pathways that regulate the EMT process, therefore whether CXCL8 promoted the activation of these pathways was further investigated. As shown in Fig. 5C and D, rh-CXCL8 treatment promoted phosphorylation of AKT, STAT3 and SMAD2/3, whereas its effect on β -catenin appeared to be minimal although this was not quantitatively assessed, suggesting that CXCL8 may be involved in the progression of EMT by regulating the activation of AKT, STAT3 and SMAD2/3. The present study revealed several signaling pathways that may be involved in the action of CXCL8. Since activation of AKT and STAT3 by CXCL8 has been reported (35-37), which is consistent with the findings of the present study, the TGF- β /SMAD signaling pathway was the focus of the present study.

CXCL8/CXCR1 promotes the EMT process by activating SMAD2/3. CXCR1 was knocked down to study the activating effect of CXCL8 on SMAD2/3. As shown in Fig. 6A, the protein expression levels of p-SMAD2/3 were significantly reduced when CXCR1 was knocked down, suggesting that CXCL8 may activate SMAD2/3 by binding to CXCR1. The regulatory role of CXCL8/CXCR1 in the ERS state was further analyzed. As shown in Fig. 6B, the expression levels of p-SMAD2/3 were significantly increased in TG-treated ESCC cells, whereas knockdown of CXCR1 partially alleviated this effect.

Analysis revealed that 10 ng/ml CXCL8 significantly promoted the expression of SNAI2 and ZEB1 and whether this effect was achieved through its activation of SMAD2/3 was further investigated. KYSE-150 and TE-1 cells were treated with 10 ng/ml TGF- β 1 for 48 h (38). As shown in Fig. 6C and D, increased mRNA and protein expression levels of SNAI2 and ZEB1 were detected in TGF- β 1-treated cells, potentially indicating the regulatory effect of TGF- β /SMAD signaling pathway on the expression of SNAI2 and ZEB1. A binding site for SMAD3 was identified in the promoter region of SNAI2, and dual-luciferase reporter assay confirmed that exogenous addition of TGF- β 1 promoted transcription of SNAI2 (Fig. 6E). Two binding sites for SMAD3 were also found in the promoter region of ZEB1, but ZEB1 transcription was not affected by exogenous incorporation of TGF- β 1 (Fig. 6F). miR-3146, miR-3163 and miR-3171 were predicted to be transcriptionally regulated by SMAD3 and further regulated the expression of ZEB1 by binding to its 3'-UTR (Fig. 6G).

CXCL8 facilitates ESCC progression in vivo. To evaluate the oncogenic role of CXCL8 *in vivo*, KYSE-150 cells stably overexpressing CXCL8 or control cells were Xeno transplanted subcutaneously into the flanks of mice. Quantitative analysis at 30 days post-inoculation revealed a statistically significant augmentation in both tumor volume and weight in the CXCL8-overexpressing group compared with control group (Fig. 7A-C), suggesting a pro-tumorigenic function of CXCL8. To further validate the role of the CXCL8-CXCR1/2-SMAD2/3-SNAI2/ZEB1

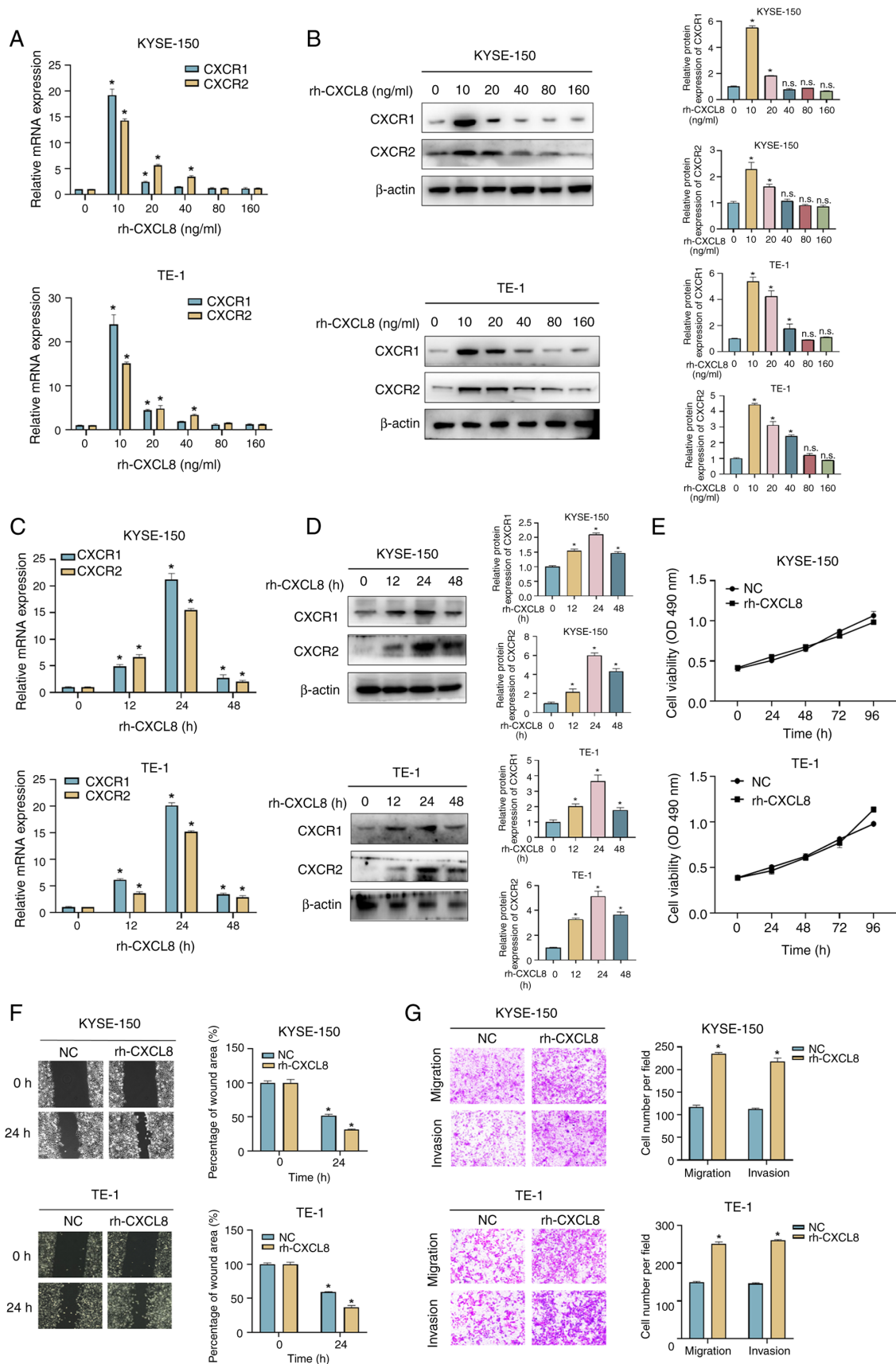


Figure 3. CXCL8 facilitates migration and invasion of ESCC cells. Evaluation of (A) mRNA and (B) protein expression levels of CXCR1 and CXCR2 in KYSE-150 and TE-1 cells treated with 0, 10, 20, 49, 80 and 160 mg/ml rh-CXCL8 for 24 h. Evaluation of (C) mRNA and (D) protein expression levels of CXCR1 and CXCR2 in KYSE-150 and TE-1 cells treated with 10 ng/ml rh-CXCL8 for 0, 12, 24 and 48 h. (E) Cell proliferation was analyzed with an MTS assay in KYSE-150 and TE-1 cells treated with 10 ng/ml rh-CXCL8 for 24 h. (F) Wound healing and (G) Transwell assays were conducted to assess the migration and invasion abilities in KYSE-150 and TE-1 cells treated with 10 ng/ml rh-CXCL8 for 24 h. All data are expressed as mean $\pm$ SD, n=3/group. *P<0.05 vs. 0 ng/ml rh-CXCL8-treated group, rh-CXCL8-treated 0 h group, NC-treated 0 h group or NC group. n.s., not significant; rh-CXCL, recombinant human-C-X-C motif chemokine ligand; CXCR, chemokine receptor; NC, negative control.

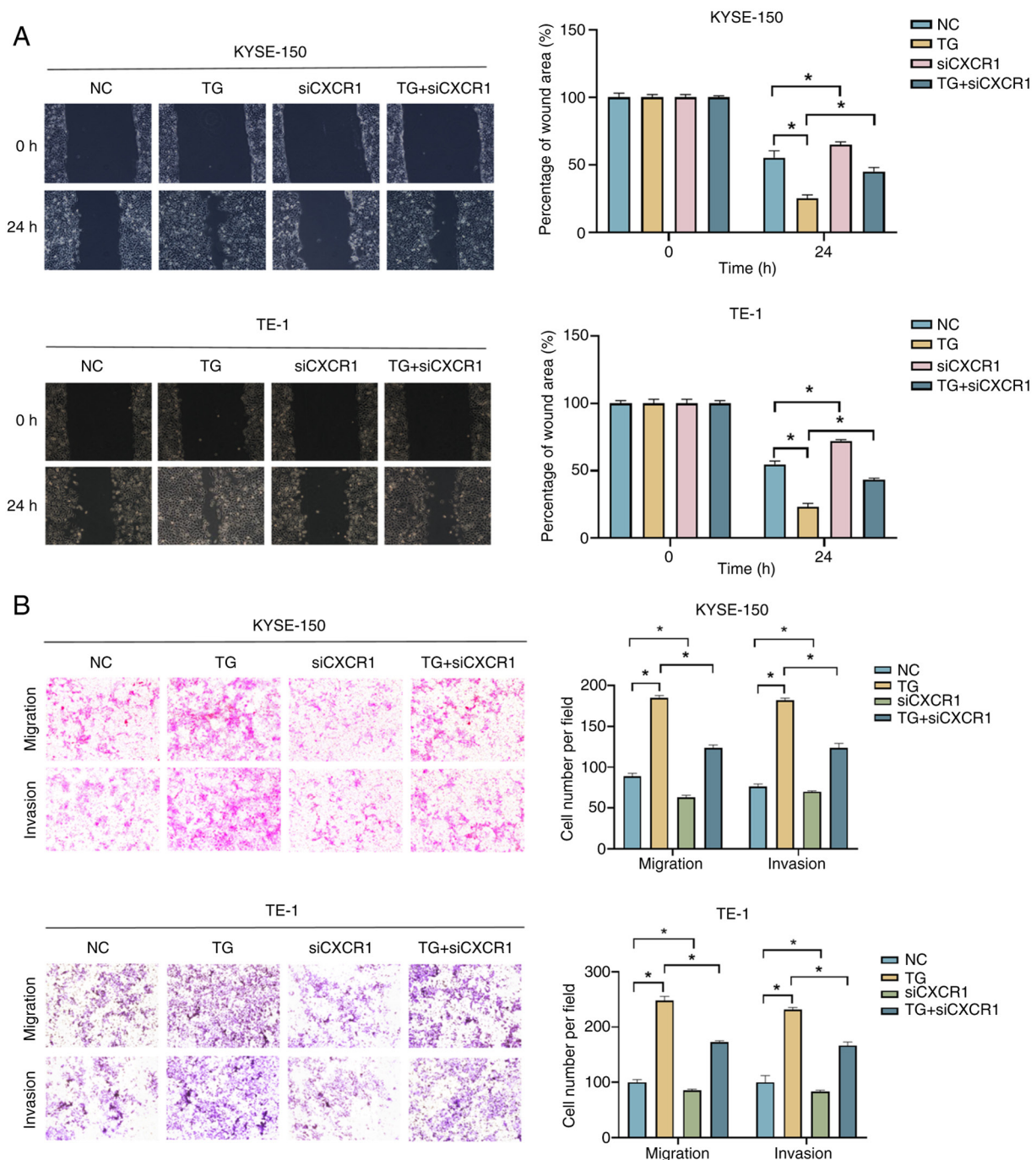


Figure 4. Effect of CXCR1 knockdown on esophageal squamous cell carcinoma cell migration and invasion under an endoplasmic reticulum stress state. (A) Wound healing and (B) Transwell assays were conducted to explore the migration and invasion ability of KYSE-150 and TE-1 cells that underwent various treatments (magnification, x100). All data are expressed as mean $\pm$ SD, n=3/group. *P<0.05 vs. NC-treated 24 h group, TG-treated 24 h group, NC-treated group or TG-treated group. NC, negative control; TG, thapsigargin; si, small interfering; CXCR, chemokine receptor.

axis in tumor metastasis, the protein expression levels of CXCR1/2, p-SMAD2/3, SNAI2 and ZEB1 were examined in CXCL8-overexpressing ESCC tissues. Immunohistochemical analyses revealed enhanced positive staining for CXCR1/2, p-SMAD2/3, SNAI2 and ZEB1 in the CXCL8-overexpression group compared with control group (Fig. 7D).

The aforementioned results suggested that CXCL8, which was highly secreted by ESCC cells in the ERS state, promoted the EMT process by activating SMAD2/3 after binding to its receptors. Activated SMAD2/3 directly or indirectly regulated the transcription of SNAI2 and ZEB1 (Fig. 7E).

Discussion

The significance of ERS in tumor progression is well recognized, where the activation of the UPR intricately governs the fate of tumor cells (39,40). In different types of human cancer, the activation of distinct elements within the UPR cascade was poised to yield diverse cellular outcomes across nearly all stages of tumor cell development (14,17,41-43). In addition, a previous study proposed that the UPR may influence key characteristics defining cancer, encompassing angiogenesis, metastasis, genome stability, inflammatory

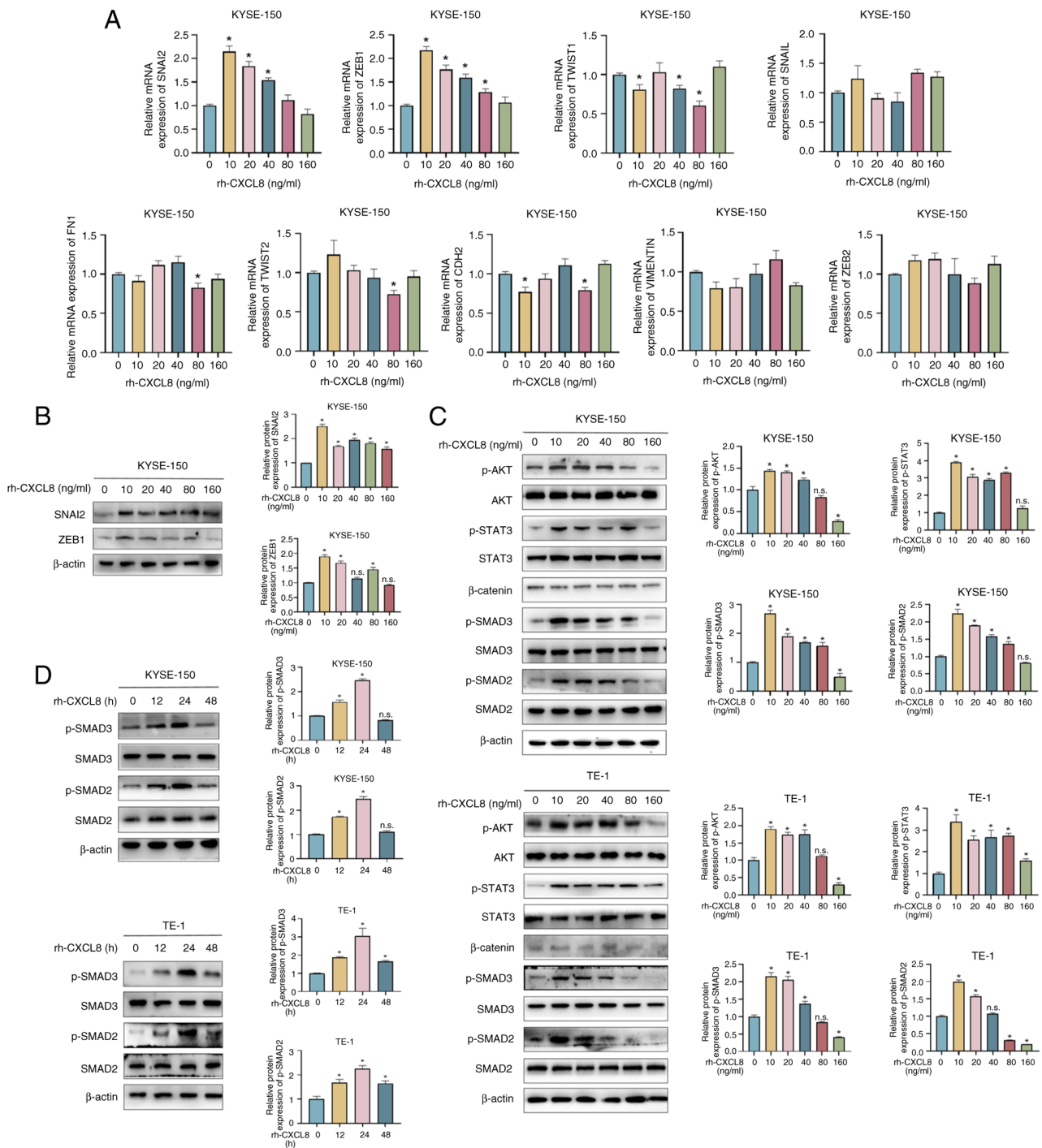


Figure 5. CXCL8 participates in the EMT process by regulating the expression of SNAI2 and ZEB1. KYSE-150 cells were treated with specified concentrations of rh-CXCL8 for 24 h, and then (A) mRNA expression of SNAI2, ZEB1, TWIST1, SNAIL, FN1, TWIST2, CDH2, VIMENTIN and ZEB2 was detected by RT-qPCR, and (B) protein expression of SNAI2 and ZEB1 was detected by western blotting. (C) KYSE-150 and TE-1 cells were treated with specified concentrations of rh-CXCL8 for 24 h and the protein expression levels of EMT-related pathway genes were examined by Western blot assay. (D) KYSE-150 and TE-1 cells were treated with 10 ng/ml rh-CXCL8 for indicated times, and the protein expression of SMAD2/3 and p-SMAD2/3 was detected by Western blot assay. All data are expressed as mean $\pm$ SD, $n=3$ /group. * $P<0.05$ vs. 0 ng/ml rh-CXCL8-treated group or rh-CXCL8-treated 0 h group. n.s., not significant; rh-CXCL, recombinant human C-X-C motif chemokine ligand; EMT, epithelial-mesenchymal transition.

responses and resistance to drugs (39). As for the role of ERS in the emergence and advancement of ESCC, studies have revealed that the ERS-induced PERK/eIF2 α /CHOP pathway might impede proliferation and induce apoptosis (44,45). The activation of the IRE1/JNK pathway in ESCC is implicated in regulating apoptosis and autophagy (46). Notably, the IRE1 α /AKT/mTOR signaling axis has been identified as

an inducer of apoptosis in ESCC cells (47). Nevertheless, to the best of our knowledge, the involvement of CXCL8 in the ERS of ESCC and its specific functional role have not been reported. The present study revealed that CXCL8 was one of the most significantly upregulated genes in ESCC cells with ERS, and may be induced by the IRE1 α -XBP1 and PERK-ATF4 pathways. CXCL8-induced activation of

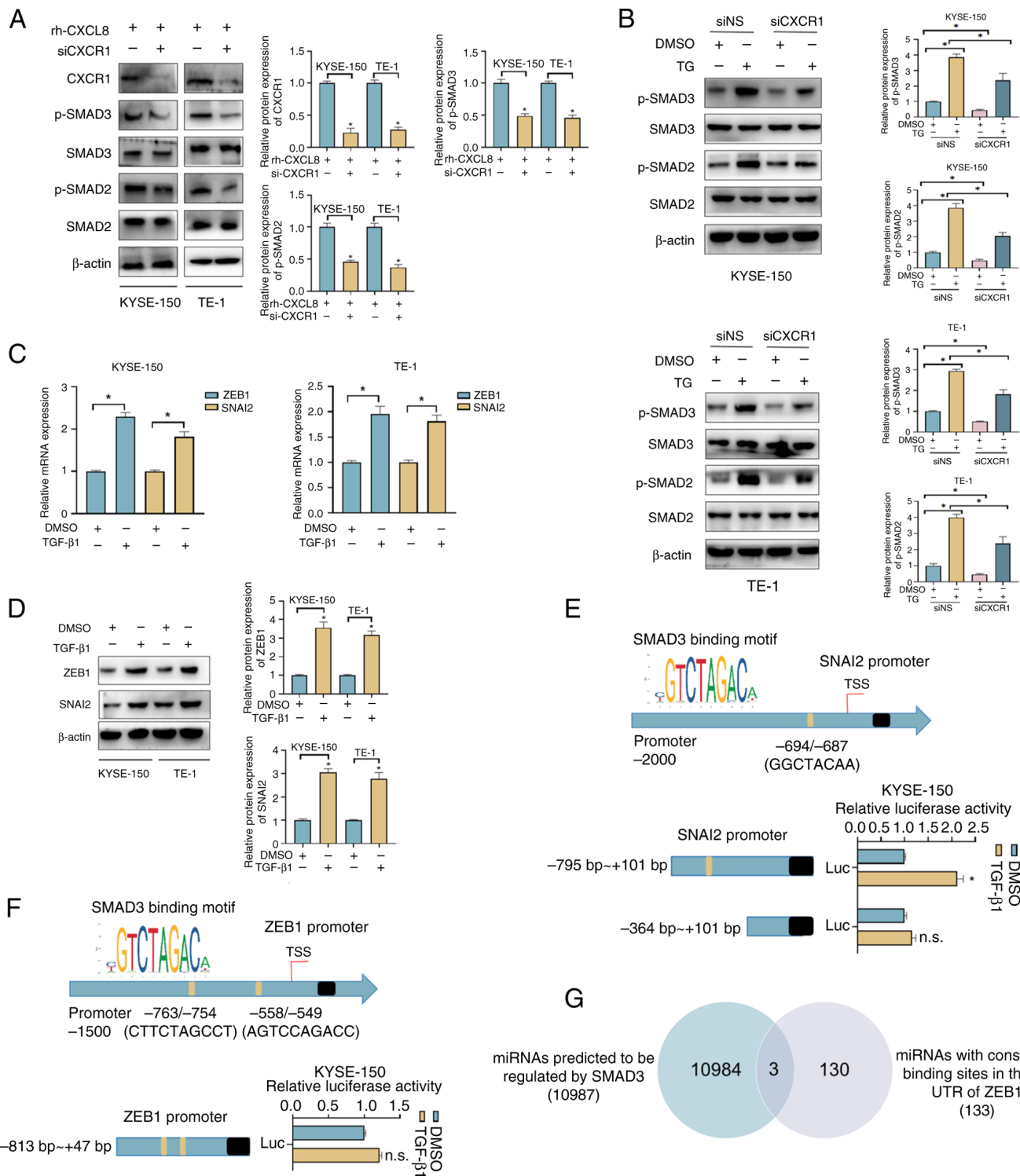


Figure 6. CXCL8 regulates the expression of SNAI2 and ZEB1 through phosphorylated activation of SMAD2/3. (A) Influence of knocking down CXCR1 on the protein expression of SMAD2/3 and p-SMAD2/3 in KYSE-150 and TE-1 cells, treated with 10 ng/ml rh-CXCL8 for 24 h. (B) Influence of knocking down CXCR1 on protein expression of SMAD2/3 and p-SMAD2/3 in TG (100 nM; 24 h) treated KYSE-150 and TE-1 cells. (C) mRNA and (D) protein expression levels of ZEB1 and SNAI2 were examined in KYSE-150 and TE-1 cells treated with TGF- β 1 (10 ng/ml; 48 h) using reverse transcription-quantitative PCR and western blot analysis. Dual-luciferase reporter assays were conducted in KYSE-150 cells to detect the transcriptional regulatory effect of SMAD3 on (E) SNAI2 and (F) ZEB1. (G) Venn diagram showing the bioinformatics identification of SMAD3-regulated miRNAs that target ZEB1. All data are expressed as mean $\pm$ SD, n=3/group. *P<0.05 vs. rh-CXCL8 group, DMSO + siNS group, TG + siNS group or DMSO group. n.s., not significant; rh-CXCL, recombinant human-C-X-C motif chemokine ligand; CXCR, chemokine receptor; p-, phosphorylated; TG, thapsigargin; si, small interfering; NS, non-silencing; Luc, luciferase; miRNA, microRNA; UTR, untranslated region.

SMAD2/3 and SMAD2/3 subsequently exerted direct or indirect regulation of SNAI2 and ZEB1 and promoted the progression of EMT in ESCC cells.

CXCL8, recognized as a multifaceted chemokine, encompasses diverse functions in biological processes and has been implicated in numerous diseases including HIV (48),

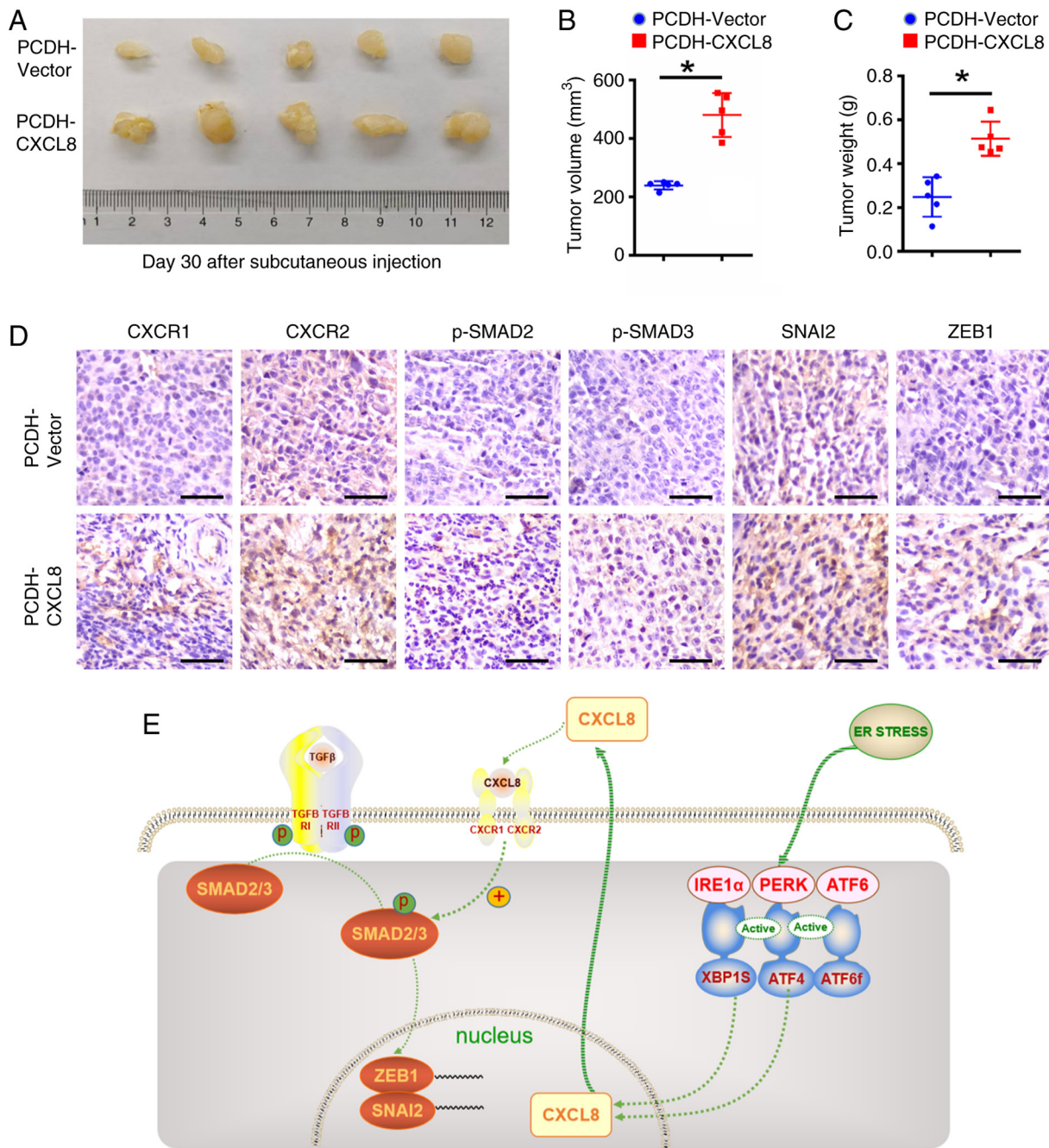


Figure 7. Animal experiments validate the role of the CXCL8-CXCR1/2-SMAD2/3-SNAI2/ZEB1 axis in tumor growth and metastasis. (A) Image of subcutaneous xenograft tumors following injection of CXCL8-overexpressing KYSE-150 cells (n=5) or control cells (n=5) in BALB/c-nude mice. The volume (B) and weight (C) of harvested xenograft tumors were measured (n=5). (D) Representative immunohistochemical images of CXCR1/2, p-SMAD2/3, SNAI2 and ZEB1 expression in CXCL8-overexpressing ESCC tissues and corresponding control tissues (n=5). Scale bar, 50 μ m. (E) Mechanistic diagram of CXCL8 in ESCC cells under an ER stress state. All data are expressed as mean $\pm$ SD. *P<0.05, vs. PCDH-Vector group. CXCL, C-X-C motif chemokine ligand; IRE1 α , inositol-requiring enzyme 1 α ; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; ATF, activating transcription factor; XBP, X-box binding protein; ER, endoplasmic reticulum; p-, phosphorylated; ESCC, esophageal squamous cell carcinoma.

inflammatory bowel disease (49), reperfusion injury (50) and chronic obstructive pulmonary disease (51,52). The increased expression of CXCL8 has also been reported in several types of cancer. For example, elevated CXCL8 enhances the tumorigenic properties of human colon cancer LoVo cells by activating specific signaling pathways (23); CXCL8 serves as a pivotal mediator in the tumor-stroma-inflammation network of triple-negative breast cancer (24) and CXCL8 functions as an autocrine growth regulator in human lung cancer pathogenesis (25).

Mechanistically, the present study uncovered a previously unrecognized role of CXCL8 in driving ESCC progression by engaging in ERS-associated cascades, challenging the conventional perspective on its pro-metastatic functions. Functional analyses demonstrated that tumor-derived CXCL8 served in a dual capacity. It operated in a paracrine manner, modifying the immune cell composition within the tumor microenvironment. Simultaneously, CXCL8 acted in an autocrine manner, promoting oncogenic signaling, angiogenesis and fostering

pro-metastatic traits such as invasion and resistance (53). However, the functional role and underlying mechanisms of CXCL8 in the ERS state tumors remain poorly investigated, with limited reports in only a few types of cancer. In breast cancer cells, CXCL8 has been identified as one of the secretory factors displaying the highest fold change after treatment with TG and it might be induced via the PERK-CEBP δ pathway (54). In thyroid tumors, TG treatment markedly increased the expression of CXCL8 at both the mRNA and protein levels (55). In non-small cell lung adenocarcinoma A549 cells, the transcription factors ATF4 and P65 downstream of the UPR directly orchestrated the transcription of CXCL8 (56). The present study revealed that the upregulation of CXCL8 in the ERS state ESCC cells was not only regulated by the PERK/ATF4 signaling pathway as observed in non-small cell lung adenocarcinoma (56), but also regulated by the IRE1 α /XBP1 pathway. The evident upregulation of CXCL8 by the two pathways promoted ESCC migration and invasion through participating in the EMT process.

The augmentation of CXCL8-CXCR1/2 expression has been observed in several tumors. CXCL8/CXCR1/2 promoted androgen-independent prostate cancer cell growth and proliferation ability by activating cyclin D1 expression (57). In pancreatic cancer (PaCa), CXCL8-CXCR2 notably increased cancer cell angiogenesis, proliferation and invasion ability, and CXCR2 was an anti-angiogenic target in PaCa (58). CXCR1 also promotes proliferation, migration and invasion of gastric cancer *in vitro* and *in vivo* (59). CXCL8 enhances angiogenesis of glioblastoma endothelial cells via CXCR2 signaling (60). The present study revealed that CXCL8-CXCR1/2 could facilitate the migration and invasion of ESCC cells *in vitro*. The promotion of tumor progression by CXCL8 occurs due to its binding to CXCR1 and CXCR2 receptors, indicating that targeting this interaction could be a promising therapeutic strategy for ESCC.

The CXCL8-CXCR1/2 axis has been reported to be involved in tumor progression through activating several signaling pathways, including PI3K/AKT (36,61), JAK2/STAT3 (37), MAPK (35,62), phospholipase C (37) and Rho-GTPase (37). In the present study, aside from the aforementioned pathways, the phosphorylated activation of SMAD2/3 by CXCL8/CXCR1 was observed. The activated SMAD2/3 directly or indirectly modulated the transcription of EMT markers SNAI2 and ZEB1 to further participate in the EMT process. The direct transcriptional regulatory effect of p-SMAD2/3 on SNAI2 observed in the present study was consistent with the study by Brandl *et al* (63) in panc1 cells, as for the indirect regulation of p-SMAD2/3 on ZEB1, there may be intermediate genes or mechanisms involved. For example, in the process of renal fibrosis, the miR-200 family regulates TGF- β 1-induced renal tubular EMT through the SMAD pathway by targeting ZEB1 (64). Based on bioinformatics analysis using the TransmiR v3.0 database and TargetScan Release 7.1, miR-3146, miR-3163 and miR-3171 were predicted to be regulated by SMAD3 and target the 3'-UTR of ZEB1 to modulate its expression. However, further studies need to be carried out to verify this prediction.

In summary, the findings of the present study highlighted that CXCL8, originating from the IRE1 α -XBP1 and PERK-

ATF4 pathways in ERS, facilitated the EMT process in ESCC cells through the CXCL8-CXCR1/2-SMAD2/3-SNAI2/ZEB1 axis. This underscored the potential therapeutic strategy of targeting the CXCL8-CXCR1/2 axis to intervene in ERS signaling, hopefully improving therapeutic efficacy in ESCC treatment.

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Availability of data and materials

The sequencing data generated in the present study may be found in the NCBI SRA database under accession number PRJNA1256794 or at the following URL: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1256794>. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

WG and LL contributed to the conception and design of the study, data acquisition, analysis and interpretation, supervision of the experiments, and revision of the manuscript. JW carried out the experiments, analyzed the data and drafted the paper. FS and JL collected the data and prepared the tables and figures. HX, XY and FL carried out the statistical analysis. All authors read and approved the final manuscript. WG and LL confirm the authenticity of all raw data.

Ethics approval and consent to participate

All animal experiments in the present study were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals and relevant institutional guidelines. The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Fourth Hospital of Hebei Medical University (approval no. IACUC-4th Hos Hebmu-2023001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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