Technology in Cancer Research & Treatment
Volume 20: 1–9

DOI: 10.1177/15330338211038492
journals.sagepub.com/home/tct
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NANOG Promotes Cell Proliferation, Invasion, and Stemness via IL-6/STAT3 Signaling in Esophageal Squamous Carcinoma

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Abstract

Background: Cancer cells have properties similar to those of stem cells, including high proliferation and self-renewal ability. *NANOG* is the key regulatory gene that maintains the self-renewal and pluripotency characteristics of embryonic stem cells. We previously reported that knockdown of the pluripotent stem cell factor NANOG obviously reduced the proliferation and drug-resistance capabilities of esophageal squamous cell carcinoma (ESCC). In this study, we gained insights into the potential regulatory mechanism of NANOG, particularly in ESCC. **Methods:** NANOG was ectopically expressed in the Eca-109 cell line via pcDNA3.1 vector transfection. The mRNA expression of different genes was detected using quantitative real-time polymerase chain reaction, and protein quantification was performed by western blotting. The enzyme-linked immunosorbent assay was used to detect the expression of interleukin 6 (IL-6). The capabilities of proliferation, migration, and invasion were investigated using cell count and Transwell assays. The tumor sphere-forming assay was used to investigate the sphere formation capacity of cancer stem cells. **Results:** The expression of NANOG promoted the cell proliferation and sphere formation capacity of cancer stem cells in a dose-dependent manner. IL-6-mediated activation of signal transducer and activator of transcription 3 (STAT3) was closely related to the expression of NANOG in ESCC. Consistently, the target genes of STAT3, including *CCL5*, *VEGFA*, *CCND1*, and *Bcl-xL*, were upregulated upon the overexpression of NANOG. **Conclusion:** These results revealed that the expression of NANOG promotes cell proliferation, invasion, and stemness via IL-6/STAT3 signaling in ESCC.

Keywords

esophageal cancer, NANOG, cell proliferation, IL-6, STAT3

Abbreviations

ESCs, embryonic stem cells; CSCs, cancer stem cells; ESCC, esophageal squamous cell carcinoma; ON cells, NANOG overexpression cells; , ATP binding cassette subfamily G member 2 (ABCG2); IL-6, interleukin 6; JAKs, Janus kinases; p-STAT3, phospho-STAT3; STAT3, signal transducer and activator of transcription 3; qPCR, quantitative real-time PCR

Received: January 20, 2021; Revised: July 6, 2021; Accepted: July 22, 2021.

Introduction

Esophageal cancer is the seventh most common cancer and the fourth leading cause of cancer-related deaths, with 5-year survival rates as low as 13%.[1] Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer in Asia, ranking fourth by incidence in China.[2] Currently, the treatment of esophageal cancer is mainly based on esophagectomy combined with radio- and chemotherapy; some natural products such as curcumin are expected to be used in adjuvant

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treatment.[3] Therefore, it is vital to elucidate the molecular mechanisms of ESCC development, with the goal of early detection and therapy to inhibit further tumor progression.

Cancer cells often share many key biological properties with embryonic stem cells (ESCs), including tumor formation and self-renewal ability.[4-8] These properties are often attributed to the expression of pluripotency genes,[9-12] such as those of NANOG (Nanog homeobox), OCT3/4 (POU class 5 homeobox 1), and SOX2 (SRY-box transcription factor 2), which are essential transcription factors for maintaining ESC totipotency.[13,14] Numerous studies have shown that many pluripotency factors are expressed in solid tumors and participate in tumor development.[15-21] NANOG is one such mediator that is expressed in various cancers, such as ovarian,[22] breast,[23] and prostate cancers,[18] and is enriched in cancer stem cells (CSCs).[19] Previously, we investigated the correlation between the expression of NANOG and the malignant characteristics of ESCC.[24] We found that NANOG mRNA and protein were highly expressed in ESCC cell lines. Further, mRNA silencing technology was used to knock down NANOG expression in ESCC. We found that the clonal formation, proliferation, and drug resistance of Eca-109 cells decreased upon the downregulation of NANOG expression. Moreover, NANOG deficiency downregulated the expression of ATP binding cassette subfamily G member 2. Therefore, in this study, we mainly focused on the tumor-promoting effect and related mechanism of NANOG in ESCC.

Materials and Methods

Cell Culture and Transfection

Eca-109, KYSE-150, and TE-1 cells were purchased from the Shanghai cell bank, Chinese Academy of Sciences. The complete medium (DMEM with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin [Hyclone]) configuration was used for cell culture. shRNA against human NANOG have been described by Deng et al.[24] Ectopic expression plasmids (control and NANOG) were constructed by Shanghai GeneChem Co., Ltd Lipofectamine 2000 (Invitrogen) was used for cell transfection. After 16 to 24 h, a selective medium (supplemented with 2.0 μg/mL puromycin) was used to remove un-transfected cells. In this study, commercial immortalized cell lines were used, and no human or animal experiments were conducted.

Tumor Sphere-Forming Assay

Eca-109 cells were plated in super-low-adherence dishes (NEST), and the medium (DMEM-F12, B27, epidermal growth factor [20 ng/mL]), and basic fibroblast growth factor [20 ng/mL]) was replaced every 3 days. Spheres larger than 2 mm in diameter were counted.

Cell Migration and Invasion

Transwell chambers (8 μm pores, Corning) were coated with and without Matrigel for migration and invasion, respectively. Cells (5×10^4) were suspended in serum-free media and seeded onto the upper chambers; the lower chambers were supplemented with 20% fetal bovine serum. After 24 h, the cells on the lower chamber were fixed in 4% paraformaldehyde for 15 min and stained with crystal violet staining solution. The cells were then counted under a microscope. The experiments were repeated thrice.

Enzyme-Linked Immunosorbent Assay (ELISA)

The Human IL-6 ELISA Kit (MULTI SCIENCES) was used to measure the expression of interleukin 6 (IL-6) in the medium. The culture medium was collected 3 days after cell culture (13 000 r/min, 5 min). ELISA was performed according to the manufacturer's instructions. A microplate reader was used to measure the absorbance at 450 nm.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) Analysis

Referring to the operating instructions, TRIzol (Invitrogen) was used for the extraction of RNA. M-MLV reverse transcriptase (Invitrogen) was used for reverse transcription of RNA. qPCR was performed with EvaGreen Supermix (Bio-Rad), using an IQ-5 Real-Time polymerase chain reaction (PCR) machine (Bio-Rad). The primer sequences used are shown in Table 1.

Protein Extraction and Western Blot Analysis

The spheroid or adherent Eca-109 cells were collected and lysed in cell lysis buffer. Then, the lysates were vortexed every 5 min for 25 min on ice, sonicated, and centrifuged at 12 000*g* and 4°C for 10 min. The protein samples were separated using 10% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for western blot analysis. The primary antibodies used were anti-NANOG (Santa Cruz, sc-134218, 1:2000), signal transducer and activator of transcription 3 (STAT3; HuaBio, ET1607 to 38, 1:1000), phospho-STAT3 (p-STAT3; HuaBio, ET1603 to 40, 1:1000), IL-6 (HuaBio, EM1701 to 45, 1:1000), phospho-Janus kinase 2 (p-JAK2; HuaBio, ET1607 to 34, 1:500), glyceraldehyde 3-phosphate dehydrogenase (Abcam, ab8245, 1:10000), PTEN (HuaBio, RT1519, 1:1000), and Phospho-PTEN(S380) (HuaBio, ET1701 to 46, 1:500). The HRP-conjugated anti-rabbit/mouse secondary antibody was used to enable detection. The ECL-Plus detection system (Bio-Rad) was used to visualize the bands.

Statistical Analysis

Results were expressed as means ± SD. The 2-tailed Student's *t*-test was used to compare means between groups. *P*-values less than .05 were considered statistically significant.

Table 1. Primers for qPCR analysis.

Genes	Forward (5′-3′)	Reverse (5′-3′)
NANOG	ACCTATGCCTGTGATTTGTGG	AGTGGGTTGTTTGCCTTTGG
GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG
Bcl-xl	GACATCCCAGCTCCACATC	GTTCCCATAGAGTTCCACAAAAG
CCND1	CATCTACACCGACAACTCCATC	TCTGGCATTTTGGAGAGGAAG
c-Myc	TTCGGGTAGTGGAAAACCAG	AGTAGAAATACGGCTGCACC
MCL1	AAGGACAAAACGGGACTGG	ATATGCCAAACCAGCTCCTAC
VEGFA	AGTCCAACATCACCATGCAG	TTCCCTTTCCTCGAACTGATTT
Snail	ACAAGCACCAAGAGTCCG	ATGGCAGTGAGAAGGATGTG
MMP9	ACGTGAACATCTTCGACGCCATC	TCAGAGAATCGCCAGTACTTCCC
CCL5	TGCCCACATCAAGGAGTATTTC	CCATCCTAGCTCATCTCCAAAG
IL-6	CCACTCACCTCTTCAGAACG	CATCTTTGGAAGGTTCAGGTTG

Abbreviations: qPCR, quantitative real-time; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *Bcl-xl*, B-cell lymphoma-extra-large; VEGFA, vascular endothelial growth factor A; CCND1, cyclin D1; IL-6, interleukin 6; MMP9, matrix metalloproteinase 9; CCL5, C-C chemokine ligand 5.

Results

NANOG Expression Positively Correlates with Cell Proliferation, Migration, and Cancer Stem-Like Characteristics in ESCC

We previously provided preliminary evidence that NANOG is highly expressed in ESCC, and that NANOG knockdown dramatically reduces cell proliferation.[24] To clarify the role of NANOG in ESCC, we constructed an ectopic NANOG vector (pcDNA3.1-NANOG) and transfected different doses of ectopic NANOG in the Eca-109 cell line (ON cells). The specificity of pcDNA3.1-NANOG was verified using the rise in mRNA and protein levels of NANOG in different ESCC cell lines (Figure 1A and B). We found that the cell proliferation of Eca-109 cells positively correlated with the dose of ectopically expressed NANOG (Figure 1B). The total number of cells increased upon the overexpression of NANOG.

NANOG is an important transcription factor that maintains the pluripotency of ESCs. There is accumulating evidence that NANOG plays a critical role in tumorigenesis.[25] In this study, we determined the roles of NANOG in ESCC migration and cancer stem-like properties. The sphere-forming assay was used to determine the relationship between the expression of NANOG and the sphere-forming ability in ESCC. In this experiment, we observed that the sphere-forming ability of ESCC was closely related to the expression of NANOG. Spheroid body formation increased significantly in the NANOG overexpression group (ON) and decreased in the knockdown group (shN1) (Figure 1C and F left). Moreover, as shown in Figure 1D and F (right), knocking down NANOG significantly reduced the ability of Eca-109 cells to migrate through Transwell pores, whereas overexpression of NANOG increased migration. These results indicate that NANOG expression is consistently required for ESCC cell proliferation, migration, and cancer stem-like properties ($*P<.05$; $**P<.01$; $***P<.001$; $****P<.0001$).

NANOG Promotes Cancer Cell Characteristics in ESCC by Activating IL-6/STAT3 Signaling

Cell proliferation, self-renewal, and EMT are regulated by various signaling pathways such as the TGF-β, Notch, STAT3, Wnt/β-catenin, and Hedgehog pathways.[26] We examined whether NANOG is related to key factors in these signaling pathways. We found that the expression of IL-6 changed in a quantitative manner with the expression of NANOG (Figure 2A and B). The levels of IL-6 mRNA were determined using qPCR experiments (Figure 2C), and the level of IL-6 secreted into the growth medium was detected using an ELISA kit (Figure 2E). ($*P<.05$; $**P<.01$; $***P<.001$). For most tumors, inflammation is a risk factor associated with tumor development and metastasis.[26] The inflammatory cytokine IL-6 has been demonstrated to promote metastasis in a variety of tumor models.[27] In addition, IL-6 increases the expression of anti-apoptotic proteins in ovarian cancer cells by activating the STAT3 signaling pathway.[28]

More importantly, studies have shown that Oct4 can regulate STAT3 expression in embryonic stem cells.[29] Therefore, we hypothesized that NANOG, which is also an important stem cell regulatory transcription factor, plays a role in the cell proliferation, invasion, and CSC properties of ESCC by regulating IL-6/STAT3. Hence, we detected the expression of total STAT3 and p-STAT3 (Y705) in Eca-109 ON cells, Eca-109 CON (control) cells (Figure 2A and D), and Eca-109 shN1 cells (Figure 2B). Consistently, NANOG promoted cell proliferation and CSC properties through IL-6/STAT3 in TE-1 and KYSE-150 cells (Supplemental Figure S1A to D). The results showed that the expression of p-STAT3 and p-JAK2 protein positively correlated with NANOG expression, including the loss and overexpression conditions. However, NANOG expression had little effect on the total STAT3 level. These findings suggested that NANOG promoted cell proliferation, invasion, CSC properties, and resistance ability through IL-6/STAT3 activation in ESCC.

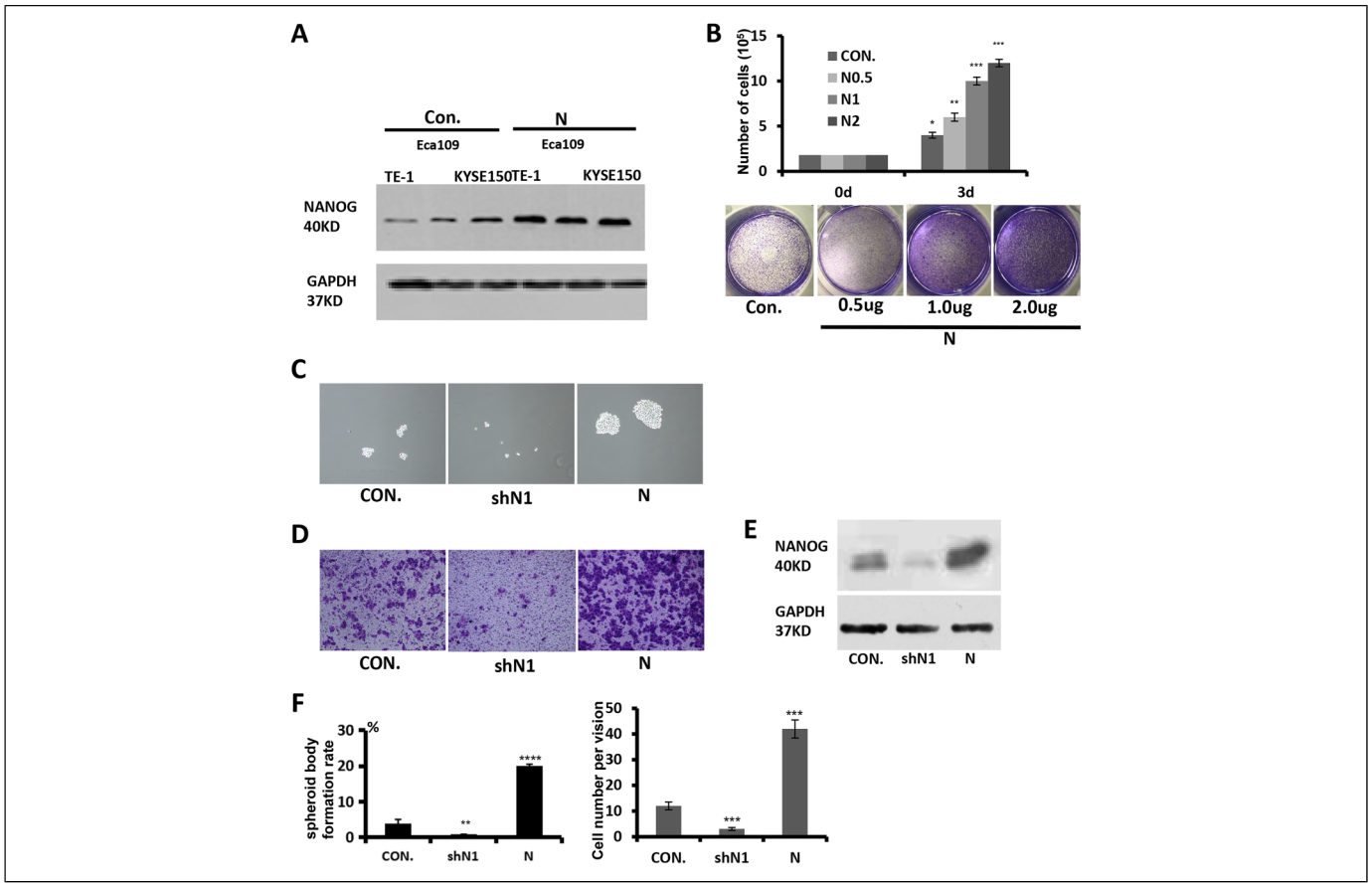

Figure 1. NANOG expression positively correlates with cell proliferation, migration, and cancer stem-like properties in ESCC. (A) Western blotting was performed to analyze the ectopic expression of NANOG in ESCC. The protein concentrations of the samples were detected using the BCA assay. (B) Cell numbers after using a series of doses of NANOG expression in Eca-109 cells. Bottom panel: bright-field microscopy of cells stained with crystal violet solution on day 5 after NANOG overexpression. Scale bar: 200 μm. (C) Spheroid bodies are derived from Eca-109 CON, shN1, and ON cells. (D) Matrigel-coated Transwell assays were performed to determine the capability of cell invasion. Cells were stained, counted, and photographed (100×). (E) The expression of NANOG in Eca-109 CON, shN1, and ON cells was detected using protein blot analysis. (F) Comparison of rates of spheroid body formation of Eca-109 ON, CON, and shN1 groups (left). The number of Eca-109 ON, CON, and shN1 cells passing through the matrix (right). All the results were normalized to the blank, based on 3 repetitions (*$P<.05$; **$P<.01$; ***$P<.001$; ****$P<.0001$).

Abbreviations: ESCC, esophageal squamous cell carcinoma; BCA, Bicinchoninic acid; ON cells, NANOG overexpression cells.

Target Genes of STAT3 are Differentially Expressed Upon NANOG Expression

Multiple genes associated with tumor growth and metastasis that are target genes of STAT3 play an important role in the occurrence and metastasis of tumors.[30] We evaluated the related genes in Eca-109 cells under overexpression or knockdown of NANOG. Among these target genes, we chose a group of representative genes whose functions are known: Myelocytomatosis viral oncogene homolog (*MYC*), *Bcl-xL*, *Mcl1* (apoptosis-related), *Snail* (migration and invasion-related), *MMP9*, *CCL5* (resistance), *CCND1* (cell cycle-related), and *VEGFA* (angiogenesis-related). The results showed that the mRNA expression of *CCL5*, *CCND1*, and *VEGFA* significantly increased in a dose-dependent manner when NANOG was overexpressed in Eca-109 cells (Figure 3A to C). It is notable that *CCL5*, *CCND1*, and *VEGFA* in most tumors are associated with the proliferation and invasion-ability genes and are significantly overexpressed.[31-34] Consistently, *Bcl-xL*, *Mcl1*, *MYC*, *MMP9*, and *Snail* were differentially expressed upon NANOG expression (Figure 3D to H). Snail, CCL5, CCND1, MYC, and VEGFA levels increased upon NANOG overexpression, whereas MMP9 and Mcl1 levels changed inconsistently in TE-1 and KYSE-150 cells (Supplemental Figure S2A to G) (*$P<.05$; **$P<.01$; ***$P<.001$; ****$P<.0001$). These results revealed that diverse downstream target genes were dynamically regulated by NANOG expression via the IL-6/STAT3 pathway in ESCC.

Inhibition of IL-6/STAT3 Signaling Blocks Cell Proliferation, Invasion, and Cancer Stem-Like Properties in Eca-109 ON Cells

To further confirm whether NANOG promoted cell proliferation, invasion, and cancer stem-like properties via the IL-6/

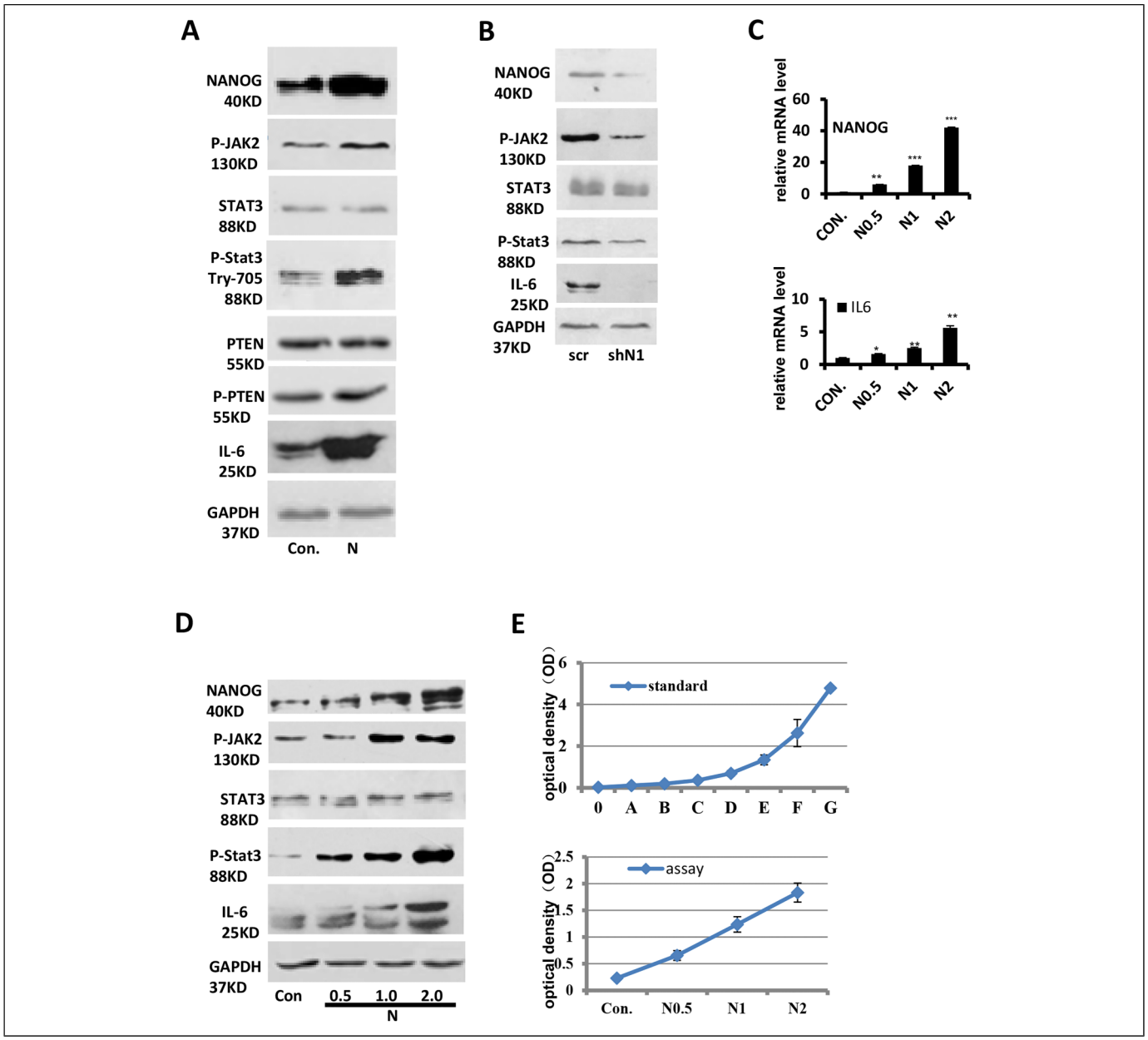

Figure 2. NANOG promotes cancer cell characteristics in ESCC by activating IL-6/STAT3 signaling. (A and B) Expression of IL-6/STAT3, etc was detected by western blotting (A. overexpression and B. knockdown). (C) qPCR tests were used to determine the mRNA expression of NANOG and IL-6 for ectopic expression of different doses of NANOG. (D) Comparison of p-JAK2, total STAT3, p-STAT3, and IL-6 expression by immunoblotting for ectopic expression of different NANOG doses in Eca-109 cells. (E) The level of IL-6 secreted into the growth medium upon ectopic NANOG expression (lower panel) was detected using an ELISA kit, and compared with that of standard samples (upper panel) (*$P <$ .05; **$P <$ .01; ***$P <$ .001).

Abbreviations: ESCC, esophageal squamous cell carcinoma; STAT3, signal transducer and activator of transcription 3; IL-6, interleukin 6; qPCR, quantitative real-time PCR; ELISA, enzyme-linked immunosorbent assay; p-JAK2, phospho-Janus kinase 2.

STAT3 pathway, we blocked this pathway using antibodies of the IL-6 receptor or a STAT3 inhibitor (S31 to 201) in Eca-109 ON cells.[35,36] We found that blocking of antibodies and inhibition of STAT3 phosphorylation both suppressed cell proliferation, cancer stem-like properties, and invasion effects in Eca-109 ON cells (Figure 4A to D) (**$P <$ .01; ***$P <$ .001). These findings clearly indicated that the effects of NANOG were specifically due to IL-6/STAT3 signaling in ESCC.

Discussion

Previously, we have demonstrated that the pluripotent stem cell regulation factor NANOG is highly expressed in ESCC and participates in the development of esophageal cancer.[24] In this work, our aim was to determine how NANOG implements its regulating role in ESCC. We constructed a vector for the ectopic expression of NANOG. We found that overexpression

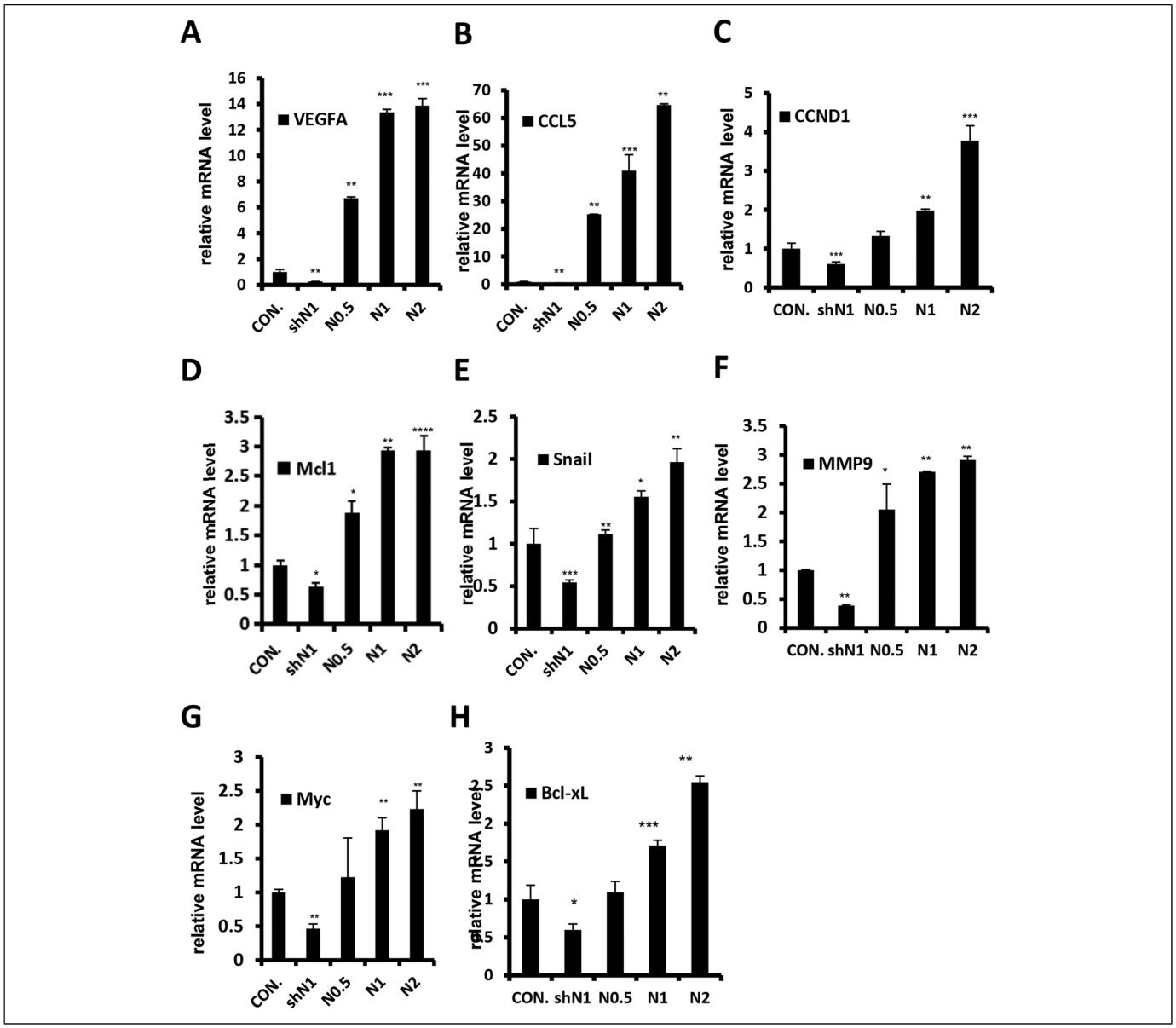

Figure 3. The downstream genes of STAT3 were differentially expressed upon ectopic NANOG expression. (A–H) qPCR tests were performed to evaluate the mRNA expression of multiple target genes of STAT3 from Eca-109 ON cell samples. (A) *VEGFA*; (B) *CCL5*; (C) *CCND1*; (D) *Mcl1*; (E) *Snail*; (F) *JAK1*; (G) *MYC*; (H) *Bcl-xL*. (*$P < .05$; **$P < 0.01$; ***$P < .001$; ****$P < .0001$).

Abbreviations: STAT3, signal transducer and activator of transcription 3; qPCR, quantitative real-time PCR; VEGFA, vascular endothelial growth factor A; CCND1, cyclin D1; CCL5, C-C chemokine ligand 5; JAK1, phospho-Janus kinase 1; MYC, myelocytomatosis viral oncogene homolog; *Bcl-xl*, B-cell lymphoma-extra-large.

of NANOG promoted cell proliferation, invasion, and sphere formation in Eca-109 cells. It is notable that cell proliferation and invasion ability play an important role during tumor recurrence and metastasis. Consistently, the molecular experiments uncovered a novel mechanism: pluripotent transcription factor NANOG can act directly on tumor cell characteristics by activating IL-6/STAT3 signaling in ESCC. The data presented herein, combined with our previous results, reveal the function and mechanism of action of NANOG in ESCC. Inflammation is a risk factor in most tumors and is closely related to tumor progression and metastasis.[37] When the IL-6 ligand binds to its receptor (coupled with gp130), signaling is activated. This binding leads to the activation of STAT3 by inducing the autophosphorylation and activation of Janus kinases (JAKs). In fact, changes in the downstream genes of STAT3 are the major causes of IL-6-related effects.[27,35] The activation of STAT3 is important for normal cells, but the signal is strictly controlled. However, the abnormal expression of downstream genes (such as *CCL5*, *CCND1*, *Snail*, *Twist*, and *VEGF*) promotes cell proliferation and prevents apoptosis, whereas the activity of STAT3 signaling is out of control in various tumor cells.[31-33,38] The expression of anti-apoptosis-related proteins

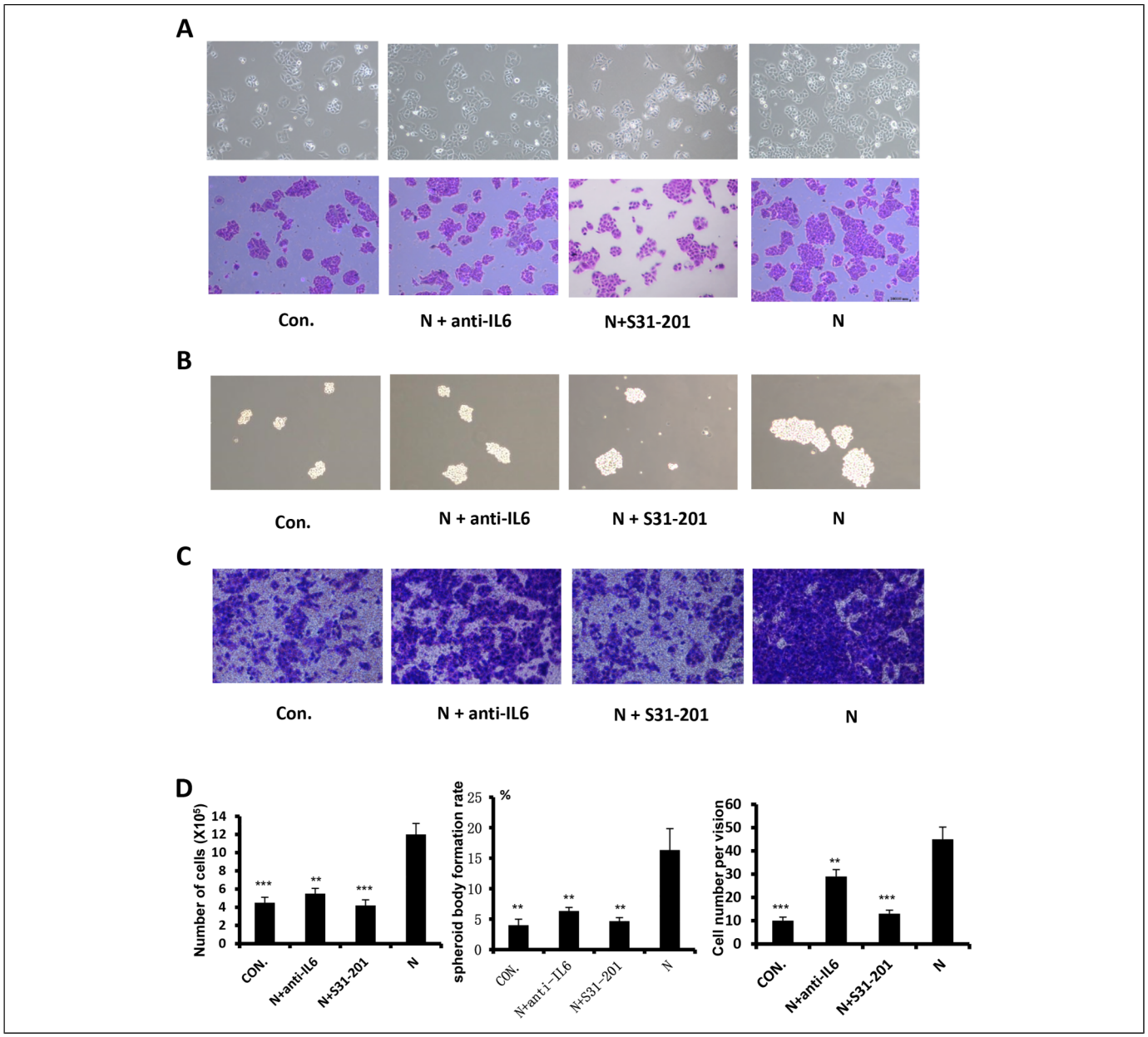

Figure 4. Inhibition of IL-6/STAT3 blocked cell proliferation, invasion, and cancer stem-like properties in Eca-109 ON cells. (A) Microscopy of cell cloning of Eca-109 CON and ON cells with or without anti-IL-6 (N + S31 to 201) (upper panel: bright-field; lower panel: staining with Crystal Violet solution.) (B) Spheroid bodies derived from Eca-109 CON and ON cells with or without anti-IL-6 (N + S31 to 201). (C) Invasive ability of Eca-109 CON and ON cells with or without anti-IL-6 (N + S31 to 201) that were stained and photographed (100×). (D) Columnar analysis diagram of Eca-109 CON and ON cells with or without anti-IL-6 (N + S31 to 201). Cell numbers (left); spheroid body formation rate (center); and number of cells passing through the Matrigel (right) (**$P < .01$; ***$P < .001$).
Abbreviations: STAT3, signal transducer and activator of transcription 3; IL-6, interleukin 6.

has been proven to increase via the Il-6/STAT3 pathway in ovarian cancer cells.[28] This study has demonstrated that the expression of IL-6 and p-STAT3 changes in a dose-dependent manner along with the expression of NANOG. Consistently, the downstream genes of STAT3, including *CCL5*, *CCND1*, and *VEGF*, were also differentially expressed upon NANOG expression. Strikingly, we found that blocking of the IL-6 receptor and inhibition of STAT3 phosphorylation both suppressed cell proliferation, cancer stem-like properties, and invasion effects in Eca-109 ON cells. In other words, NANOG promoted cell proliferation, invasion, and CSC properties via IL-6/STAT3 signaling in ESCC.

Upon further research, numerous studies have shown that noncoding RNAs such as long noncoding RNAs, microRNAs, and circular RNAs play an important role in gastrointestinal cancers, including esophageal cancer.[39-42] Therefore, we hypothesize that NANOG, an important pluripotency-related transcription factor, affects the occurrence

and development of esophageal cancer through the targeted regulation of different noncoding RNAs or as a target of different noncoding RNAs. This will be our next important research direction.

Authors' Note

DL and GF designed the experiments, and DL and GF wrote the paper. DL, XPZ, XXC, RX, DQX, ZC, and KL performed the experiments.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Bureau of Science and Technology and Intellectual Property Nanchong City, China (grant nos. 18SXHZ0375, 19SXHZ0353).

Ethics Approval

In this study, commercial immortalized cell lines were used and no human or animal experiments were involved.

Informed Consent

Not applicable, because this article does not contain any studies with human or animal subjects.

Trial Registration

Not applicable, because this article does not contain any clinical trials.

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Supplemental Material

Supplemental material for this article is available online.

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