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ORIGINAL ARTICLE

NANOG regulates epithelial-mesenchymal transition and chemoresistance through activation of the STAT3 pathway in epithelial ovarian cancer

Suqing Liu[1] · Jing Sun[1] · Bin Cai[1] · Xiaowei Xi[1] · Liu Yang[1] · Zhenbo Zhang[1] ·
Youji Feng[1] · Yunyan Sun[1]

Abstract NANOG is a key transcription factor that is
overexpressed and plays an important role in various cancers.
Its overexpression is associated with highly tumorigenic,
drug-resistant, and poor prognosis. However, the underlying
mechanism of action of NANOG in ovarian cancer remains
unclear. Epithelial-mesenchymal transition (EMT), which is a
critical process in cancer invasion and metastasis, is also as-
sociated with drug resistance. We determined whether
NANOG is associated with EMT and chemoresistance in ep-
ithelial ovarian cancer cells. NANOG expression was in-
creased in epithelial ovarian cancer cells (HEY and SKOV3)
compared with normal epithelial ovarian cells (Moody). Low
expression of NANOG increased the expression of E-cadherin
and decreased the expression of vimentin, β-catenin, and
Snail. Furthermore, the cell migration and invasion abilities
were decreased. The multidrug resistance genes MDR-1 and
GST-π were also downregulated when NANOG was lowly
expressed. The cells that were transfected with the si-
NANOG plasmid were more sensitive to cisplatin compared
with the cells that were transfected with empty vector. The
data demonstrated that Stat3 was correlated with NANOG-
mediated EMT and drug resistance. The silencing of Stat3
expression abrogated NANOG-mediated EMT changes and
increased the sensitivity of the cells to chemotherapy. These
results suggest that NANOG mediates EMT and drug

resistance through activation of the Stat3 pathway in epithelial
ovarian cancer.

Introduction

Ovarian cancer is one of the most common malignant tumors
of the female reproductive system. Ovarian cancer has the
fifth highest mortality rate among all types of cancer. Over
21,980 new cases and over 14,270 deaths occurred in the
USA in 2014. Approximately 62 % of patients were in late
cancer stages prior to being diagnosed. The 5-year relative
survival rate is only 44 % [1]. Cytoreductive surgery, followed
by combination chemotherapy, is the most common manage-
ment for ovarian cancer patients [2], but distant metastasis and
drug resistance are the major factors affecting prognosis.
Therefore, studying the mechanisms of tumor cells distant
metastasis and drug resistance may provide a new perspective
for the treatment of ovarian cancer.

NANOG is a key transcription factor that plays an impor-
tant role in maintaining self-renewal and pluripotency in em-
bryonic stem cells [3, 4]. NANOG is overexpressed in various
cancer-affected tissues, including testicular germ cells and tes-
tis [5], liver [6], lung [7], prostate [8], breast [9, 10], cervix
[11, 12], and ovary [13, 14]. NANOG is highly expressed in
ovarian cancer cells and also impacts patient prognosis [15].
NANOG-mediated EMT changes and invasion/metastasis
through the Stat3/Snail signaling pathway have been observed
in various tumors [16–18]. However, NANOG-mediated mo-
lecular mechanisms in ovarian cancer have yet to be
investigated.

✉ Yunyan Sun
sunyy2003@126.com

1   Department of Obstetrics and Gynecology, Shanghai General
Hospital, Shanghai Jiao Tong University School of Medicine,
Shanghai, China 201600

Epithelial-mesenchymal transition (EMT) is a progressive conversion of epithelial cells into migratory cells; this process is characterized by loss of the epithelial phenotype, such as loss of the connection to the basement membrane, and acquisition of a mesenchymal phenotype, such as migration, invasion, and resistance to apoptosis. EMT may play an important role in the progression and metastasis of epithelial cell malignant tumors. Chemoresistant ovarian cancer cells have an EMT phenotype and are highly invasive [19]. Furthermore, NANOG expression levels are positively correlated with the expression level of Snail, an EMT-related transcription factor. NANOG and Snail are expressed at low levels in low-grade tumors and are highly expressed in high-grade tumors [20–22]. Thus, we investigated the roles of NANOG, EMT, and chemoresistance in epithelial ovarian cancer cells. Our results demonstrated that high NANOG expression levels promote EMT and chemoresistance through Stat3 signaling in epithelial ovarian cancer cells.

Materials and methods

Clinical samples

Archived formalin-fixed, paraffin-embedded tissue blocks of ovarian samples were retrieved from the Department of Obstetrics and Gynecology, Shanghai Jiao Tong University School of Medicine, Shanghai General Hospital, including 6 cases of benign cystadenomas, 2 cases of borderline tumor, and 12 cases of epithelial carcinomas for studying NANOG protein expression. Pathological diagnoses of the above ovarian lesions were made by two gynecological pathologists based on the World Health Organization classification.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described [23]. Briefly, NANOG expression was detected using a rabbit monoclonal anti-human NANOG IgG (ab10925; Abcam, Cambridge, UK). The sections were incubated with anti-NANOG (1:200 dilution) in a moisture chamber overnight at 4 °C followed by a 30-min incubation with biotinylated secondary antibody. Staining intensity was scored as 0 (negative), 1 (faint), 2 (moderate), or 3 (strong). The percentage of positively stained cells and the intensity of the staining in these sections were assessed in a blinded manner. The percentage of positive cells was rated as 0 (<5 %), 1 (5–25 %), 2 (26–50 %), 3 (51–75 %), or 4 (>75 %). NANOG reactivity was graded on a score ranging from 0 to 12 based on the product of staining intensity (0 to 3) and percentage of the cells stained (0 to 4). Individual IHC scores for each case were used for the statistical analysis. All IHC slides were reviewed independently by two investigators.

Cell culture

The epithelial ovarian cancer cell lines HEY and SKOV3 and the normal ovarian cell line Moody were cultured as previously described [23]. HEY, SKOV3, and Moody cells were cultured in a humidified incubator with 5 % CO_2 at 37 °C in DMEM/F12 medium supplemented with 10 % fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Transient transfection

The siRNA-NANOG plasmid and empty vector were constructed by GeneChen (Songjiang, Shanghai, China). Transfection of the plasmid into HEY or SKOV3 cells was performed using Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. To determine the effect of Stat3, cells were pretreated with a Stat3 inhibitor WP1066 (Millipore, Bedford, MA, 10 µM) for 1 h and then transfected with plasmid. The siRNA sequence corresponding to the NANOG gene was 5′-CCGGCTGTAAAGAATCTT CACCTATCTCGAGATAGGTGAAGATTCTTTACAG TTTTTG-3′. The efficiency of gene downregulation was measured via qRT-PCR and Western blot analyses.

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized with the PrimeScript™ RT reagent kit (Takara, Kyoto, Japan) by adding 1 µg of total RNA to a 20-µl reaction system. The cDNA template (2 µl) was used in a 20-µl reaction volume with SYBR® Premix Ex Taq™ (Takara, Dalian, China) for real-time PCR. All procedures were performed according to the manufacturer's recommendations. GAPDH served as an internal normalization control. The primers used for the specific amplification of the NANOG and GAPDH genes were synthesized by Sangon Biotech (Shanghai, China). The primer sequences are listed in Table 1. Then, the $2^{-\Delta\Delta CT}$ method was used to determine the relative gene expression levels, and each experiment was repeated at least three times.

Western blot analysis

Western blots were performed as previously described [24]. Briefly, 30 µg protein samples were loaded onto 7.5–10 % SDS-PAGE gels, transferred to PVDF (Millipore, Bedford, MA, USA) membranes, blocked with 5 % skim milk for 1 h, and incubated overnight with specific primary antibodies at 4 °C, followed by incubation with an appropriate secondary

Table 1 Primer sequences used to amplify the target genes

Gene	Primer sequence (5′ → 3′)	Annealing temperature (°C)
NANOG	Forward: CCTCCTCCCATCCCTCATAG	58
	Reverse: CAACCATACTCCACCCTCCA	58
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC	58
	Reverse: TGGTGAAGACGCCAGTGGA	58

antibody at room temperature for 1 h. The bands were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). The following primary antibodies were purchased from Abcam (Cambridge Science Park, Cambridge, UK): NANOG, Stat3, phosphor-Stat3, P-Glycoprotein, and GST-π. Antibodies to detect EMT, including E-cadherin, vimentin, β-catenin, and Snail, were purchased from Cell Signaling Technology (Danvers, MA, USA). The experiment was replicated at least three times.

Transwell migration and invasion assays

Cell migration and invasion assays were conducted as previously described [25, 26]. Briefly, for the transwell migration assays, HEY and SKOV3 (1×10^5) cells in 100 μl of serum-free medium were plated into the upper chambers of a 24-well plate Transwell system with a polycarbonate filter membrane (8-μm pore size) (Corning, NY, USA). The lower chamber was filled with 100 μl of DMEM/F12 medium supplemented with 10 % FBS. For the transwell invasion assays, the membrane was coated with BD Matrigel. After incubation for 24 h at 37 °C, the migratory and invasive cells were stained with Crystal Violet Staining Solution (Beyotime, Shanghai, China). The cells were then counted under a microscope. The experiment was replicated at least three times.

Cell survival assay

Drug resistance was determined using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The cells were trypsinized and seeded in 96-well plates (4000 cells/well) for experimental assessment 24 h after transfection. After being grown for 24 h, the cells were incubated with cisplatin (Sigma, St. Louis, MO, USA) at various concentrations. After 48 h of treatment, 10 μl of CCK-8 solution was added and the cells were incubated for 2 h. Then, the optical density (OD) of the cell lysates at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was performed in a 96-well plate with three replicate wells, and the experiment was replicated at least three times. After treatment, the cell survival rate was measured.

Statistical analyses

Statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY, USA). All data are presented as means ± standard (SD) deviations. The difference between the experimental and control groups was analyzed using Student's t test, and $P < 0.05$ was considered significant.

Results

EMT was involved in epithelial ovarian cancer cells

EMT plays an important role in the progression and development of carcinomas. To investigate whether EMT played a role in epithelial ovarian cancer, epithelial markers, E-cadherin, mesenchymal markers, vimentin, relative protein, β-catenin, and the transcription factor Snail were examined through Western blot analyses (Fig. 1). Compared with a normal ovarian cell line (Moody), the expression of E-cadherin was dramatically downregulated ($P < 0.001$), whereas the expression levels of vimentin, β-catenin, and Snail were significantly upregulated in HEY and SKOV3 ovarian cancer cells ($P < 0.001$). These data all suggest that EMT was involved in ovarian carcinoma.

NANOG was highly expressed in epithelial ovarian cancer, but its expression was downregulated by siRNA

Ovarian tissue samples from 20 patients were used in this study, and the expression of NANOG was analyzed by immunohistochemical (IHC) staining. As is shown in Table 2, no cases of benign cystadenomas had high levels of NANOG protein, whereas one of two cases (50 %) of borderline tumors and 10 of 12 cases of epithelial ovarian cancer (83.3 %) showed high expression of NANOG protein. Significantly increased NANOG was observed in epithelial ovarian cancer compared with that observed in benign cystadenomas and borderline tumors ($P < 0.05$), and the distinct brown staining was located predominantly in the cytoplasm of positive cells (Fig. 2a). Furthermore, NANOG mRNA and protein levels were increased in HEY and SKOV3 cells ($P < 0.01$) (Fig. 2b, c). We hypothesized that high NANOG expression levels were involved in the progression of epithelial ovarian
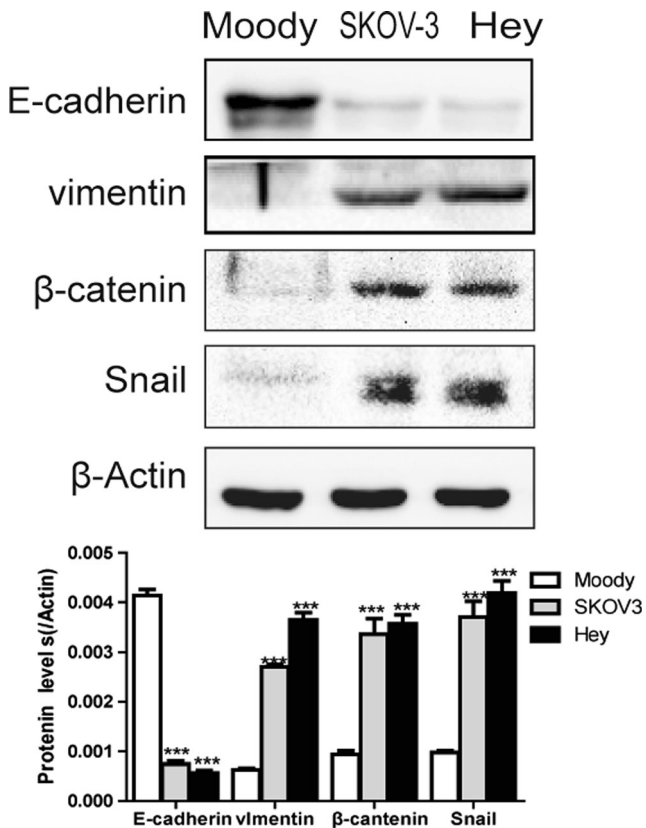
Fig. 1 Epithelial-mesenchymal transition (EMT) occurred in epithelial ovarian cancer cells. Compared with Moody cells, E-cadherin expression was reduced, while the levels of vimentin, β-catenin, and Snail were enhanced in epithelial ovarian cancer cells. These results suggest that EMT occurs in epithelial ovarian cancer cells. β-Actin was used as a loading control. Data were quantified by densitometric analyses and were expressed as the means ± standard deviations from at least three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with normal ovarian cells

cancer and that the inactivation of this protein would reverse a subset of tumor behaviors. NANOG was expressed at low levels after transfection with the si-NANOG plasmid in HEY and SKOV3 cells. The efficiency of downregulation was measured through qRT-PCR and Western blot analyses (Fig. 2d, e). Compared with control cells, NANOG mRNA and protein expression levels were significantly downregulated ($P < 0.05$).

Table 2 Expression of NANOG in distinct tumor tissue types

Tissue type	Total	High level	Low level	%	P value
Cystadenomas	6	0	6	0	0.01
Borderline tumors	2	1	1	50	
Epithelial carcinomas	12	10	2	83.3	

An IHC score less than 3 was considered low expression; a score of 4–12 was considered high expression. Fisher's exact test, $P = 0.01$

% percentage of high level NANOG expression

NANOG RNAi inhibited EMT, as well as migration and invasion, in epithelial ovarian cancer cells

To elucidate the effect of NANOG RNAi on epithelial ovarian cancer cells, we examined the expression levels of E-cadherin, vimentin, β-catenin, and Snail after transfection. In the cells transfected with the si-NANOG plasmid, the expression of E-cadherin was upregulated, whereas the expression levels of vimentin, β-catenin, and Snail were downregulated ($P < 0.05$) (Fig. 3a). These results suggest that NANOG is necessary for the EMT process in epithelial ovarian cancer cells. Furthermore, to determine whether NANOG was involved in the migration and invasion of epithelial ovarian cancer cells, the migratory and invasive abilities of HEY and SKOV3 cells transfected with the si-NANOG plasmid or empty vectors were estimated with the Transwell assay (Fig. 3b). The number of migratory and invasive transfected cells was dramatically decreased compared with the control cells ($P < 0.001$). Our results suggest that NANOG induces EMT, migration, and invasion in epithelial ovarian cancer cells.

NANOG mediated drug resistance in epithelial ovarian cancer cells

EMT can modulate cancer progression and metastasis and is also implicated in the onset of drug resistance [27]. To determine whether NANOG was involved in drug resistance in epithelial ovarian cancer, we quantified the expression levels of the chemoresistance genes MDR-1 and GST-π (Fig. 4a). P-glycoprotein protein is encoded by the MDR-1 gene. The expression levels of MDR-1 and GST-π were significantly decreased after NANOG was downregulated. To further test our hypothesis, we treated si-NANOG or si-control cells (HEY or SKOV3) with different concentrations of cisplatin (Fig. 4b). The survival rate was higher in the cells transfected with the si-NANOG plasmid ($P < 0.05$). These data confirm our hypothesis that NANOG mediates drug resistance in epithelial ovarian cancer cells.

NANOG induced EMT and drug resistance through the Stat3 signaling pathway in epithelial ovarian cancer

Involvement of the Stat3 signaling pathway has been confirmed in the progression of many tumors [28–31]. To explore whether NANOG induced EMT and drug resistance through the Stat3 pathway in epithelial ovarian cancer, we also examined the expression levels of total Stat3 and phosphor-Stat3. As shown in Fig. 5a, the expression levels of Stat3 and NANOG were positively correlated. After the expression of NANOG was decreased, the expression levels of total and phosphor-Stat3 were also downregulated ($P < 0.05$). To
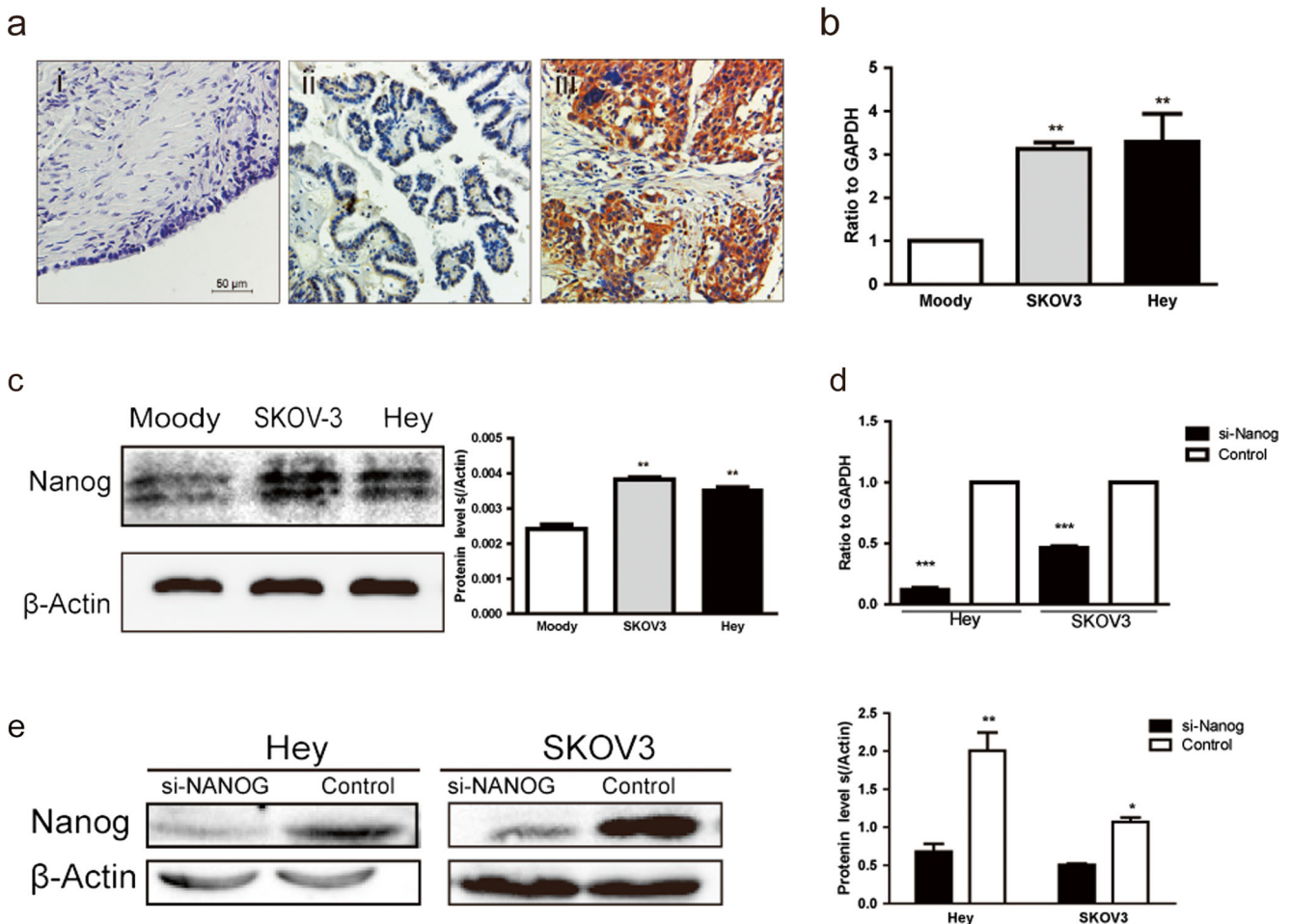
Fig. 2 NANOG was highly expressed in epithelial ovarian cancer, and its expression was downregulated by siRNA. Immunohistochemical staining showed that NANOG was highly expressed in epithelial ovarian cancer, minimally expressed in borderline tumors and benign cystadenoma (**a**, benign cystadenoma (*i*), borderline tumor (*ii*), epithelial ovarian carcinoma (*iii*)). NANOG mRNA (**b**) and protein (**c**) expression in two epithelial ovarian cancer cell lines (HEY and SKOV3) and one normal ovarian cell line (Moody) were examined by qRT-PCR and Western blot analyses. NANOG was expressed at low levels after the transfection of the epithelial ovarian cancer cells with the si-NANOG plasmid. The efficiency of the downregulation was measured by qRT-PCR (**d**) and Western blot (**e**) analyses. The amount of β-actin protein present in the cells of different treatment groups served as a loading control. Data were quantified by densitometric analyses and were expressed as the means ± standard deviations from at least three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with control cells

further explore the effect of the Stat3 signaling pathway, we treated cells with a Stat3 inhibitor (WP1066, 10 μM) for 1 h before plasmid transfection. DMSO was used as a control. NANOG expression decreased after Stat3 expression was blocked. To determine whether NANOG mediated EMT and drug resistance through the Stat3 pathway, we examined the expression levels of MDR-1, GST-π, E-cadherin, and vimentin (Fig. 5b, c). Compared with cells that were treated with DMSO, MDR-1, and GST-π expression levels were suppressed in the WP1066 group (whether transfected with si-NANOG or si-control), but the suppression was greater in the si-NANOG group. Furthermore, the highest expression level of E-cadherin and the lowest expression level of vimentin were also observed in the si-NANOG-transfected cells that were treated with WP1066. Overall, our data suggest that NANOG mediates EMT and drug resistance through the Stat3 pathway in epithelial ovarian cancer cells.

Discussion

Recently, some scholars have postulated the theory of cancer stem cells (CSCs), which states that a small subpopulation of tumor cells exhibit a capacity for self-renewal and are responsible for tumor maintenance and metastasis [32]. EMT has been identified as a key phenomenon that is tightly linked to CSC-like properties, and EMT is often accompanied by an increase in cancer stem cells [33–35]. A previous study has demonstrated that in ovarian cancer, stem-like side-population cells are tumorigenic and chemoresistant and that NANOG is
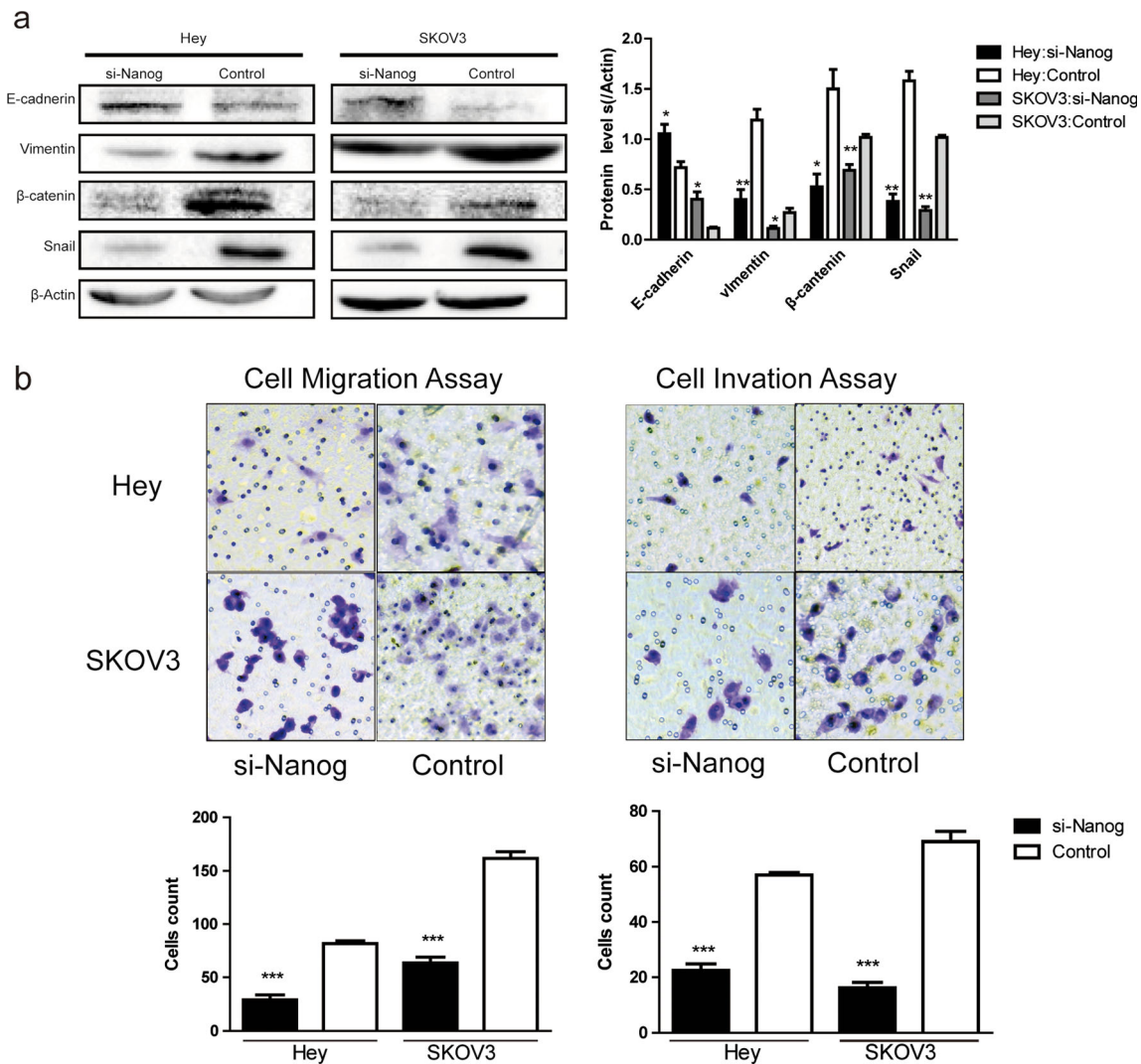
Fig. 3 NANOG RNAi inhibited EMT as well as migration and invasion in epithelial ovarian cancer cells. NANOG was expressed at low levels after transfection with the si-NANOG plasmid in HEY and SKOV3 cells; in the transfected cells, the expression of E-cadherin was increased, whereas the expression levels of vimentin, β-catenin, and Snail were decreased compared with cells that were transfected with empty vector (**a**). Data were quantified by densitometric analyses and were expressed as the means ± standard deviations from at least three independent experiments. **b** The migratory and invasive abilities of the HEY and SKOV3 cells that were transfected with the si-NANOG and control cells were examined with the transwell assay. The numbers of migrating and invading cells were significantly decreased in the si-NANOG-treated cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with cells from the control group

overexpressed [36]. Based on these findings, we determined whether NANOG was associated with EMT and chemoresistance in epithelial ovarian cancer cells.

NANOG is one of the key factors that renders the reprogramming of adult cells into germline-competent induced pluripotent stem cells [37]. NANOG is expressed not only in germ cell tumors but also in many other tumors including ovarian cancer [12, 13]. In this study, we also found that NANOG was highly expressed in epithelial ovarian cancer and could enhance the migration, invasion, and drug-resistant abilities of tumor cells. Furthermore, positive expression of NANOG is associated with highly tumorigenic cancers and poor prognosis of patients with ovarian carcinoma [14, 15, 36]. All of these findings suggest that NANOG may play

an important role in the development of differentiation and progression of ovarian carcinoma. However, the question of how NANOG regulates ovarian cancer cell activation is not well understood.

It is well known that EMT is crucial for cancer metastasis. EMT status is significantly associated with peritoneal metastasis and with both the progression-free and overall survival of patients with ovarian cancer [33]. Ectopic NANOG overexpression enhanced ovarian cancer cell migration and invasion and also led to a decrease in E-cadherin expression [38]. Previous studies showed that high levels of Snail expression correlate with metastasis and high levels of NANOG expression in tumor cells [21]. In our study, the knockdown of NANOG with specific siRNA significantly enhanced the expression

Fig. 4 NANOG mediated drug resistance in epithelial ovarian cancer cells. The expression of chemoresistance genes was examined by Western blot analysis (**a**). P-glycoprotein protein is encoded by the MDR-1 gene. The expression levels of NANOG, MDR-1, and GST-π were positively correlated. Data were quantified by densitometric analyses and were expressed as the means ± standard deviations from at least three independent experiments. After NANOG expression was downregulated, the cell survival rate was decreased after treatment with different concentrations of cisplatin (**b**)
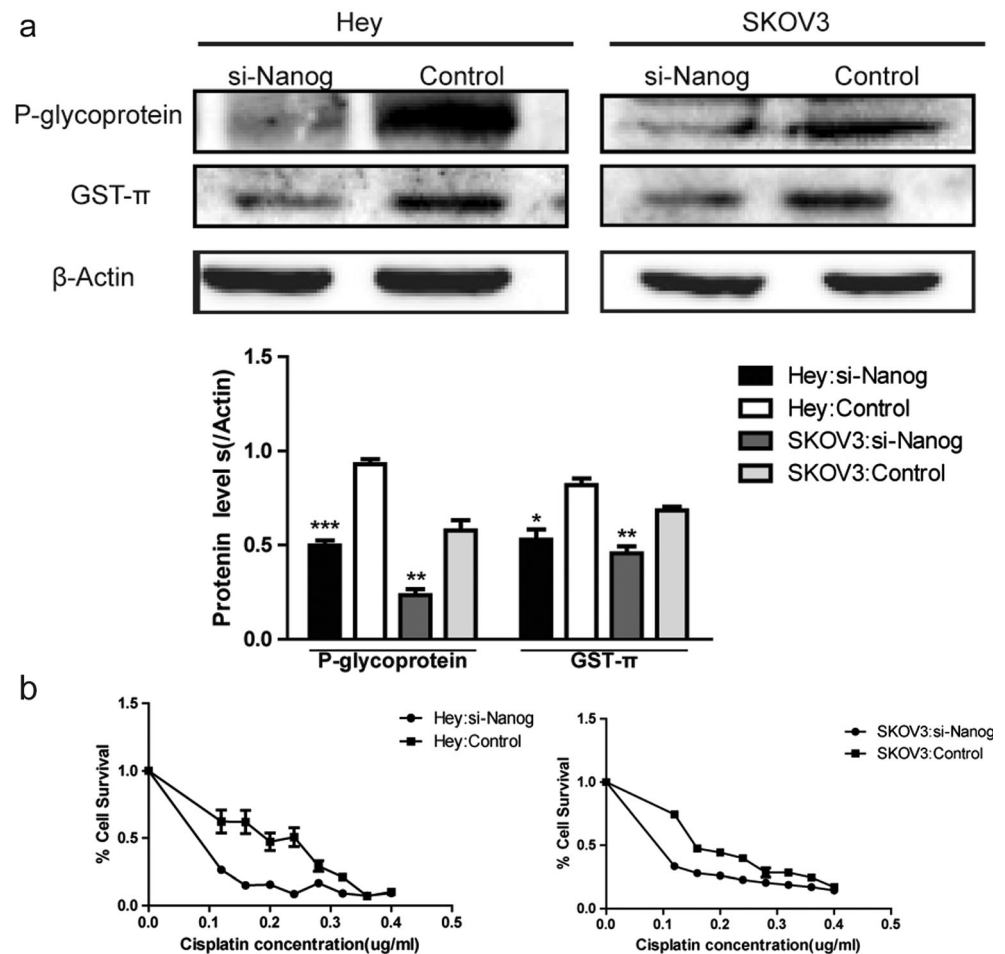
level of E-cadherin and reduced the expression level of vimentin, β-catenin, and Snail, which demonstrated that overexpression of NANOG induced EMT in epithelial ovarian cancer. Furthermore, the migration and invasion abilities of tumor cells were decreased after NANOG expression was downregulated. Thus, our data may explain the association between NANOG expression and distant metastasis in epithelial ovarian cancer. Indeed, the induction of NANOG overexpression promoted the migration of CSCs in vitro, and NANOG was associated with poor prognosis in patients with lung or liver cancer [7, 17]. It is possible that NANOG is crucial for the tumorigenic process in ovarian cancer cells. We are interested in how NANOG expression promotes the EMT process and metastasis of epithelial ovarian cancer.

In many malignant tumors, the failure of chemotherapy is partially associated with the abnormal expression of the MDR-1 gene, and knockdown of the MDR-1 gene can restore the sensitivity of tumor cells to drugs [39–42]. GST-π is another chemoresistance gene that is highly expressed in ovarian cancer cells [43]. We therefore used MDR-1 and GST-π to investigate drug resistance. The expression levels of MDR-1 and GST-π were significantly decreased after NANOG

expression was downregulated, as low levels of NANOG restored cisplatin sensitivity in epithelial ovarian cancer cells.

Distant metastasis and drug resistance are major factors that affect the prognosis of patients with ovarian cancer. EMT, a critical process of cancer invasion and metastasis, is associated with drug resistance [19, 27]. A previous study has reported a significant increase in the expression levels of N-cadherin and vimentin and a decrease in the expression levels of E-cadherin in cisplatin-treated ovarian cancer OVCA433 cells compared with untreated cells [44]. NANOG can induce EMT and chemoresistance in various cancers, and our results support a similar conclusion for epithelial ovarian cancer.

Abnormal Stat3 signaling appears to play a critical role in oncogenesis [45]. In particular, phosphorylated Stat3 has been observed in human malignancies including epithelial ovarian carcinomas [46]. Previous studies showed that NANOG and Stat3 are functionally coupled during oncogenesis and that the activation of Stat3 induced EMT in head and neck tumors [16, 47]. In this study, we observed that NANOG expression was positively correlated with levels of total and phosphor-Stat3and that NANOG mediates EMT and drug resistance through activation of the Stat3 pathway in epithelial ovarian
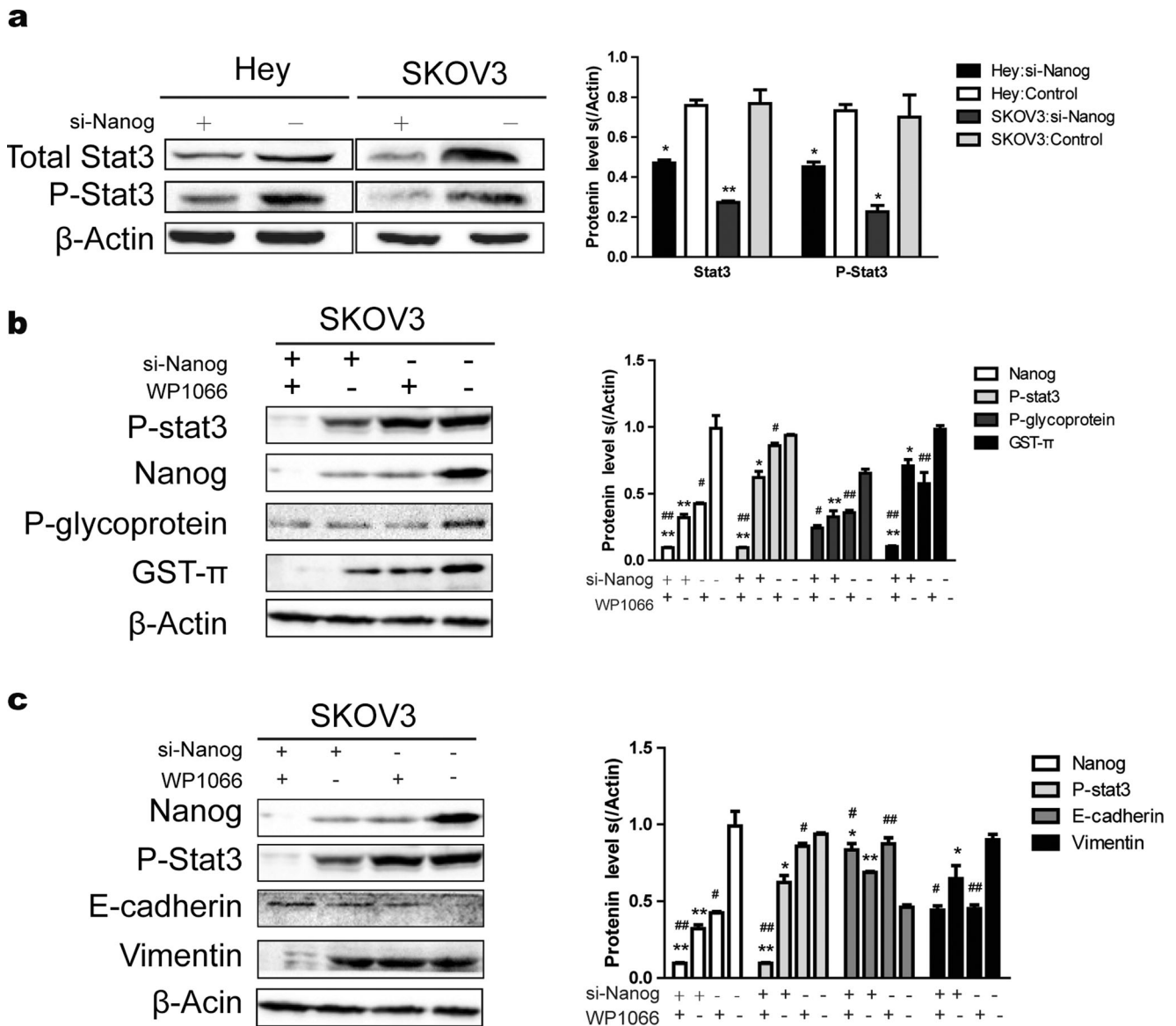
a

b

c

Fig. 5 NANOG induced EMT and drug resistance through the Stat3 signaling pathway in epithelial ovarian cancer. **a** In HEY and SKOV3 cells, the expression levels of total stat3 and phospho-stat3 were downregulated after transfection with si-NANOG. To further explore the effect of Stat3, we treated SKOV3 cells with a Stat3 inhibitor (WP1066,10 μM) for 1 h before the plasmid transfection. DMSO was used as a control. **b** Compared with cells that were treated with DMSO, the expression levels of MDR-1 and GST-π were suppressed in the WP1066 group, whether the cells were transfected with si-NANOG or si-control (but the suppression was more obvious in the former). **c** Western blots showed the expression levels of epithelial markers and mesenchymal markers in SKOV3 cells with different treatments. The highest expression level of E-cadherin and the lowest expression level of vimentin were observed in si-NANOG-transfected cells that were treated with WP1066. Data were quantified via densitometric analyses and were expressed as the means ± standard deviations from at least three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicated statistical significance compared with si-control cells; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ indicated statistical significance compared with cells treated by DMSO

cancer cells. Yin X et al. also concluded that NANOG regulates EMT through activation of Stat3 in hepatocellular carcinoma. However, in another study, the knockdown of NANOG had no effect on phosphor-Stat3 [17]. Wen Z et al. proposed that specific phosphorylation of the tyrosine 705 residue was needed for cellular transformation by Stat3 [48]. Upon application of the Stat3 inhibitor WP1066, the expression of NANOG was decreased. However, other research has shown that WP1066 had no effect on NANOG expression [21]. Further research is required to determine whether phosphor-Stat3 is involved in the progression of EMT and drug resistance induced by NANOG and relatives of the two genes.

Our results suggested that low expression of NANOG can reverse EMT and increase the sensitivity of epithelial ovarian cancer cells to chemotherapy. It would be interesting to further investigate the mechanism of NANOG-mediated EMT and

chemoresistance by the overexpression of NANOG in vivo and in vitro. Furthermore, the underlying mechanistic link between EMT and chemoresistance in ovarian cancer requires further research. Finally, NANOG in ovarian cancer may be a new target for future therapeutic treatment.

Compliance with ethical standards   All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and national research committees, with the 1964 Declaration of Helsinki and its later amendments or with comparable ethical standards.

Conflicts of interest   None

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