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Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcome in ovarian cancers

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Abstract

Ovarian cancer is the most lethal of all gynecological malignacies and the identification of novel prognostic and therapeutic targets for ovarian cancer is crucial. It is believed that only a small subset of cancer cells are endowed with stem cell properties, which are responsible for tumor growth, metastatic progression and recurrence. NANOG is one of the key transcription factors essential for the maintaining self-renewal and pluripotency in stem cells. This study investigated the role of NANOG in ovarian carcinogenesis and showed overexpression of NANOG mRNA and protein in the nucleus of ovarian cancers compared with benign ovarian lesions. Increased nuclear NANOG expression was significantly associated with high grade cancers, serous histological subtypes, reduced chemosensitivity, and poor overall and disease-free survival. Further analysis showed NANOG is an independent prognostic factor for overall and disease-free survival. Moreover, NANOG was highly expressed in ovarian cancer cell lines with metastasis-associated property and in clinical samples of metastatic foci. Stable knockdown of NANOG impeded ovarian cancer cell proliferation, migration and invasion, which was accompanied by an increase in mRNA expression of E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1 and FOXB1. Conversely, ectopic NANOG overexpression enhanced ovarian cancer cell migration and invasion along with decreased E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1 and FOXB1 mRNA expression. Importantly, we found Nanog-mediated cell migration and invasion involved its regulation of E-cadherin and FOXJ1. This is the first report revealing the association between NANOG expression and clinical outcome of patients with ovarian cancers, suggesting NANOG to be a potential prognostic marker and therapeutic molecular target in ovarian cancer.

Keywords: NANOG, cell migration and invasion, prognostic marker, therapeutic molecular target, ovarian cancer

Introduction

Ovarian cancer is a common gynecological cancer world-wide and contributes to high mortality, despite advances in treatment modalities.[1] The poor prognosis is due to a lack of symptoms at early stages until widespread metastasis develops and the high rates of chemoresistance found in patients with advanced diseases.[2] In consequence, it is vital to identify novel prognostic markers and therapeutic targets for ovarian cancer.

NANOG is one of the core transcription factors expressed in pluripotent embryonic stem (ES) cells but not in somatic organs.[3, 4] NANOG plays essential roles in maintaining self-renewal and the undifferentiated state of pluripotent stem cells during early embryonic development. Besides controlling such "stemness" properties, the role of NANOG in tumorigenesis has attracted attention.[5]

Increasing evidence has suggested that most tumors are heterogeneous. Of which, a small subset of cells, known as cancer stem cells, arise from mutated adult stem/progenitor cells possessing stem-like properties, which are responsible for tumor growth, metastasis, chemoresistance, and thus cancer recurrence. Only by targeting these population of cells which exhibit a number of important phenotypic, biological and functional characteristics associated with normal stem cells can one ultimately cure the disease.[6, 7] Therefore, cancer stem cell markers, which are good therapeutic targets in common cancers, are being vigorously investigated.[8, 9]

Recent studies have identified and characterized a self-renewing subpopulation of cancer-initiating cells in ovarian cancers endowed with stem-like properties and induced NANOG expression.[9-11] In addition, NANOG expression has also been found

in an ovarian cancer cell line and is involved in multidrug resistance.[12] In this study, we investigated the prognostic significance of NANOG in ovarian cancer and assessed for the first time the functional roles and putative downstream targets of NANOG in ovarian cancer. Our results suggest that NANOG may be one of cancer stem cell markers that play a central role in the progression of ovarian cancers. As such, NANOG could also be an important prognostic marker for identifying patients who respond better to current treatment regimes as well as a therapeutic target for ovarian cancer treatment.

Results

NANOG is overexpressed in the nucleus of ovarian cancers and associated with tumor aggressiveness, metastasis and chemosensitivity. By immunohistochemistry, no nuclear NANOG immunostaining was detected in benign cystadenomas, whereas weak to moderate expression was found in borderline tumors and ovarian cancers respectively (Figure 1a). In terms of the percentage of positive cells, around 2 to 20% cancer cells were stained in borderline tumors and ovarian cancers respectively (Table 1). The differential nuclear NANOG immunoreactivity (i.e. histoscore as mentioned in Methods) among the three diagnostic categories, including benign, borderline and carcinomas, were statistically significant ($p=0.031$) (Table 1). Moreover, statistically higher nuclear NANOG immunoreactivity was found in metastatic foci than their corresponding primary carcinomas ($p=0.005$) (Figure 1a and Table 1). High nuclear NANOG immunoreactivity was significantly associated with poor histological grade, serous histological subtypes and chemosensitivity (all $p<0.05$; Table 1). Similar trend was also obtained when percentage, but not intensity, of NANOG stained cells was used for analyses (Table 1). Significantly higher NANOG mRNA levels were also found in ovarian cancers than in benign cystadenomas as detected by qPCR ($p=0.046$) (Figure. 1b i). The result also revealed that the mRNA expression of SOX-2 and

OCT-4, two other core stem cell transcription factors,[13] was also overexpressed in ovarian cancers (Figure 1b ii and iii).

NANOG overexpression is associated with poor prognosis of ovarian cancer patients. Univariate analysis revealed that NANOG was significantly associated with shorter overall ($p=0.001$) and disease-free ($p=0.002$) survival (Figure 2). Similar trends were also observed when either intensity or percentage of NANOG immunoreactive cells was used for analyses (data not shown). By multivariate analysis, NANOG, disease stage and chemosensitivity remained significant predictors for overall survival, whereas NANOG, disease stage and debulking remained significant predictors for disease-free survival (all $p<0.05$, Supplementary Table 3)

NANOG is overexpressed in ovarian cancer cell lines and localized in the nucleus of cancer cells. By qPCR, NANOG mRNA expression was higher in five (OVCA 433, OVCAR-3, PA-1, SKOV-3 and SW626) and lower in two (OVCA 420 and TOV112D) out of twelve ovarian cancer cell lines compared to the three normal HOSE cell lines. In particular, NANOG mRNA expression in SKOV-3 and OVCAR-3, two cell lines produced from metastatic ovarian cancers, was at least 6- to 8-fold higher than the normal HOSE cell lines (Figure 3a). Moreover, we found that NANOG mRNA expression was about 9-fold higher in 2008-C13 (cisplatin-resistance) than in the 2008 (cisplatin-sensitive) cell lines (Figure 3b). Subcellular expression of NANOG in nuclear and cytoplasmic fractions of OVCAR-3 was also determined by immunoblotting. Concurring with the immunohistochemical findings, NANOG protein expression was predominately found in the nuclear fraction, with no detectable expression in the cytoplasmic fraction (Figure 3c).

Knockdown of NANOG impedes ovarian cancer cell proliferation, migration and invasion, down-regulates SOX-2 and up-regulates E-cadherin and caveolin-1 mRNA expression. Stable knockdown of NANOG in OVCAR-3 was detected at both mRNA and protein levels (Figure 4a). We found that stable knockdown of NANOG significantly retarded cell proliferation after 12 d (Figure 4b). Moreover, Transwell migration and invasion assays revealed significantly reduced migration and invasion ($p<0.05$) in shNANOG OVCAR-3 compared with that in control (Figure 4c). In addition, specific transient (siNanog#1 and #2; Supplementary Figure 1a) knockdown of NANOG in SKOV-3 significantly reduced migration and invasion (Supplementary Figure 1b). Next, we investigated the effect of NANOG depletion on mRNA expression of SOX-2 and OCT-4, two other core stem cell transcription factors,[13] and E-cadherin, caveolin-1 and integrin-beta(β) 1, all are possible downstream targets for cell migration and invasion.[14, 15] In fact, previous study documented that NANOG can bind to specific promoter elements of SOX-2, OCT-4, caveolin-1 and integrin-β1 in embryonic stem cells.[13] We found that depletion of NANOG expression in OVCAR-3 cells significantly decreased SOX-2, and up-regulated E-cadherin and caveolin-1, but have no virtual effect on OCT-4 and integrin-β1 mRNA expression (Figure 4d). Moreover, up-regulation of E-cadherin and caveolin-1 in protein level was also demonstrated in NANOG depleted OVCAR-3 cells (Figure 4d, inset). Transient knockdown of NANOG in SKOV-3 also significantly decreased SOX-2, and up-regulated E-cadherin, but have no virtual effect on OCT-4, caveolin-1 and integrin-β1 mRNA expression (Supplementary Figure 1c).

Knockdown of NANOG enhances FOXO1, FOXO3a, FOXJ1 and FOXB1 mRNA expression. Forkhead box (FOX) proteins are a large family of transcriptional regulators, which control a variety of biological processes leading to alteration of cell fate, thus the development and progression of cancer.[16] Since four FOX proteins, including FOXO1, FOXO3a, FOXJ1 and FOXB1, are likely targets of NANOG in embryonic stem cells,[13] we investigated their mRNA expression in NANOG depleted OVCAR-3 and SKOV-3 cells. qPCR analysis revealed that stable knockdown of NANOG in OVCAR-3 up-regulated all four FOX proteins from around 2 to 13 folds (Figure 5a) and transient knockdown of NANOG in SKOV-3 up-regulated FOXO1, FOXO3a and FOXJ1, but not FOXB1 (Supplementary Figure 1c). Among them, increasing number of studies documented that FOXO are cellular targets of antitumor drugs in malignancies, including ovarian cancer.[16, 17] As a consequence, we further explored if NANOG can regulate *FOXO1* and *FOXO3a* transcription activities. Our results showed that both *FOXO1* (Figure 5b, left panel) and *FOXO3a* (Figure 5b, right panel) promoter activities were evaluated in NANOG depleted OVCAR-3 cells.

Overexpression of NANOG promotes cell migration and invasion in association with induced SOX-2 and attenuated E-cadherin, caveolin-1, FOXO1, FOXO3a FOXJ1 and FOXB1 mRNA expression. To further study the effect and downstream targets of NANOG in ovarian cancer cell migration and invasion, ectopic overexpression of NANOG in OVCA420 was performed (Figure 6a). Significantly increased cell migration and invasion (Figure 6b) as well as up-regulation of SOX-2 and down-regulation of E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1 and FOXB1 mRNA expression (Figure 6c) was demonstrated in NANOG-overexpressing OVCA420 cells when compared with the pcDNA3.1 control.

Nanog-mediated cell migration and invasion involves E-cadherin and FOXJ1. To test if Nanog-mediated cell migration and invasion is dependent on E-cadherin and FOXJ1, NANOG depleted OVCAR-3 cells were treated with specific siRNAs of E-cadherin and FOXJ1. E-cadherin and FOXJ1 mRNA expression was reduced by 80% in siRNA-treated cells when compared with control cells (Supplementary Figure 2). Treatment with siRNAs against E-cadherin and FOXJ1 also increased basal cell migration and invasion, and rescued NANOG-reduced migration and invasion (Figure 5c). Similar results were obtained when another set of siRNAs were used (data not shown). In addition, OVCA420 cells were transiently transfected with NANOG and E-cadherin (Figure 6d). Ectopically expressed E-cadherin decreased cell migration and invasion and inhibited NANOG-mediated migration and invasion (Figure 6e).

Discussion

In this study, we showed significantly higher NANOG immunoreactivity in ovarian cancer samples when compared with borderline tumors and benign cystadenomas/inclusion cysts. Expression of NANOG mRNA and that of two other core stem cell transcription factors, SOX-2 and OCT-4, was also detected in ovarian cancer. *In vitro*, we found that SOX-2 can be regulated by NANOG in ovarian cancer cells. Interestingly, in borderline tumor and ovarian cancer clinical samples, only around 2 to 20% cancer cells were NANOG-positive, respectively. Cancer stem cells are a small population of cells found in a given malignant tissue.[8] The present finding showing a small portion of NANOG positive tumor cells in ovarian tumors concur with this property, suggesting NANOG is not only involved in ovarian cancer progression, but also may be one of cancer stem cell makers. In agreement, cancer

stem-like cells isolated from ascites derived from ovarian cancer patients,[18] prostate tumors,[19] oral squamous cell carcinoma[20] and osteosarcoma[21] also showed elevated NANOG expression.

Significantly higher NANOG immunoreactivity was detected in poorly differentiated ovarian cancers, serous histological subtypes and in metastatic foci when compared with their corresponding primary ovarian cancers. *In vitro*, NANOG mRNA expression was also particularly high in SKOV3 and OVCAR-3, which are derived from the malignant ascites of patients diagnosed with serous adenocarcinoma.[22] These serous-type tumors account for ~70% of ovarian cancers,[23] and are often associated with ascites formation and intraperitoneal metastases.[24] These findings suggest NANOG to be involved in ovarian cancer de-differentiation and metastasis which are two important cancer stem cell properties.[8, 19, 20] We also found lower NANOG mRNA expression in endometrioid tumor-derived TOV112D cell line[25] compared to the three normal HOSE cell lines which consistent with the relatively lower NANOG immunoreactivity in endometrioid ovarian cancers. More importantly, we demonstrated a significant correlation between high NANOG immunoreactivity and shorter overall and disease free survival, suggesting NANOG to be an important prognostic marker in ovarian cancer. NANOG is an independent prognostic factor for overall survival.

If high NANOG expression can be further confirmed to indicate poor prognosis, as suggested in this report, it may serve as a biomarker to assist in triage of patients with early stage ovarian cancers and decision for adjunct therapy. Ovarian cancer patients diagnosed with stage I (confined to ovary) disease do not need adjuvant chemotherapy

unless they are associated with poor prognostic parameters such as high grade cancers (high grade serous or clear cell types) or capsular involvement. Even under such situations, single agent therapy by carboplatin can be administered instead of combination with paclitaxel as in patients with higher staged disease. Moreover, maintenance molecular targeted therapy such as bevacizumab is also being investigated for patients with poor prognosis. High NANOG expression may serve as a marker for indicating combination instead of single agent chemotherapy in stage I patients as well as to select high risk patients for administering adjunct targeted therapy to improve their clinical outcome. Larger scale studies are needed to confirm such application.

Ovarian cancer-initiating cells isolated from primary tumors with overexpressed NANOG and other stem cell markers was shown to enhance chemoresistance to the ovarian cancer chemotherapeutics cisplatin or paclitaxel.[10] Cancer stem-like cells isolated from osteosarcoma also showed evaluated NANOG expression along with chemoresistance.[21] Moreover, HA treatment of ovarian and breast cancer cells induced Stat-3 bound to NANOG and favored Stat-3-specific transcriptional activation leading to *MDR1* gene expression and multidrug resistance.[12] Thus, increasing evidence showed that conventional anticancer therapies are mostly unable to remove cancer stem cell clones and instead help cancer stem cell expand and/or select for resistant cancer stem cell clones, leading to cancer patient relapse.[8] In this study, significantly higher NANOG immunoreactivity in chemoresistant ovarian cancer samples and cell lines was detected. We also found up-regulation of FOXO1 and FOXO3a transcription activities and mRNA levels after stable knockdown of NANOG in ovarian cancers as well as down-regulation of FOXO1 and FOXO3a

mRNA expression in NANOG-overexpressing cells, suggesting NANOG to be a negative regulator of FOXO1 and FOXO3a. Given that FOXO transcription factors are cellular targets of anticancer drugs in multiple cancers[16, 17] and low FOXO3a expression is associated with poor prognosis in ovarian cancer patients,[26] it is possible that NANOG affect chemosensitivity through transcription regulation of FOXO1 and FOXO3a which will be studied in near future.

Functionally, we found knockdown of NANOG reduced ovarian cancer cell proliferation, migration and invasion along with up-regulated E-cadherin and caveolin-1, whilst ectopic overexpression of NANOG led to increased cell migration and invasion along with down-regulated E-cadherin. We have previously reported positive effect of NANOG on choriocarcinoma cell migration and invasion.[27] In ovarian cancer cells, simultaneous expression of caveolin-1 and E-cadherin found to stabilize adherens junctions through inhibition of src-related kinases.[28] In contrast, depletion of E-cadherin promoted ovarian cancer metastasis through induced α5-integrin expression.[15] *In vivo*, NANOG expression pattern is opposite to E-cadherin expression in ovarian cancers where reduced E-cadherin expression was found at metastatic sites when compared with their primary ovarian tumors.[29] Moreover, a significantly shorter survival was found in ovarian cancer patients with negative E-cadherin expression.[30] Importantly, our rescue experiments demonstrated Nanog-mediated cell migration and invasion in E-cadherin dependent manner.

FOXJ1 has been found to suppress inflammation through repression of NF-κB.[31] Besides being as an important mediator of immune response, NF-κB is also involved in regulating tumor growth, apoptosis and metastasis, thus tumorigenesis. FOXJ1 was

hypermethylated in breast tumor cell lines and clinical samples, suggesting being a putative tumor suppressor gene.[32] While the mechanisms through which FOXJ1 suppress tumor growth remains unknown, our findings implicate FOXJ1 as one of the downstream mediators of NANOG in regulating cell migration and invasion and suggest that FOXJ1 suppress tumor progression though regulation on cellular processes in metastasis.

In conclusion, our *in vivo* and *in vitro* findings demonstrated NANOG, an important stem cell related transcription factor, to be involved in ovarian tumorigenesis probably through regulating chemosensitivity, cell proliferation, migration and invasion. NANOG is a negative regulator of E-cadherin and FOXJ1 in mediating ovarian cancer cell migration and invasion. Importantly, NANOG is a potential prognostic marker and molecular therapeutic target in ovarian cancer.

Materials and Methods

Clinical samples. Archived formalin-fixed paraffin-embedded tissue blocks in ovarian cancer, with the corresponding clinical follow-up data were retrieved from Department of Pathology, the University of Hong Kong, Queen Mary Hospital including six benign cystadenomas (age range 20-35 years, mean age 34.8 years), seven borderline tumors (age range 20-46 years, mean age 28.9 years), 97 carcinomas (age range 32-83 years, mean age 50.5 years) with different histological subtypes and 43 corresponding metastatic foci of ovarian cancers for studying NANOG protein expression. Among patients with ovarian cancers, 80 received chemotherapy including platinum/paclitaxel after surgery, and the median follow-up period was 63 months (range, 4-209 months). Twenty-eight randomly selected ovarian tumors

clinical samples with available frozen blocks including three benign cystadenomas and twenty-five ovarian cancers were also retrieved for studying mRNA expression of NANOG, SOX-2 and OCT-4. The use of these samples was approved by the Institutional Ethical Review Board. The diagnosis of each sample was assessed by pathologists and ensured to have more than 70% tumor cells.

Cell lines, subcellular protein extraction and treatment. Three immortalized human normal ovarian epithelial cell lines, HOSE 6-3, HOSE 11-12 and HOSE 17-1, and fourteen ovarian cancer cell lines, DOV13, ES2, OC316, OVCA 420, OVCA 433, OVCAR-3, PA-1, SKOV-3, SW626, TOV21G, TOV112D, 2008 and 2008-C13 were cultured as previously described.[33, 34] HOSE 6-3, HOSE 11-12, HOSE 17-1, OVCA 420 and OVCA 433 were provided by Prof. S.W. Tsao (Department of Anatomy, the University of Hong Kong). OVCAR-3, SKOV-3 and SW626 were from ATCC (Manassas, VA). 2008 and 2008-C13 cells were established by Dr. S. B. Howell (University of California at San Diego, La Jolla, CA) and provided by Dr. Z. H. Siddik (M. D. Anderson Cancer Center).[35, 36] Cytoplasmic and nuclear extracts from OVCAR-3 cells were isolated using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).[33, 34]

Stable knockdown of NANOG in OVCAR-3. Small hairpin RNA (shRNA) constructs targeting human NANOG (pRS-shNANOG with puromycin resistant gene; Origen, Rockville, MD) were stably transfected into OVCAR-3 cells using Lipofectamine 2000 (Invitrogen, San Diego, CA), while pRS empty vector (pRS-shControl; Origen) transfected cells were used as control.[27, 33] Stable clones were selected with puromycin (1.875μg/ml).

Transient knockdown of NANOG in SKOV-3 and E-cadherin and FOXJ1 in OVCAR-3. Cells were transfected with two siRNAs of NANOG, E-cadherin and FOXJ1 (Ambion, TX, USA) using SilentFect™ (Bio-Rad Laboratories, Hercules, CA) per manufacturer's instructions for 48 hours before cell counting and cell plating. Silencer® Select Negative Control siRNA (Ambion) was used as control.

Ectopic overexpression of NANOG and E-cadherin in OVCA420. The pcDNA3.1-NANOG and pcDNA3.1-E-cadherin plasmids were obtained from Addgene (www.addgene.org). Cells were transfected with NANOG and E-cadherin or the control vector using PolyJet™ DNA In Vitro Tranfection Reagent (SignaGen Laboratories, Rockville, MD) for 48 hours before cell counting and cell plating.

Immunohistochemistry. Immunohistochemical staining was performed as described.[27, 34] Antibody against NANOG (ab21603; Abcam, Cambridge, MA) at a dilution of 1:50 was applied to deparaffinized sections and tested using EnVision+ Dual Link System (K4061; Dako, Carpinteria, CA). Antigen recovery was performed by heating in a pressure cooker using 10mM EDTA (pH 8.0). Elimination or replacement of the primary antibody with preimmune IgG serum was acted as a negative control. Both the intensity and percentage of stained epithelial cells were evaluated semiquantitatively. Staining intensity was scored as 0 (negative), 1 (faint), 2 (moderate), and 3 (strong). The percentage of positive cells was rated as 0 (<5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (>75%). Only nuclear staining was considered as positive. The immunoreactivity was assessed by multiplying the staining

intensity by the percentage of stained cells to give a composite "histoscore". High and low expression levels of NANOG were defined by the "histoscores" cut off at mean.

Real-time PCR (qPCR). Total RNA extracted from cancer cell lines was reverse transcribed by SuperScript Reverse Transcriptase (Invitrogen). qPCR was done using ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) as described.[27, 33] Primer sequences were listed in Supplementary Table 1.

Immunoblotting. 20 μg protein lysate was resolved by SDS-PAGE, eletroblotted to polyvinylidene difluoride membrane, and hybridized with corresponding antibodies.[27, 34] Antibodies used in this study were listed in Supplementary Table 2.

Cell proliferation was determined by cell count method. Cells (3×10^4) were seeded in T150 culture flasks and maintained in growth medium.[34] Cell number was counted using trypan blue dye exclusion with hematocytometer at day 12.

***In vitro* migration and invasion assays.** *In vitro* migration and invasion assays were done as previously described.[27, 34] OVCAR-3 cells (1.25×10^5) were plated on the upper side of a Transwell chamber. Cells migrated through an 8-μm pore size membrane (migration assay) and Matrigel–coated membrane (invasion assay were assessed respectively. After 24 hours (migration assay) or 48 hours (invasion assay), cells on the upper compartment of the membrane were removed and the migrated or invaded cells at the lower surface of the membrane were fixed, stained, and counted.

Luciferase reporter assay. Control and shNANOG OVCAR-3 cells were transiently transfected with pGL3-Basic empty vector (negative control), pGL3-Basic-FOXO1A-Luc (bp -1609/+230) or pGL3-Basic-FOXO3-Luc (bp 1480/-25) reporter plasmids. pRL-SV40-Luc was used as internal control. Cells were lysed 48h post-transfection. Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as previously described.[37] Transfection efficiency was normalized by *Renilla* luciferase activities.

Statistical Analysis. Statistical analysis was performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mann-Whitney test and Kruskal–Wallis rank test were used for comparison of data between two groups and among multiple groups respectively. Kaplan–Meier analysis and log-rank test were used for survival analysis. Multivariate survival analysis was done using Cox regression analysis. P values < 0.05 were considered as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Overexpressed NANOG in ovarian cancer associated with tumor aggressiveness, metastasis and chemosensitivity. (a) Immunohistochemical staining of NANOG in serous benign cystadenomas (i), serous borderline tumors (ii), serous carcinomas (iii) with corresponding metastatic foci (iv), mucinous benign cystadenomas (v), mucinous borderline tumors (vi), mucinous carcinomas (vii), endometrial carcinomas (viii), clear cell carcinomas (ix), chemosensitive (x) and chemoresistant (xi) ovarian carcinomas. *Insets* highlight regions with higher magnification. (b) qPCR analysis of (i) NANOG, (ii) SOX-2 and (iii) OCT-4 mRNA in ovarian tumors as shown in scatter plots with a line at mean. The fold change of the target gene expression was calculated with respect to the mean expression of the target gene in benign cystadenomas.

Figure 2. Increase in NANOG expression was significantly associated with poor overall (a) and disease-free (b) survival in the univariate analysis (cut off at mean).

Figure 3. Overexpressed NANOG in ovarian cancer cell lines and localization of NANOG in the nucleus of ovarian cancer cells. (a) NANOG mRNA expression in HOSE cell lines and ovarian cancer cell lines as determined by qPCR (Bars, means±SD of three experiments; *, $p<0.05$; **, $p<0.005$ compared with HOSE 6-3, Mann-Whitney test). (b) NANOG mRNA expression in chemosensitive (2008) and chemoresistant (2008-C13) cell lines as determined by qPCR (**, $p<0.005$ compared with 2008). (c) NANOG in subcellular protein fractions of OVCAR-3 (T: total cell lysate, C: cytoplasmic fraction, N: nuclear fraction).

Figure 4. NANOG depletion reduced OVCAR-3 cell migration and invasion, down-regulated SOX-2, and up-regulated E-cadherin and caveolin-1. (a) Stable knockdown of NANOG mRNA and protein in OVCAR-3 as detected by qPCR and immunoblotting (inset) respectively. (b) Cell proliferation rate of OVCAR-3 in control and shNANOG after 14 days displayed as fold change compared to control; n=3; **, $p<0.005$. (c) *In vitro* migration and invasion assays using Transwell membrane without or with Matrigel coating respectively. Upper panel: representative images of migrating or invading cells. Lower panel: Cell migration or invasion presented as percentage of control; n=3; **, $p<0.005$, Mann-Whitney test. (d) mRNA expression of SOX-2, OCT-4, E-cadherin, caveolin-1 and integrin-β1 in control and shNANOG OVCAR-3 as determined by qPCR analysis. Bars, means±SD of three experiments; *, $p<0.05$; **, $p<0.005$, Mann-Whitney test. Protein expression of E-cadherin, caveolin-1 in control and shNANOG OVCAR-3 as determined by immunoblotting (inset).

Figure 5. NANOG depletion up-regulated FOXO1, FOXO3a, FOXJ1, FOXD3 and FOXB1 and enhanced FOXO1 and FOXO3a promoter activities. Nanog-mediated cell migration and invasion involved E-cadherin and FOXJ1. (a) mRNA expression of FOXO1, FOXO3a, FOXJ1 and FOXB1 in control and shNANOG OVCAR-3 as determined by qPCR analysis. Bars, means±SD of three experiments; *, $p<0.05$; **, $p< 0.005$, Mann-Whitney test. (b) *FOXO1* (left panel) and *FOXO3a* (right panel) promoter activities in control and shNANOG OVCAR-3 cells as determined by dual luciferase assays. Bars, means±SD of three experiments; *, $p<0.05$, Mann-Whitney test. (c) *In vitro* migration (left panel) and invasion (right panel) assays in control and

shNANOG OVCAR-3 cells combined with siRNAs of E-cadherin and FOXJ1. Cell migration or invasion presented as percentage of control; n=3; *, $p<0.05$, **, $p<0.005$.

Figure 6. NANOG overexpression enhanced OVCA420 cell migration and invasion, up-regulated SOX-2, and down-regulated E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1, FOXD3 and FOXB1. Nanog-mediated cell migration and invasion involved E-cadherin. (a) Ectopic overexpression of NANOG mRNA in OVCA420 as detected by qPCR. (b) *In vitro* migration and invasion assays using Transwell membrane without or with Matrigel coating respectively. Cell migration or invasion presented as percentage of control; n=3; **, $p<0.005$, Mann-Whitney test. (c) mRNA expression of SOX-2, OCT-4, E-cadherin, caveolin-1, integrin-β1, FOXO1, FOXO3a, FOXJ1 and FOXB1 in control and NANOG-overexpressing OVCA420 as determined by qPCR analysis. Bars, means±SD of three experiments; *, $p<0.05$; **, $p<0.005$, Mann-Whitney test. (d) Ectopic overexpression of NANOG (left panel) and E-cadherin (right panel) mRNA in OVCA420 as detected by qPCR. (e) *In vitro* migration (left panel) and invasion (right panel) assays in control and NANOG-overexpressing OVCA420 cells combined with ectopic overexpression of E-cadherin. Cell migration or invasion presented as percentage of control; n=3; *, $p<0.05$, **, $p<0.005$.

Supplementary Figure 1. Transient knockdown of NANOG impeded SKOV-3 cell migration and invasion, down-regulated SOX-2, and up-regulated E-cadherin, FOXO1, FOXO3a and FOXJ1. (a) Transient knockdown of NANOG mRNA in SKOV-3 as detected by qPCR. (b) *In vitro* migration and invasion assays using Transwell membrane without or with Matrigel coating respectively. Cell migration or invasion presented as percentage of control; n=3; **, $p<0.005$, Mann-Whitney test. (c)

mRNA expression of SOX-2, OCT-4, E-cadherin, caveolin-1, integrin-β1, FOXO1, FOXO3a, FOXJ1 and FOXB1 in control and siNANOG SKOV-3 as determined by qPCR analysis. Bars, means±SD of three experiments; *, $p<0.05$; **, $p<0.005$, Mann-Whitney test.

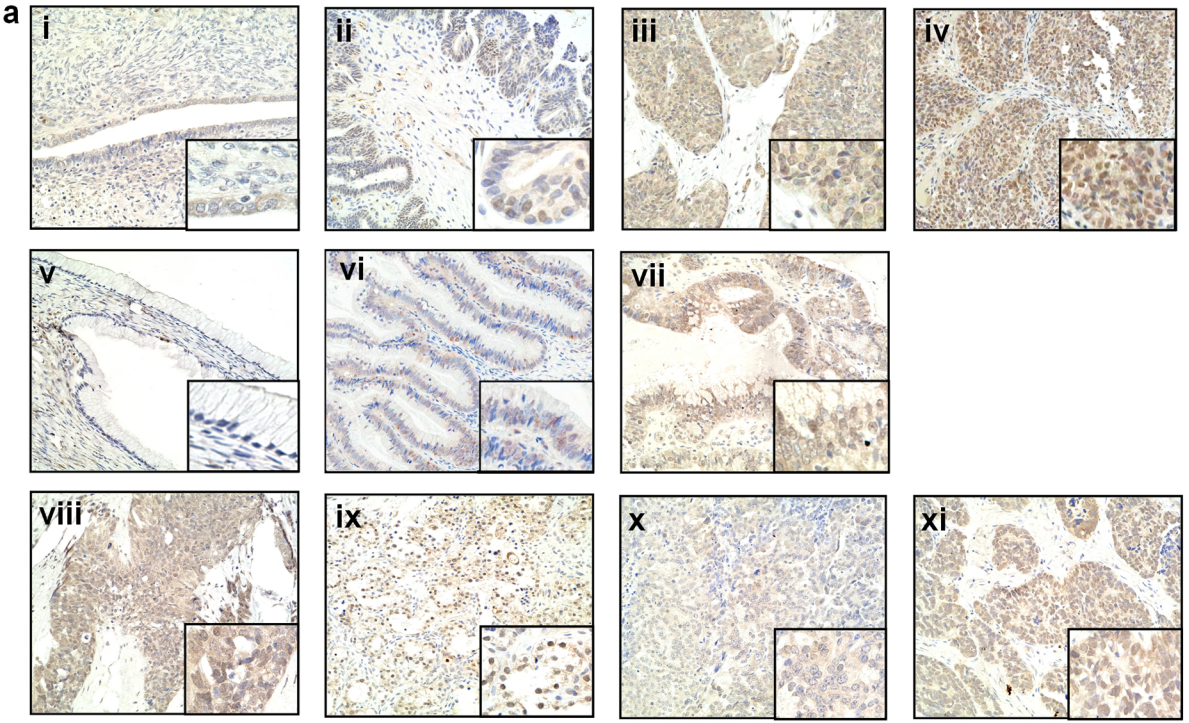
Supplementary Figure 2. Transient knockdown of mRNA expression of E-cadherin (let panel) and FOXJ1 (right panel) in OVCAR-3 cells detected by qPCR.

Table 1. Correlation of the intensity or percentage of NANOG immunoreactive cells and NANOG histoscore with different diagnostic categories and clinicopathological parameters in ovarian cancer.

Characteristics	Case (n)	NANOG (intensity)		NANOG (percentage)		NANOG (histoscore)	
		Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
Diagnostic categories							
Benign	6	0±0		0±0		0±0	
Borderline	7	0.29±0.49		2.1±3.9		0.29±0.49	
Carcinomas	97	1.02±1.16	0.018*	21.7±30.7	0.019*	1.07±1.68	0.031*
Carcinomas‡	24	0.62±0.98		13.9±25.4		0.59±0.93	
Metastatic foci‡	43	1.42±1.24	0.004†	28.1±32.4	0.015†	2.23±2.86	0.005†
Stage (FIGO)							
I	35	1.08±1.08		20.8±29.5		0.97±1.41	
II	14	0.64±1.08		5.4±10.7		0.42±0.97	
III	30	1.07±1.26		23.2±30.4		1.50±2.27	
IV	13	1.23±1.30	0.464*	26.9±33.9	0.207*	1.20±1.39	0.304*
Early (I-II)	49	0.96±1.09		16.5±26.5		0.81±1.31	
Late (III-IV)	43	1.12±1.26	0.659†	24.3±31.1	0.386†	1.41±2.03	0.340†
Histological grade (FIGO)							
1	19	0.84±1.21		4.0±7.2		0.24±0.46	
2	42	1.26±1.21		26.0±30.7		1.45±1.83	
3	34	0.76±1.02	0.119*	22.2±33.6	0.043*	1.10±1.82	0.041*
Low (1)	19	0.84±1.21		4.0±7.2		0.24±0.46	
High (2-3)	76	1.04±1.15	0.372†	24.3±31.8	0.031†	1.29±1.82	0.040†
Histology							
Serous	37	1.26±1.19		28.4±31.5		1.50±1.81	
Clear Cell	22	0.86±1.13		20.9±33.9		0.96±1.38	
Mucinous	7	1.14±1.46		17.9±25.8		1.56±3.08	
Endometrioid	31	0.84±1.07	0.374*	13.2±24.6	0.171*	0.67±1.28	0.183*
Serous	37	1.26±1.19		28.4±31.5		1.50±1.81	
Non-serous	60	0.88±1.12	0.089†	16.6±28.2	0.031†	0.88±1.60	0.035†
Chemosensitivity§							
Sensitive	65	0.91±1.11		16.0±26.9		0.76±1.38	
Resistant	15	1.33±1.23	0.162†	39.3±34.2	0.020†	2.38±2.55	0.019†
Debulking¶							
Optimal	56	1.11±1.17		20.6 ±29.0		1.09±1.67	
Suboptimal	10	0.60±0.70	0.261†	16.5±30.5	0.550†	0.78±1.77	0.423†
Cycles of chemotherapy							
≤ 6	55	0.86±1.08		17.9±29.0		0.97±1.70	
> 6	12	1.33±1.61	0.508†	23.3±38.5	0.752†	1.53±2.48	0.661†

*Kruskal–Wallis rank test; †Mann-Whitney test; ‡Randomly selected primary carcinomas with matched metastatic foci. §Chemosensitive-patients remained disease free more than 6 months after completion of first-line chemotherapy; ¶Optimal debulking referred to minimal residual disease (≤ 1 cm in maximal diameter). Those with significant P-values were underlined.

Figure 1


a
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi

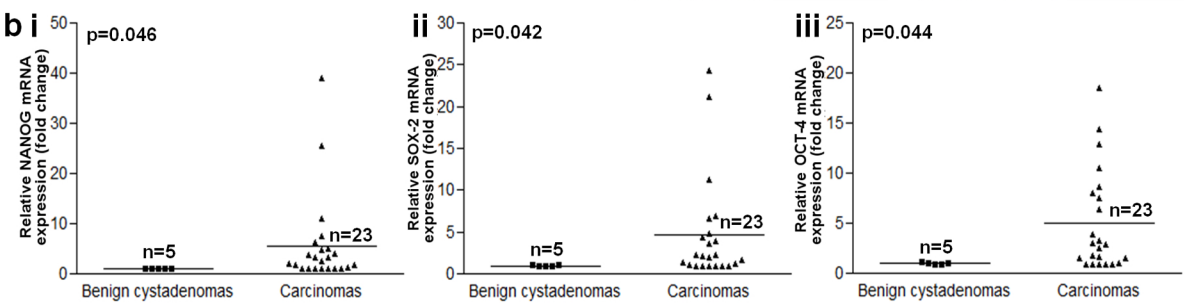

b i
p=0.046
Relative NANOG mRNA expression (fold change)
n=5
n=23
Benign cystadenomas
Carcinomas
ii
p=0.042
Relative SOX-2 mRNA expression (fold change)
n=5
n=23
Benign cystadenomas
Carcinomas
iii
p=0.044
Relative OCT-4 mRNA expression (fold change)
n=5
n=23
Benign cystadenomas
Carcinomas

Figure 2

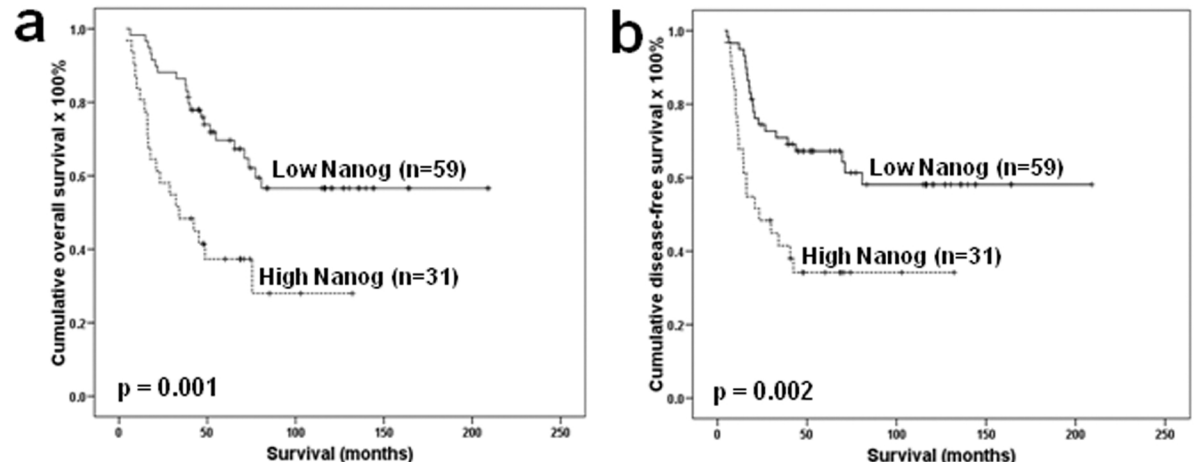

a
Cumulative overall survival x 100%
1.0
0.8
0.6
0.4
0.2
0.0
Low Nanog (n=59)
High Nanog (n=31)
p = 0.001
0
50
100
150
200
250
Survival (months)
b
Cumulative disease-free survival x 100%
1.0
0.8
0.6
0.4
0.2
0.0
Low Nanog (n=59)
High Nanog (n=31)
p = 0.002
0
50
100
150
200
250
Survival (months)

Figure 3

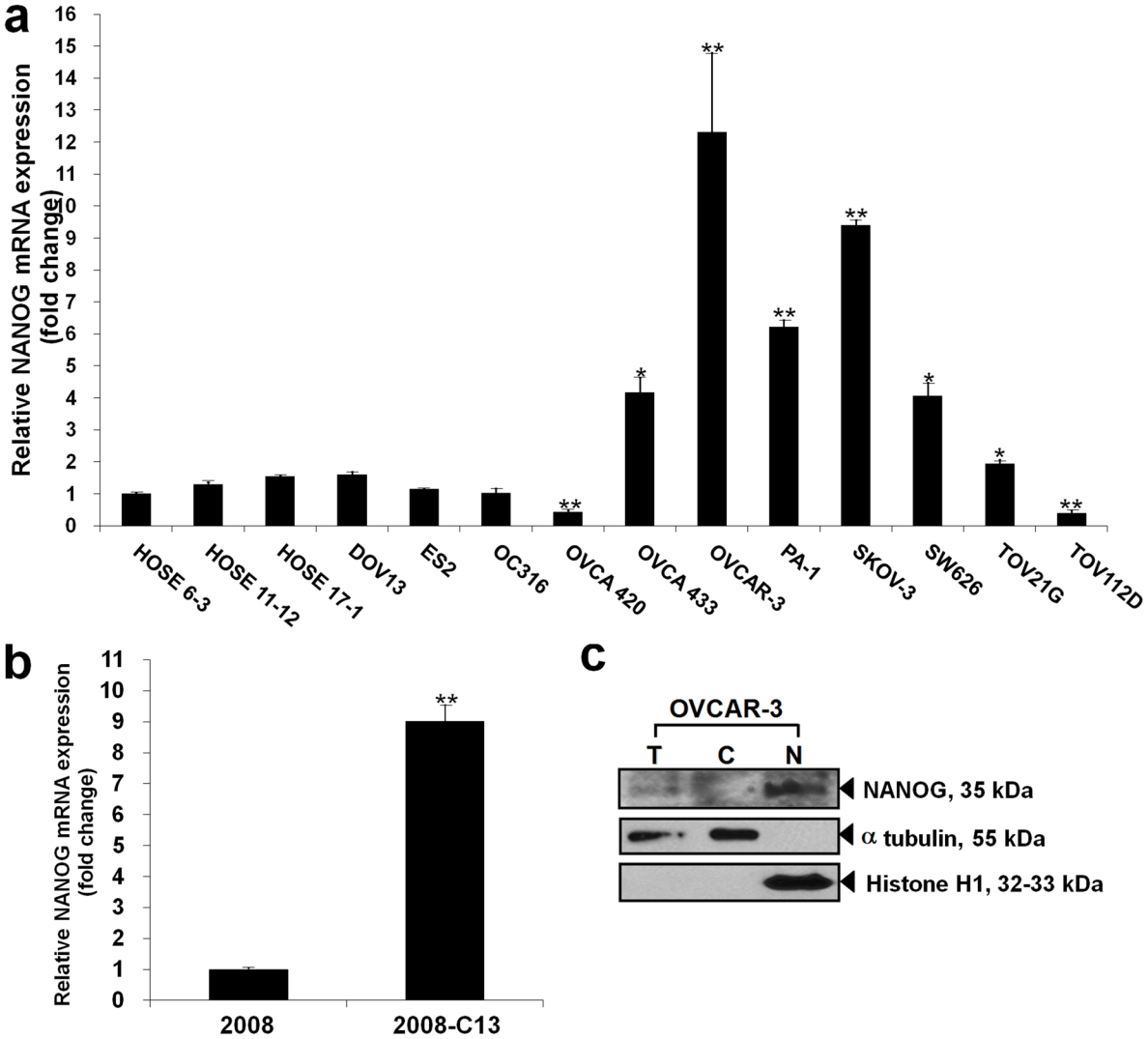

a
Relative NANOG mRNA expression (fold change)
HOSE 6-3
HOSE 11-12
HOSE 17-1
DOV13
ES2
OC316
OVCA 420
OVCA 433
OVCAR-3
PA-1
SKOV-3
SW626
TOV21G
TOV112D
b
Relative NANOG mRNA expression (fold change)
2008
2008-C13
c
OVCAR-3
T
C
N
NANOG, 35 kDa
α tubulin, 55 kDa
Histone H1, 32-33 kDa

Figure 4

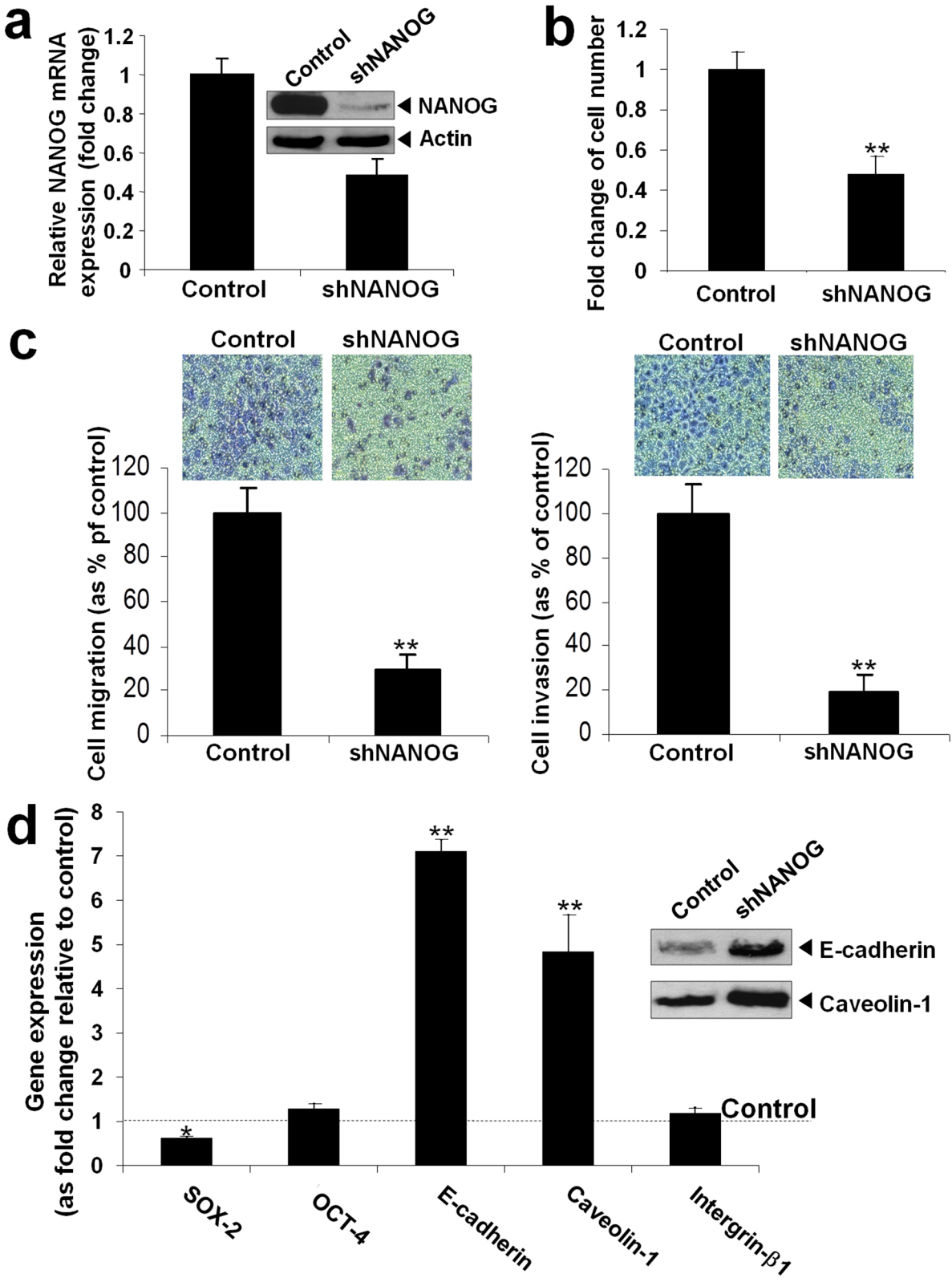

a
Relative NANOG mRNA expression (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
Control
shNANOG
Control
shNANOG
NANOG
Actin
b
Fold change of cell number
1.2
1
0.8
0.6
0.4
0.2
0
**
Control
shNANOG
c
Control
shNANOG
Cell migration (as % pf control)
120
100
80
60
40
20
0
**
Control
shNANOG
Control
shNANOG
Cell invasion (as % of control)
120
100
80
60
40
20
0
**
Control
shNANOG
d
Gene expression
(as fold change relative to control)
8
7
6
5
4
3
2
1
0
*
**
**
Control
SOX-2
OCT-4
E-cadherin
Caveolin-1
Intergrin-β1
Control
shNANOG
E-cadherin
Caveolin-1

Figure 5

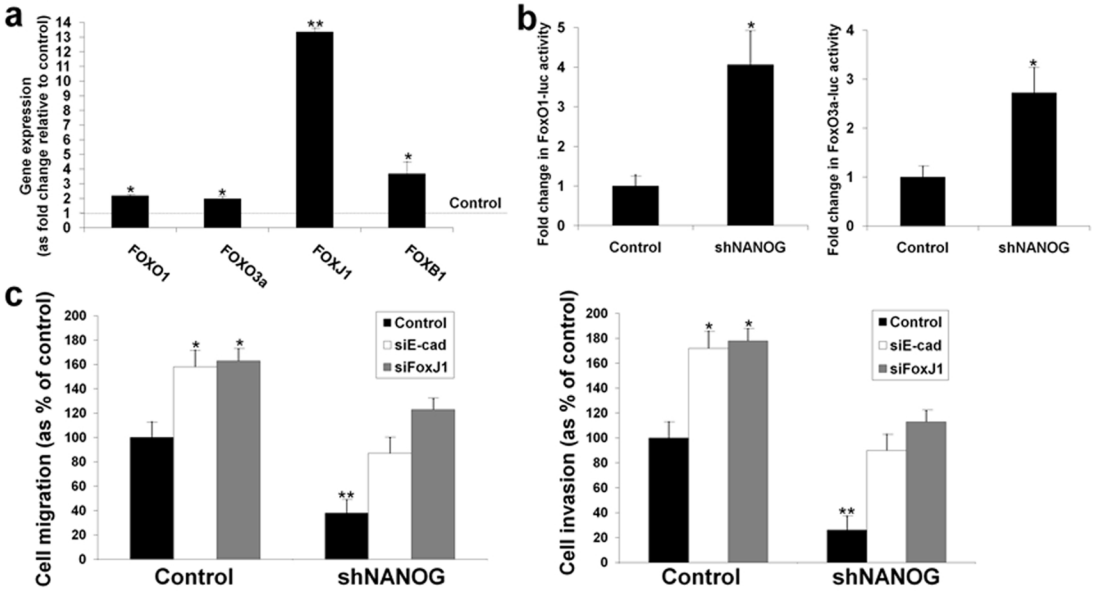
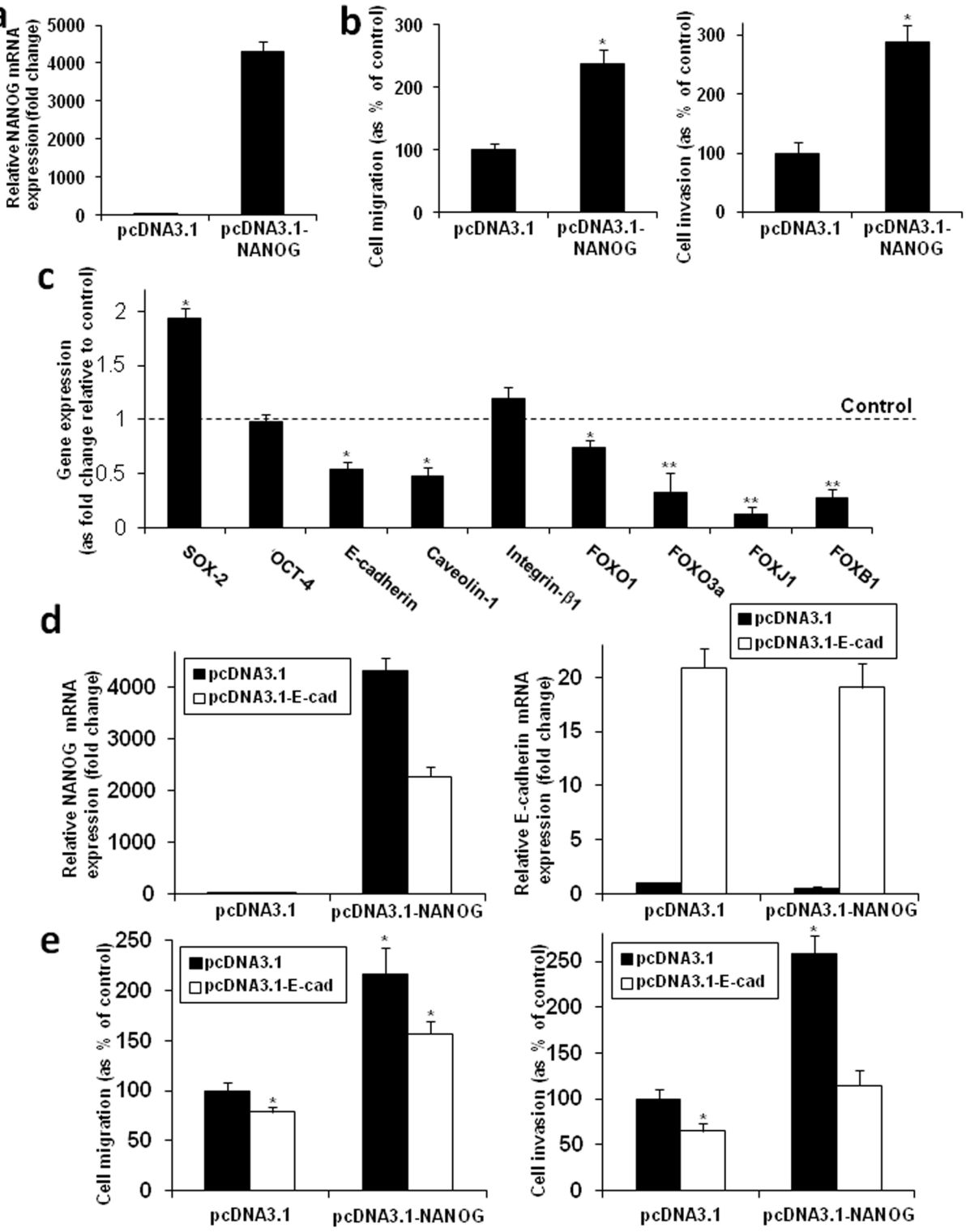

a
Gene expression
(as fold change relative to control)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
**
*
*
*
Control
FOXO1
FOXO3a
FOXJ1
FOXB1
b
Fold change in FoxO1-luc activity
5
4
3
2
1
0
*
Control
shNANOG
Fold change in FoxO3a-luc activity
4
3
2
1
0
*
Control
shNANOG
c
Cell migration (as % of control)
200
180
160
140
120
100
80
60
40
20
0
Control
siE-cad
siFoxJ1
*
*
**
Control
shNANOG
Cell invasion (as % of control)
200
180
160
140
120
100
80
60
40
20
0
Control
siE-cad
siFoxJ1
*
*
**
Control
shNANOG

Figure 6


a
Relative NANOG mRNA expression (fold change)
5000
4000
3000
2000
1000
0
pcDNA3.1
pcDNA3.1-NANOG
b
Cell migration (as % of control)
300
200
100
0
pcDNA3.1
pcDNA3.1-NANOG
Cell invasion (as % of control)
300
200
100
0
pcDNA3.1
pcDNA3.1-NANOG
c
Gene expression (as fold change relative to control)
2
1.5
1
0.5
0
Control
SOX-2
OCT-4
E-cadherin
Caveolin-1
Integrin-β1
FOXO1
FOXO3a
FOXJ1
FOXB1
d
pcDNA3.1
pcDNA3.1-E-cad
Relative NANOG mRNA expression (fold change)
4000
3000
2000
1000
0
pcDNA3.1
pcDNA3.1-NANOG
Relative E-cadherin mRNA expression (fold change)
20
15
10
5
0
pcDNA3.1
pcDNA3.1-NANOG
e
pcDNA3.1
pcDNA3.1-E-cad
Cell migration (as % of control)
250
200
150
100
50
0
pcDNA3.1
pcDNA3.1-NANOG
Cell invasion (as % of control)
250
200
150
100
50
0
pcDNA3.1
pcDNA3.1-NANOG