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NANOG regulates epithelial–mesenchymal transition and chemoresistance in ovarian cancer

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Synopsis

A key transcription factor associated with poor prognosis and resistance to chemotherapy in ovarian cancer is NANOG. However, the mechanism by which NANOG functions remains undefined. It has been suggested that epithelial-to-mesenchymal transition (EMT) also contributes to development of drug resistance in different cancers. We thus determined whether NANOG expression was associated with EMT and chemoresistance in epithelial ovarian cancer cells. NANOG expression was increased in epithelial ovarian cancer cell lines compared with its expression in normal epithelial ovarian cell lines. NANOG expression in SKOV-3 or OV2008 cells directly correlated with high expression of mesenchymal cell markers and inversely with low expression of epithelial cell marker. RNAi-mediated silencing of NANOG in SKOV-3 reversed the expression of mesenchymal cell markers and restored expression of E-cadherin. Reversibly, stable overexpression of NANOG in Moody cells increased expression of N-cadherin whereas down-regulating expression of E-cadherin, cumulatively indicating that NANOG plays an important role in maintaining the mesenchymal cell markers. Modulating NANOG expression did not have any effect on proliferation or colony formation. Susceptibility to cisplatin increased in SKOV-3 cells on down-regulating NANOG and reversible results were obtained in Moody cells post-overexpression of NANOG. NANOG silencing in SKOV-3 and OV2008 robustly attenuated *in vitro* migration and invasion. NANOG expression exhibited a biphasic pattern in patients with ovarian cancer and expression was directly correlated to chemoresistance retrospectively. Cumulatively, our data demonstrate that NANOG expression modulates chemosensitivity and EMT resistance in ovarian cancer.

Key words: epithelial-to-mesenchymal transition, NANOG, ovarian cancer.

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INTRODUCTION

The gynaecological cancer with highest incidence in women is ovarian cancer, which is also a major cause of cancer mortality in women [1]. One of the prime reasons for the high mortality associated with ovarian cancer is diagnosis at late stages of the disease, high relapse rate following surgical resection and systemic chemotherapy [2,3]. It is not very apparent if the aetiology of ovarian cancer is intrinsic or imported [4].

It was reported that cluster of differentiation 24 (CD24) and NANOG co-localize in the walls of the ovarian cysts in 25% of normal appearing ovaries in post-menopausal women [4]. Other studies have indicated presence of cancer stem cells

(CSCs) in ovarian cancer [5–7]. These CSCs attributed to resistance to chemo- and radiotherapy, as well as potent tumorigenicity and relapse rates observed in ovarian carcinoma [8–10]. Interestingly, even though epithelial-to-mesenchymal transition (EMT) – a process associated with primary tumour cells acquiring highly motile mesenchymal phenotype – has not been associated with ovarian cancer progression, CSCs in ovarian cancer have been reported to have traits of EMT [11].

However, whether NANOG expression is correlated to the EMT traits and induction of chemoresistance in ovarian cancer have not been clearly identified. Therefore, enhanced molecular understanding of putative EMT traits in ovarian cancer is imperative in order to develop more potent therapeutic strategies against ovarian cancer.

Abbreviations: BME, basement membrane extract; CD24, cluster of differentiation 24; CSC, cancer stem cell; DMEM, Dulbecco's Modified Eagle's medium; EMT, epithelial-to-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; IP, immunoprecipitation; OE-FF luc, firefly luciferase; STAT3, signal transducer and activator of transcription 3.

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MATERIALS AND METHODS

Cell culture

Moody, T80, SKOV-3 and OV2008 cell lines were purchased from the A.T.C.C. and cultured in Dulbecco's Modified Eagle's medium (DMEM) medium supplemented with 10% FBS (Lonza) and penicillin/streptomycin. Cells were maintained at 37 °C under a humidified atmosphere of 5% carbon dioxide.

Cell lysis and Western blot

Cells were lysed in immunoprecipitation (IP) lysis buffer (Life Technologies) supplemented with Mini protease inhibitor cocktail (Roche Diagnostics). Lysates were resolved by SDS/PAGE and probed with indicated antibodies (Abcam). The blots were subsequently stripped and re-probed with antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#ab-9485, Abcam) each time to ensure equal loading.

Gene construction and transfection

The pcDNA3.1-*NANOG* plasmid was obtained from Addgene. Silencer Select siRNAs targeting *Renilla* luciferase or *NANOG* were obtained from Life Technologies. Cells (4×10^4) were transiently transfected with indicated plasmids or siRNAs using Lipofectamine 3000 (Life Technologies). Cells were harvested 72 h after transfection and analysed as indicated.

Cell proliferation assay

Cell proliferation was performed using the MTT assay kit (Sigma-Aldrich). Results were expressed in terms of absorbance (A), as mean \pm S.D.

Soft agar colony formation assay

Indicated cells (10^3) were re-suspended in 3 ml of DMEM containing 0.3% agar and layered on to 6 ml of 0.5% agar beds created in 60 mm dishes. Cells were subsequently grown for 2 weeks before being counted by a colony counter. Only colonies greater than 50 μ m in diameter were counted as positive. Data were represented as mean \pm S.D.

In vitro transwell migration and invasion assays

Cultrex 96-well cell migration and Cultrex 96-well basement membrane extract (BME) cell invasion assay kits (R&D Systems) were used respectively. Images were obtained at 10 \times magnification.

Drug treatment

Moody cells were either not transfected or transfected with expression plasmid encoding firefly luciferase or *NANOG*. SKOV-3 cells were either not transfected or transfected with siRNAs tar-

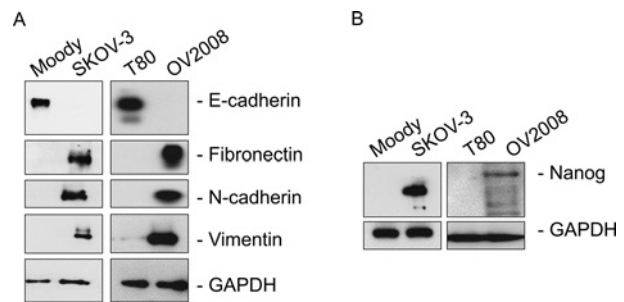


Figure 1 NANOG is overexpressed in ovarian cancer cells that express proteins' characteristics of EMT

(A) Whole-cell lysate prepared from epithelial ovarian cancer cells, SKOV-3 and OV2008, and normal epithelial ovarian cells, Moody and T80, were probed with indicated antibodies against known epithelial (E-cadherin) and mesenchymal cell (vimentin and fibronectin) markers. The blots were probed with anti-GAPDH antibody to confirm equal loading. (B) Same as (A) except for that the blot was probed with NANOG antibody.

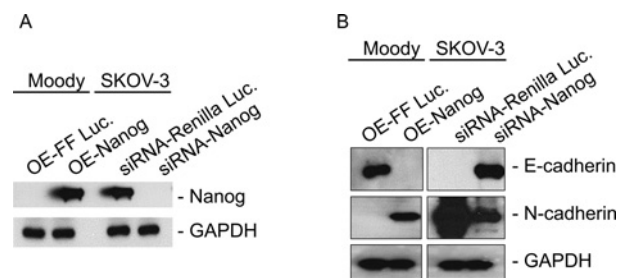


Figure 2 NANOG expression regulates the expression of EMT markers in ovarian cancer cells

(A) Successful ectopic overexpression in Moody cells and RNAi-mediated silencing of NANOG expression in SKOV-3 were confirmed by Western blot analysis. The blot was stripped and probed with GAPDH antibody to serve as loading control. (B) Whole-cell lysates in indicated cells were probed for expression of E-cadherin and N-cadherin. The blots were probed with anti-GAPDH antibody to confirm equal loading.

geting either *Renilla* luciferase or *NANOG*. After 12 h of transfection, the cells were subjected to treatment with indicated concentrations of cisplatin (Sigma-Aldrich) for 72 h. Following treatment, cell viability was measured by the MTT assay.

Immunohistochemistry analysis and scoring

Approval was obtained from the Institutional Review Board of The Second Hospital of Hebei Medical University. Curated ovarian cancer tissue specimens from 18 patients were obtained from the Department of Pathology at our hospital. Each case has information available about the patient's history of chemoresistance. Tissue specimens were stained for NANOG expression (ab#9485, Abcam) at 1:250 dilution. The stained slides were scored by a pathologist blinded to the chemoresistance history as percent of NANOG positive cells with a range of 0 to 100. The percent scores were then compared with the history of chemoresistance in these 18 patients.

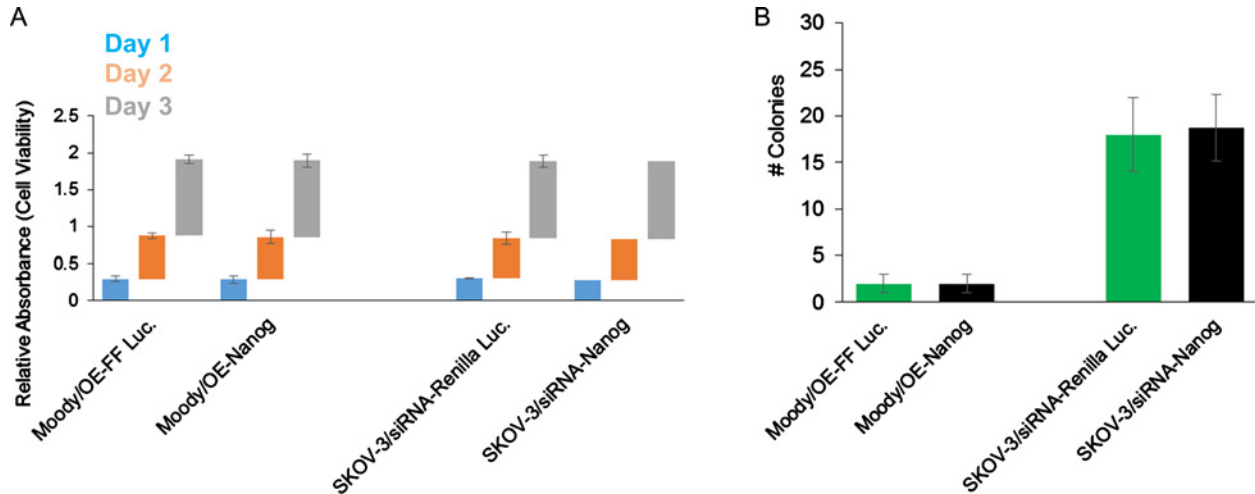


Figure 3 Modulating NANOG expression did not affect *in vitro* cell proliferation and colony formation (A) Cell viability was measured over 3 days in Moody cells transfected with Firefly luciferase or NANOG expression construct or in SKOV-3 cells transfected with shRNA targeting either *Renilla* luciferase or NANOG. (B) Moody cells transfected with Firefly luciferase or NANOG expression construct or in SKOV-3 cells transfected with shRNA targeting either *Renilla* luciferase or NANOG were grown for 2 weeks before being counted by a colony counter. Only colonies greater than 50 μm in diameter were counted as positive. Error bars, S.D.

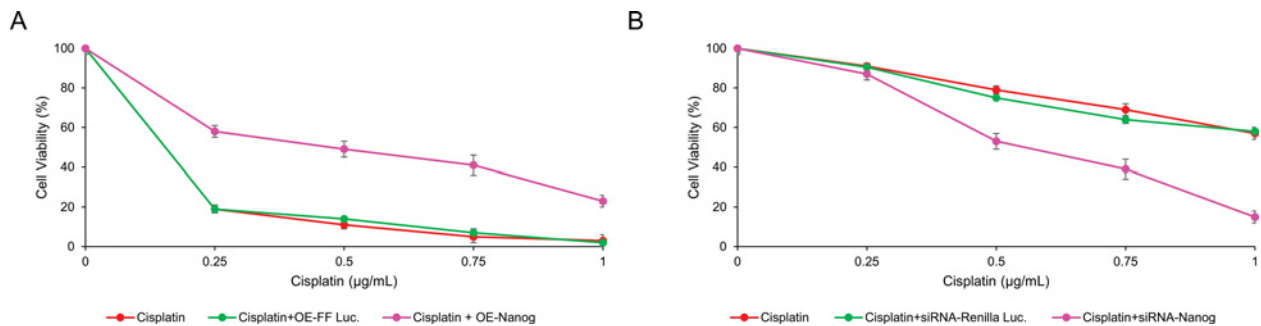


Figure 4 NANOG modulation changes susceptibility to cisplatin treatment (A) Moody cells were either untransfected or transiently transfected with firefly luciferase or NANOG for 12 h. (B) SKOV-3 cells were either untransfected or transiently transfected with shRNA targeting *Renilla* luciferase or NANOG construct for 12 h. The cells were then treated with indicated doses of cisplatin for 72 h. Cell viability was assessed by the MTT assay.

Statistical analyses

SPSS version 20.0 (IBM) was used for statistical analysis. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

To evaluate the epithelial and mesenchymal cell markers in ovarian cancer, whole-cell lysate prepared from epithelial ovarian cancer cells, SKOV-3 and OV2008, and normal epithelial ovarian cells, Moody and T80, were probed by Western blotting. As shown in Figure 1(A), SKOV-3 and OV2008 cells showed robust mesenchymal cell marker, vimentin, N-cadherin and fibronectin, expression and completely lacked in E-cadherin expression, the epithelial cell marker. In comparison, the T80 and Moody cells

had high E-cadherin expression but no detectable expression of mesenchymal cell markers (Figure 1A). This was accompanied by robust detection of NANOG in the SKOV-3 and OV2008 cells compared with the T80 and Moody cells (Figure 1B). Cumulatively, this suggested that NANOG expression is up-regulated in the ovarian cancer cells, which also expressed markers typical of mesenchymal cells.

We next determined if NANOG expression was responsible for the mesenchymal traits observed in the SKOV-3 cells. NANOG was ectopically overexpressed in the Moody cells or silenced using siRNA targeting NANOG in the SKOV-3 cells. Successful overexpression in the Moody cells and silencing in the SKOV-3 cells were verified by Western blot (Figure 2A). Whole-cell lysates from the aforementioned transfectants were then probed for expression of E-cadherin and N-cadherin. NANOG overexpression in the Moody cells induced EMT as evident by suppression

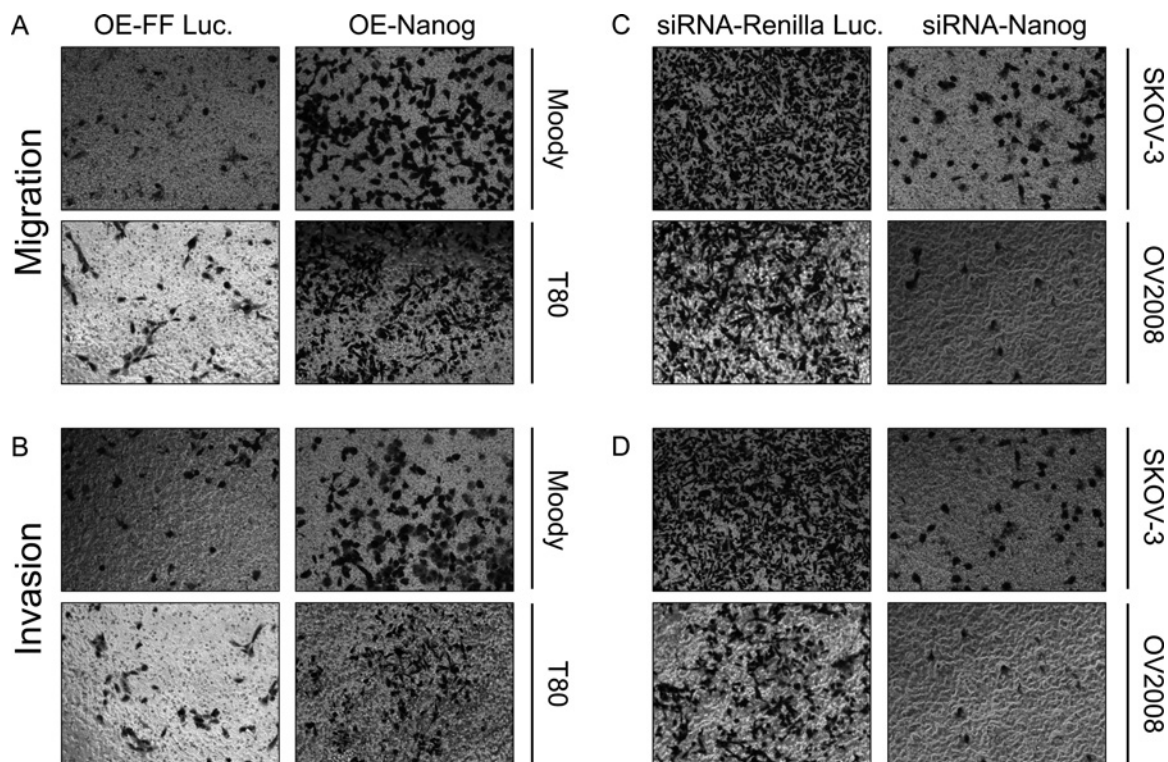


Figure 5 Modulating NANOG expression affected *in vitro* migration and invasion

Overexpression of NANOG increased *in vitro* cell migration (A) and invasion (B) in the Moody and T80 cells. RNAi-mediated silencing of NANOG decreased *in vitro* cell migration (C) and invasion (D) in the SKOV-3 and OV2008 cells respectively. The migrated and invasive cells were photographed using a microscope.

of E-cadherin expression and induction of N-cadherin expression (Figure 2B, left panels). Conversely, silencing of NANOG expression in the SKOV-3 cells reversed EMT as evident by detection of E-cadherin and decreased expression of N-cadherin (Figure 2B, right panels).

To determine whether effect of NANOG expression on EMT markers was due to intrinsic changes in cell proliferation, cell viability assays were carried out in the aforementioned transfectants. Ectopic overexpression in Moody cells (OE-FF Luc (firefly luciferase): day 1 – 0.29 ± 0.04 , day 2 – 0.58 ± 0.04 ; day 3 – 1.04 ± 0.06 /OE-NANOG: day 1 – 0.29 ± 0.05 , day 2 – 0.57 ± 0.09 ; day 3 – 1.04 ± 0.09) or silencing in SKOV-3 (siRNA-Renilla luciferase: day 1 – 0.30 ± 0.02 , day 2 – 0.54 ± 0.19 ; day 3 – 1.05 ± 0.39 /siRNA-NANOG: day 1 – 0.28 ± 0.08 , day 2 – 0.55 ± 0.20 ; day 3 – 1.05 ± 0.03) cells did not affect cell proliferation compared with the controls ($P > 0.05$ in each case) (Figure 3A). To assess the tumorigenic potential of NANOG expression, we also assessed the *in vitro* colony formation ability in the aforementioned SKOV-3 and Moody cell transfectants. As shown in Figure 3(B), ectopic overexpression of NANOG in the Moody cells or RNAi-mediated silencing of NANOG expression in the SKOV-3 cells did not induce or suppress *in vitro* colony formation in the Moody and SKOV-3 cells respectively.

To determine the therapeutic potential of NANOG expression on chemosensitivity of Moody and SKOV-3 cells to cisplatin treatment, we evaluated the effect of NANOG overexpression or silencing on the cytotoxicity of cisplatin. Overexpression of NANOG made Moody cells resistant to cisplatin treatment (Figure 4A) (IC_{50} from $11 \pm 5 \mu\text{g/ml}$ in untransfected cells, $14 \pm 1 \mu\text{g/ml}$ in luciferase overexpressing cells, to $49 \pm 4 \mu\text{g/ml}$ in NANOG overexpressing cells, $P < 0.05$). Silencing of NANOG increased chemosensitivity in SKOV-3 cells to cisplatin treatment (Figure 4B) (IC_{50} from $79 \pm 2 \mu\text{g/ml}$ in untransfected cells, $75 \pm 1 \mu\text{g/ml}$ in siRNA-Renilla luciferase cells, to $53 \pm 4 \mu\text{g/ml}$ in siRNA-NANOG cells, $P < 0.05$).

We then scored each of the individual transfectants in Moody and SKOV-3 cells, described above, for migration (Figures 5A and 5B) and invasion (Figures 5C and 5D) in standard transwell assays. Using these criteria, phase contrast imaging showed that NANOG overexpression induced *in vitro* migration (Figure 5A) and invasion (Figure 5B) in Moody cells, whereas silencing of NANOG expression suppressed migration (Figure 5C) and invasion (Figure 5D) in SKOV-3 cells. To confirm, these were not cell-type specific observations, we also assessed *in vitro* migration and invasion in T80 cells overexpressing NANOG and in OV2008 cells in which NANOG expression was silenced (Figures 5A–5D). The results suggested that NANOG

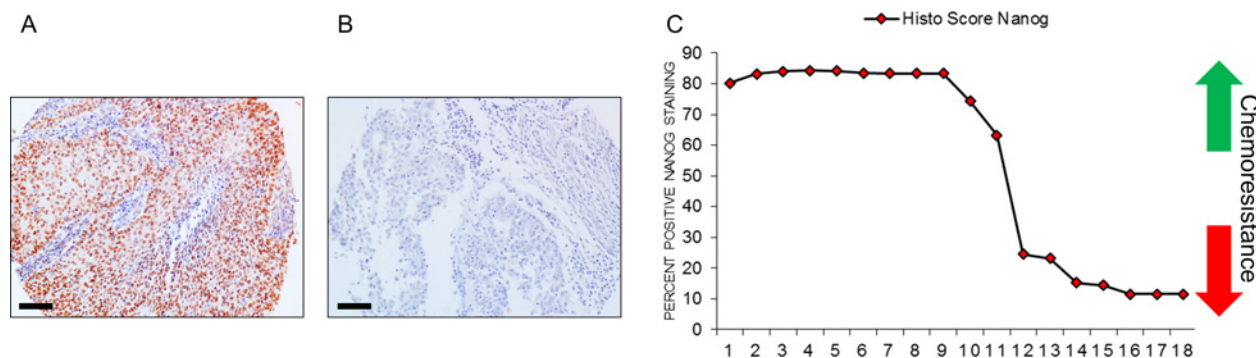


Figure 6 NANOG expression levels in patients with ovarian cancer is correlated to chemoresistance

Representative tissue specimens showing high (A) and low (B) NANOG expression; scale bar: 50 μ m. (C) Graphical representation of percent NANOG expression in the tissue specimens obtained from 18 patients. The known chemoresistance of these patients in terms of high (green arrow) and low (red arrow) is also shown.

expression levels are directly correlated to the migration and invasive potential of these cells.

We finally assessed NANOG expression by immunohistochemistry (IHC) in 18 tissue specimens obtained from patients with ovarian cancer. In 11 of the 18 cases tested, NANOG was expressed in at least 60% of the tissue core [representative case is shown in Figure 6(A)], whereas for the rest it was less than 25% [representative case is shown in Figure 6(B)]. When the patients' chemoresistance history was compared with NANOG expression, we observed that the 11 patients with high NANOG expression had some history of chemoresistance, whereas the remaining seven patients were chemosensitive (Figure 6C). Taken together, our results show that NANOG expression can be potentially used as a biomarker to predict chemosensitivity in ovarian cancer patients.

DISCUSSION

The profound induction in relative expression of NANOG in ovarian cancer cells along with its capacity to promote mesenchymal traits, *in vitro* migration and invasion, and chemoresistance together suggested that NANOG is a central determinant that drives EMT-like programmes in ovarian cancer cells that might explain the aggressive behaviour observed in ovarian CSCs. Our findings in the ovarian cancer patient samples corroborate recently reported finding that NANOG potentiates chemoresistance and EMT in ovarian cancer cells, even though in this case it was shown to be acting through signal transducer and activator of transcription 3 (STAT3) [12].

In addition, NANOG has been shown to induce EMT in gastric cancer [13], hepatocellular carcinoma [14]. Given it is a transcription factor and one that has been shown to be adequate to reprogrammed induced pluripotent cells, it is not surprising that NANOG has a role in tumorigenesis across different cancers [15]. STAT3 seems to be an important downstream effector of NANOG overexpression in cancer cells [12,16]. STAT3 is well

known to promote EMT in different cancers [17], including ovarian cancer [12].

Our findings and those of others thus calls for refining our ideas as to what EMT entails for ovarian cancer. EMT in other organs is an important pre-requisite for metastatic progression and/or chemoresistance [18,19]. In the context of ovarian cancer, we already know on the basis of our findings and that of others [12] that NANOG-mediated inheritance of mesenchymal traits induces chemoresistance in ovarian cancer. It would be interesting to determine if use of antisense oligo directed against *NANOG* in combination therapy regimens will be feasible in actual patients with ovarian cancer. In addition, it will be important to determine if NANOG-mediated EMT does have any effect on the pathogenesis of ovarian cancer.

AUTHOR CONTRIBUTION

Shan Qin designed the study, acquired and analysed the data statistically and prepared the manuscript. Xianghua Huang edited and reviewed the manuscript. Yanfang Li, Xuexia Cao and Jiexian Du acquired the data. All authors read and approved the final manuscript version that was submitted for peer review.

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