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Original Article

Silencing Nanog expression inhibits MDA-MB-231 breast stem cancer cell growth in vitro and in vivo

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Abstract: *Background:* Nanog is a transcription factor with key roles in maintaining self-renewal and pluripotency in human embryonic stem (ES) cells, and it is overexpressed in numerous malignancies. The aim of the present study was to silence Nanog gene expression by RNA interference (RNAi) and observe MDA-MB-231 breast CSC tumorigenicity and chemotherapy resistance. *Materials and methods:* We constructed a recombinant plasmid of pLvx-siNanog-ZsGreen-Puro and direct injected it into the tumors formed by MDA-MB-231 stem cells implanted in nude mice. A paclitaxel inhibition test was performed to measure resistance of MDA-MB-231 breast cancer stem cells (CSCs). Western blot and quantitative polymerase chain reaction (qPCR) analysis were used to evaluate Nanog expression. *Results:* MDA-MB-231 stem cells transfected with short interfering RNA (siRNA) constructs showed reduced resistance to paclitaxel. Similarly, western blot and qPCR analyses revealed significantly reduced Nanog levels following siRNA transfection of MDA-MB-231 stem cells. After injecting MDA-MB-231 stem cells into mice, cells transfected with Nanog siRNA formed tumors with significantly smaller volumes and weights than the negative siRNA and control groups. Finally, western blot and qPCR analyses of xenograft tumors revealed lower relative quantities of Nanog, Oct4, c-Myc, Klf4, and SOX-2 in the Nanog siRNA group. *Conclusions:* The present findings suggest that Nanog gene silencing with siRNA can reduce MDA-MB-231 breast CSC tumorigenicity and chemotherapy resistance.

Keywords: Breast cancer, transcription factor Nanog, tumor stem cells, RNA interference, mouse

Introduction

Breast cancer is the most prevalent female malignancy, threatening the health of all women [1]. Traditional treatments are often insufficient to cure the disease since the tumors are often metastatic, and new targets and novel breast cancer therapies are urgently needed. Tumor stem cells are drug resistant [2, 3] and can exclude drugs [4]. Some authors have reported that tumor stem cells are the direct cause of tumorigenesis, metastasis, and recurrence [5, 6]. Studies have shown that the stem cell transcription factors Nanog and Oct4 are less expressed or absent in cells with differential or mature organization [7, 8], but they are highly expressed in tumor tissues. Importantly, their expression is related to the degree of tumor malignancy [9, 10].

Nanog is expressed in pluripotent embryonic stem (ES) cells, as well as in breast cancer [9], retinoblastoma [11] and prostate cancer [12]. One study reported that Nanog expression in breast cancer was significantly greater than that in adjacent tissue [9]. Other researchers detected NANOGP8 (highly homologous Nanog) expression in breast cancer [13]. Besides ES cells, Nanog is highly expressed in embryonic germ (EG) and embryonic carcinoma (EC) pluripotent cells, but levels decrease after differentiation and are hardly expressed in mature tissues [14]. When Nanog is reduced in mouse and human ES cells, it leads to differentiation in an extraembryonic direction [15]. Al-Hajj and colleagues were the first to isolate breast cancer stem cells (CSCs) from breast tissue [16]. Subsequent studies have demonstrated that suspension culture can enrich breast CSCs can

be enriched [17, 18]. The microspheres are suspended in serum-free culture medium because relatively undifferentiated tumor cells are more resistant to serum-free conditions, and they form cell balls in the presence of growth factors [19].

In this study, we performed serum-free suspension culture enrichment, identified MDA-MB-231 breast CSCs, and measured Nanog expression. We then generated a RNA interference (RNAi) slow virus vector for Nanog to assess the effects of limiting its expression on tumorigenicity and chemotherapy resistance. The results indicate that Nanog may regulate stem cell markers involved in maintaining MDA-MB-231 breast CSCs in an undifferentiated phenotype. Silencing Nanog expression can inhibit the growth of breast cancer cells in nude mice and reduce resistance to the chemotherapy drug paclitaxel, offering a new targeted way to treat breast cancer.

Materials and methods

Cell culture

Human breast cancer MDA-MB-231 cell lines were provided by the cancer center of the First Affiliated Hospital of Xiamen University. The cells were cultivated in an incubator with the volume fraction of 5% CO_2 in air at 37°C. The culture medium was Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human breast cancer MDA-MB-231 cell lines were reported to contain at least 15% stem cells [20]. To obtain stem cells, MDA-MB-231 cells in the logarithmic growth phase were plated at 10^3, 10^4, 10^5, 10^6 cells/ml in serum-free DMEM/F-12 Hyclone (1:1) medium supplemented with 20 ng/ml basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA), 2% B27 (Gibco-BRL, Carlsbad, CA, USA) and 20 ng/ml epidermal growth factor (Sigma-Aldrich). Human breast cancer MDA-MB-231 stem cells were cultured in these serum-free conditions and grew as non-adherent spheroid clusters. The spheroids were dissociated and cultured in DMEM supplemented with 10% FBS. We previously used flow cytometry to demonstrate that MDA-MB-231 stem cells are homogenously CD44-positive and CD24-negative [21].

Animals and ethics

Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (45 males, 9 to 12 weeks of age, body weight 20 ± 4 g, specific pathogen-free grade) were purchased from the Experimental Animal Center of Xiamen University, Xiamen University. NOD/SCID mice were fed in an aseptic laminar flow room temperature controlled at 19-22°C and 40%-50% humidity, Noise < 50 dB. All procedures were carried out in accordance with the First Affiliated Hospital of Xiamen University (Xiamen, China) guidelines on animal experimentation, and the protocol was reviewed and approved by the Administration of Affairs Concerning Experimental Animals of Xiamen University.

RNAi silencing

RNAi experiments were performed using RNAfectin Transfection Reagent according to the manufacturer's instructions (Tiangen, Beijing, China). The following constructs were designed and synthesized by Qiagen (Hilden, Germany): Nanog siRNA (pLVX-siNanog-ZsGreen-Puro) sense: 5'-CCCAAUCCUGGAACAAUCATT-3', antisense: 5'-UGAUUGUUCCAGGAUUGGGTG-3'; a control plasmid, negative siRNA (pLVX-mCMV-ZsGreen-PGK-Puro) sense: 5'-CACCCAATCCTGGAACAATCA-3', antisense: 5'-TTCTCCGAACGTGTCACGT-3'. MDA-MB-231 stem cells were plated in 24-well plates (2×10^5 cells per well) and cultured in DMEM medium. After 24 h, those cells were transfected with 7.5 ng/μl siRNA against Nanog or non-targeting siRNA. Untransfected cells served as controls. Successfully transfected MDA-MB-231 stem cells were verified with fluorescent microscopy, and those cells were harvested 48 h after transfection to evaluate mRNA and protein expression levels.

Cell proliferation assay

Cells were seeded on 96-well plates at initial density of (1×10^5 cells/well). The cells were stained with 100 μl 3-(4.5-methylthiozol-2yl)-2.5-diphenyltetrazolium bromide (MTT) dye (0.5 mg/ml, Sigma-Aldrich) at each time point for 4 h at 37°C followed by removal of the culture medium and addition of 150 μl dimethyl sulfoxide. Absorbance was measured at 490 nm. All experiments were performed in triplicate.

Paclitaxel inhibition assay

A single cell suspension with a density of 3×10^3 MDA-MB-231 stem cells/ml was seeded in 96-well plates in serum-free DMEM. The wells were treated with paclitaxel (ASK Pharm, Nan-

jing, China) at a final volume of 200 μl, the final concentrations of paclitaxel were 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 μg/ml. The control wells contained simple cell suspension with no paclitaxel. Each concentration was tested in triplicate. After 48 h, each well was treated with 20 μl CellTiter-Blue reagent, then cultivate for 4 h at 37°C, and optical density (OD) at 570 nm was measured by fluorescence plate reader (Beckman Institute, Urbana, IL, USA). The inhibition rate was (drug uptake percentage) = (OD [control group] - OD [experimental group])/OD (control group) × 100%. Each independent experiment was repeated three times, and the half inhibitory concentration (IC50) was calculated by ProHits analysis.

Xenograft tumor model

Subcutaneous tumor xenografts were established in 9-week-old NOD/SCID mice (n = 45). The animals were divided into three groups (n = 15 each): blank control (no RNAi transfection), negative control (transfection of RNAi without any interference to the MDA-MB-231 stem cells) and Nanog siRNA group (Nanog gene sequences of RNAi). The injected MDA-MB-231 stem cells were transfected with pLVX-siNanog-ZsGreen-Puro (Nanog siRNA), pLVX-mCMV-ZsGreen-PGK-Puro (negative control), or phosphate-buffered saline (PBS, blank control). The cell suspensions (2×10^6) in a total volume of 0.2 ml were then subcutaneously injected into NOD/SCID mice. Tumor growth was monitored daily starting from the 7th day after injection. Xenograft tumor volume was estimated using the following formula: $V (mm^3) = ab^2/2$, with $b < a$. On the 14th day after inoculation, all mice were sacrificed, and the tumors were weighed.

Western blot

MDA-MB-231 stem cells and some frozen tumor tissues were collected, and the protein concentrations were measured according to the RIPA lysate manufacturer's instructions (Applygen Technologies Inc., Beijing, China). Equal amounts of protein from tissues or cells were loaded and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membranes. The mouse monoclonal antibody against human Nanog (1:1,000 dilution; ab89500) and mouse monoclonal antibody against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3,000 dilution; ab57062) were purchased from Abcam (Cambridge, UK). The PVDF membranes were incubated overnight at 4°C with the primary antibodies and then washed three times. A secondary horseradish peroxidase-labeled goat polyclonal antibody against rabbit (1:3,000 dilution; ab97200, Abcam) or a goat polyclonal antibody against mouse (1:4,000 dilution; ab-97265, Abcam) were added and incubated for 2 h at room temperature. Immunodetection was performed using an electrochemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) and the Gel Doc XR type imaging system. Band intensity was quantified using ImageJ software (National Institutes of Health, Baltimore, MD). Three independent experiments, each in triplicate, were conducted in 24-well plates.

Quantitative (q) PCR

Total RNA extraction from MDA-MB-231 stem cells and frozen tumor tissues was conducted according to the TRIzol® total RNA extraction kit manufacturer's instructions. Reverse transcription was performed from 1,000 ng total RNA using the Revert Aid First Strand cDNA Synthesis kit according to the manufacturer's instructions. The gene expression levels relative to those of GAPDH were assessed using qPCR with the ABI-7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) and SYBR-Green chemistry (Shanghai Yingjun Biotechnology Limited Company, Shanghai, China) as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The following primers were used: Nanog forward primer, 5'-TGAACCTCAGCTACAAACAG-3', and reverse primer, 5'-TGGTGGTAGGAAGAGTAAAG-3'; GAPDH forward primer, 5'-GAAGGTGAAGGTCGGAGTC-3', and reverse primer, 5'-GAAGATGGTGATGGGATTTC-3'. The reactions were run in triplicate, and the generated products were analyzed with the sodium dodecyl sulfate (SDS) software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The data were evaluated as $2^{-\Delta\Delta Ct}$ values (Ct indicates the cycle threshold). The results are expressed as the normalization ratio of the relative quantities of Nanog mRNA to those of the control, and the fold difference to the control was used for comparison.

Hematoxylin-Eosin (HE) staining

Xenograft tumors from different groups of nude mice were dissected, fixed in 0.1 g/ml neutral formalin, embedded in paraffin, cut into slices

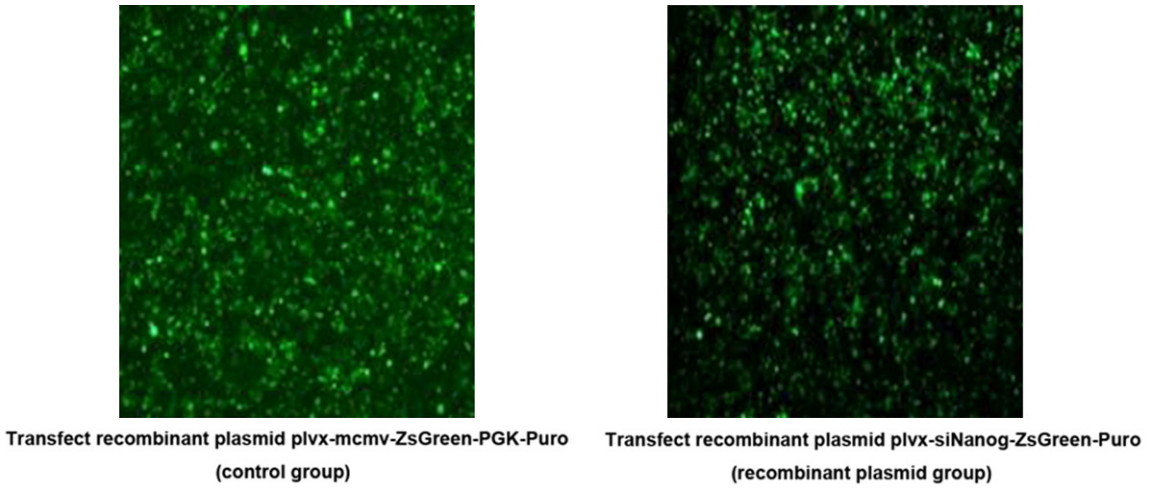

Figure 1. Recombinant plasmid plvx-siNanog-ZsGreen-Puro containing Nanog siRNA and control plasmid with plvx-mcmv-ZsGreen-PGK-Puro were transfected into human renal epithelial cells 293 (293T cells). The virus titer was 1 × 10^8 TU/ml. After 48 h, > 50% and 80% of the MDA-MB-231 stem cells exhibited green fluorescence in recombinant and control groups, respectively.

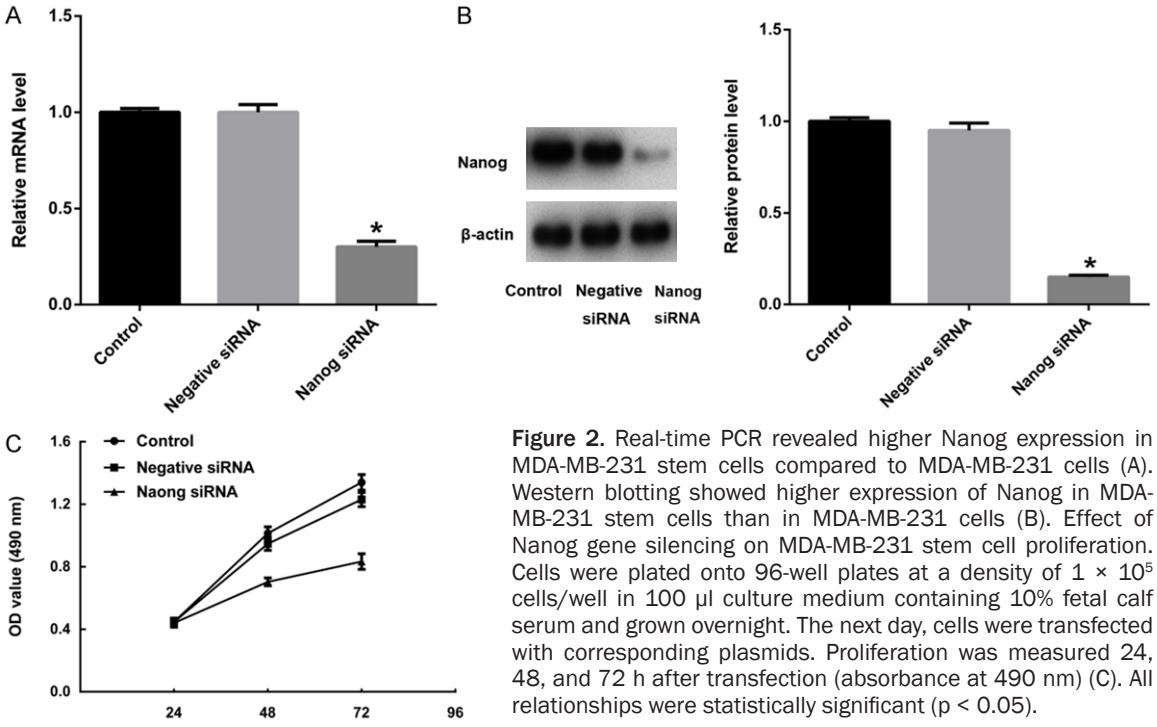

Figure 2. Real-time PCR revealed higher Nanog expression in MDA-MB-231 stem cells compared to MDA-MB-231 cells (A). Western blotting showed higher expression of Nanog in MDA-MB-231 stem cells than in MDA-MB-231 cells (B). Effect of Nanog gene silencing on MDA-MB-231 stem cell proliferation. Cells were plated onto 96-well plates at a density of 1 × 10^5 cells/well in 100 µl culture medium containing 10% fetal calf serum and grown overnight. The next day, cells were transfected with corresponding plasmids. Proliferation was measured 24, 48, and 72 h after transfection (absorbance at 490 nm) (C). All relationships were statistically significant ($p < 0.05$).

with a thickness of 3-5 µm, then stained with HE. The slides were observed under a light microscope.

Statistical methods

All results are expressed as mean ± SD. Student's two-sided *t*-tests were used to compare groups. Results were considered statistically significant at $P < 0.05$.

Results

Nanog expression was downregulated in siRNA-transfected MDA-MB-231 breast CSCs

Isolation and identification of CSCs from MDA-MB-231 breast cancer cell lines was performed as previously described [21]. We then transfected siRNA into MDA-MB-231 breast CSCs and measured Nanog expression at the mRNA

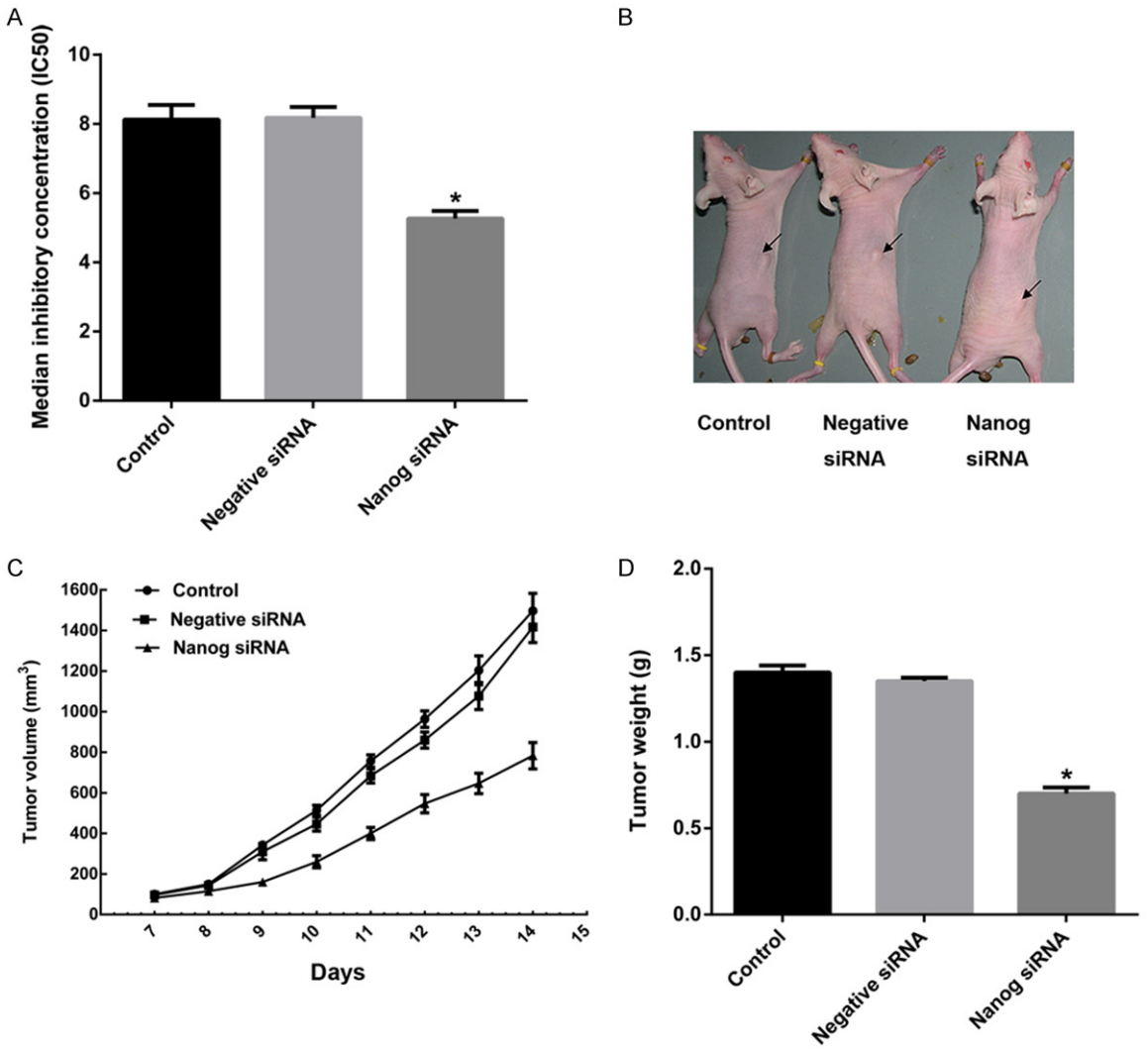

Figure 3. Paclitaxel treatment of tumor-bearing mice (16 mg/ml injection) had the greatest inhibitory effect on Nanog siRNA-transfected MDA-MB-231 stem cells. The IC50 was lowest for the animals in the Nanog siRNA group (A). Regular observation of the three groups of nude mice inoculated with MDA-MB-231 breast CSCs revealed round or oval tumors with uneven surfaces (B). Tumor volume was measured daily to calculate growth curves (C). Mice were sacrificed after 2 weeks, and the average tumor weights of the control and negative siRNA groups were greater than that of the Nanog siRNA group (D). All relationships were statistically significant ($p < 0.05$).

and protein levels. The state of transfected cells was evaluated by fluorescence microscopy, which revealed that these stem cells were successful transfected with the plasmids (**Figure 1**). Western blot and qPCR analysis showed that Nanog protein and mRNA levels were significantly decreased in MDA-MB-231 breast CSCs transfected with siRNA compared with controls (**Figure 2A**, **2B**) (both $P < 0.05$).

Nanog downregulation suppressed MDA-MB-231 stem cell growth

To further investigate the possible contribution of Nanog siRNA to MDA-MB-231 breast CSC proliferation, we examined the growth inhibition effects of siRNA on MDA-MB-231 breast CSCs in vitro using MTT assays. The cell growth curves are shown in **Figure 2C**. These results suggest that the downregulation of Nanog gene expression can suppress MDA-MB-231 breast CSC growth in vitro.

Nanog downregulation reduced drug resistance and suppressed tumor growth capability

The MDA-MB-231 stem cells transfected with siRNA became more sensitive to paclitaxel inhibition compared with controls. The paclitaxel inhibition curves for MDA-MB-231 stem cells transfected are shown in **Figure 3A**. The MDA-MB-231 stem cells transfected with the Nanog

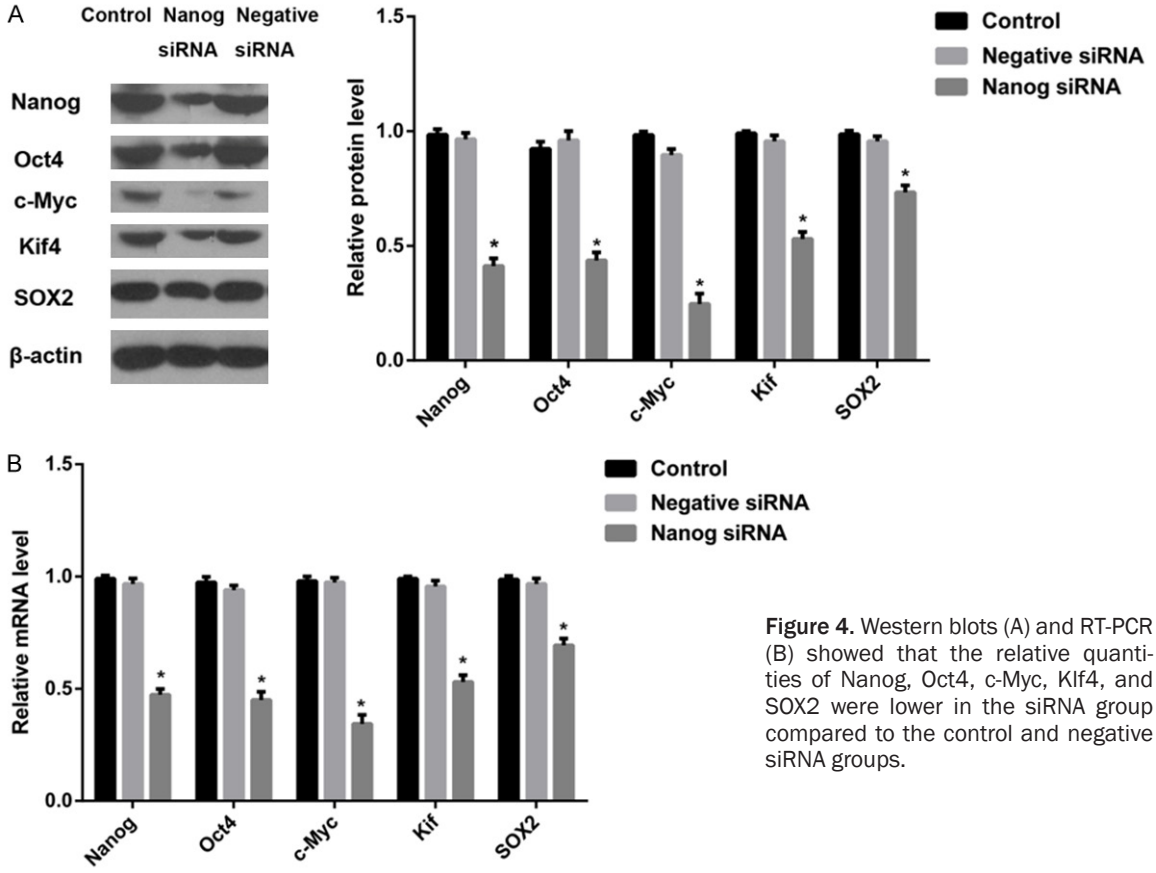

Figure 4. Western blots (A) and RT-PCR (B) showed that the relative quantities of Nanog, Oct4, c-Myc, Klf4, and SOX2 were lower in the siRNA group compared to the control and negative siRNA groups.

siRNA construct had a lower IC50 value (5.27 ± 0.22 mg/ml) than stem cells transfected with negative siRNA (8.18 ± 0.31 mg/ml) or PBS (8.13 ± 0.42 mg/ml) ($t = 7.60$, $P < 0.05$). The results show that interfering with Nanog expression can significantly enhance the sensitivity of MDA-MB-231 stem cells to chemotherapy drugs. Furthermore, it affected the tumorigenicity of MDA-MB-231 stem cells (**Figure 3B**, **3C**) as we previously described [21]. After injecting equal quantities of MDA-MB-231 stem cells (0.2×10^6 cells/animal), tumor growth was notably suppressed in the Nanog siRNA group compared with the negative siRNA or control group. Tumor implantation mice were injected with 16 mg/ml paclitaxel (in 1 ml). They were sacrificed after 14 days, and tumors were removed and weighed. The average weight of tumors in the Nanog siRNA group was significantly lower than either the negative siRNA or control group ($P < 0.05$, **Figure 3D**).

Protein and mRNA levels of Nanog, Oct4, c-Myc, Klf4, and SOX2 in xenograft tumors

The protein expression of the target genes was analyzed by western blotting. Compared to the negative siRNA group, the Nanog siRNA group had significantly lower protein levels of Nanog, Oct4, c-Myc, Klf4, and SOX2 ($P < 0.05$, **Figure 4A**). The ratio of the proteins of the target genes and β-actin varied among the groups. The mRNA expression of the target genes was analyzed by qPCR. Compared to the negative siRNA group, the Nanog siRNA group had significantly lower mRNA levels of Nanog, Oct4, c-Myc, Klf4, and SOX2 ($P < 0.05$, **Figure 4B**). The ratio of the qPCR product of the target genes and β-actin varied among the three groups. The differences between the Nanog siRNA group and negative siRNA group or control group were significant ($P < 0.05$), but the difference between the negative siRNA and control groups was not ($P > 0.05$, **Figure 4**).

HE staining of Nanog xenotransplants

Transplantation tumor tissues were stained with HE. Light microscopy revealed that tumor cell clusters were disordered, markedly atypical, variable in size, deeply stained, and with irregular mitosis. Some tumor cells were distributed as plaques, parts of the tumor centers were ischemic and necrotic, and tumor blood

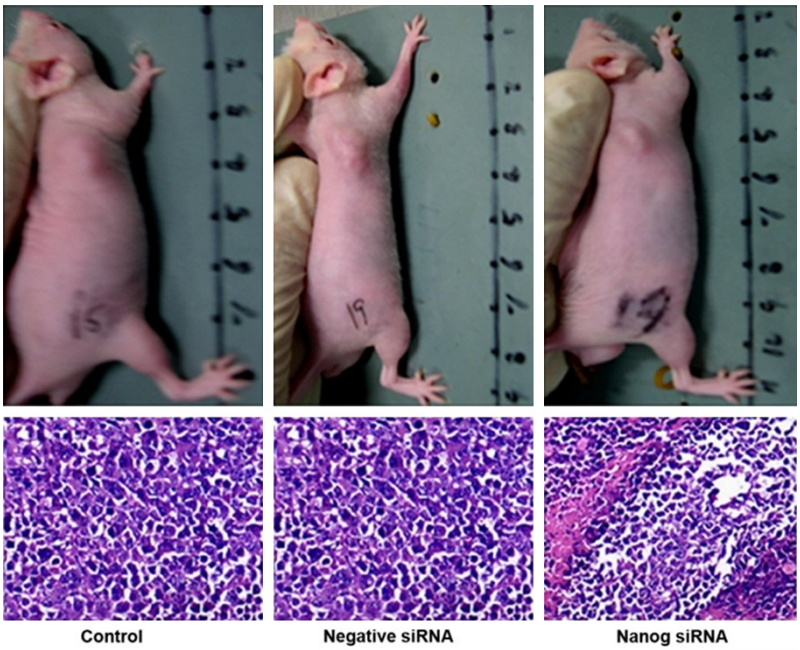

Figure 5. HE-stained transplantation tumor tissues revealed cell clusters with disordered arrangement, markedly atypical, different sizes, deep staining, and irregular mitosis. The Nanog siRNA group had fewer tumor cells compared to the negative siRNA group and control groups.

vessel-rich areas were surrounded by red blood cells and a small amount of fibrous connective tissue. The Nanog siRNA group had fewer tumor cells compared to the negative siRNA and control groups (**Figure 5**).

Discussion

Breast cancer is a common condition, and worldwide epidemiological studies have shown that breast cancer incidence and mortality rank first among female malignant tumors. There is an increasing incidence of breast cancer in China, and it has serious impacts on women's physical and mental health. The identification of tumor stem cells offers a new way to cure cancer. Breast CSCs are thought to be resistant to treatment and lead to relapse, so research into breast CSCs is of great significance. Only a small number of cells in malignant tumors have self-renewal ability and multi-directional differentiation potential, which are "stem cell" features. Hamburger first proposed the CSC theory in 1997 [22]. CSCs are specialized cells that can self-renew and differentiate into various types; they are difficult to eradicate by conventional treatments like radiation and chemotherapy, leading to tumor recurrence. CSCs have been isolated and cultured from a variety of solid tumors, and although they account for only 1%-2% of tumor cells, they are highly tumorigenic [23].

Researchers previously used PCR to screen the mouse cDNA and found an early embryo-specific NK gene specifically expressed in ES cells [24]. The gene has a homeodomain with the NK homologous gene family, indicating an important role in maintaining cell pluripotency and self-renewal, so it was named Nanog. This core transcription factor regulates the ES cell transcriptional regulatory network and maintains pluripotency by blocking differentiation. In the absence of Nanog, stem cell differentiation occurs in normal human pluripotent stem cells, and silencing Nanog can induce stem cell differentiation and apoptosis [25-27]. Nanog maintains stem cell properties (e.g., telomerase activity), so it can promote tumorigenicity [28]. Studies have shown that Nanog is not expressed in differentiated and mature cells, but it is expressed in breast, bladder, and prostate cancers and other malignancies. In poorly differentiated tumors, decreasing and increasing Nanog expression inhibit and promote tumor growth, respectively [9, 29-31]. Our studies of bone sarcoma cells using suspension growth in serum-free medium indicated that Nanog expression is closely related to tumorigenicity [32, 33]. In the present investigation, Nanog expression was high in serum-free suspension cultures of MDA-MB-231 breast CSCs. After Nanog siRNA lentivirus transfection of MDA-MB-231 breast CSCs, we observed decreased Nanog protein expression and lower mean tumor volume and weight. HE staining revealed different tumor sizes, focal scattered distribution, and ample vasculature surrounding the tumors. Tumor cells were round or polygonal epithelial cells, both large and small, with rich cytoplasm, nuclear-stained nucleolus clear atypia, and visible mitotic figures. Compared with the control group, the negative siRNA group, Nanog levels were low in the Nanog siRNA gro-

up, and there were fewer tumor cells. Collectively, our results indicate that interfering with Nanog gene expression can reduce the tumorigenicity of breast CSCs.

Effective resistance is one of the main reasons underlying treatment failure, and the existence of CSCs is a major factor in tumor chemotherapy drug resistance [2, 3]. The resistant mechanism of breast CSCs is extremely important. Nanog expression in breast CSCs is associated with taxol resistance, and our results show that paclitaxel was more effective in Nanog siRNA-transfected MDA-MB-231 breast CSCs compared to the empty vector group ($P < 0.05$). Further in vivo testing revealed significantly smaller tumor size and weight ($P < 0.05$) in the Nanog siRNA group compared to control.

We observed decreased Nanog protein levels in transplanted tumor tissue from mice treated with Nanog siRNA ($P < 0.05$), and Oct4, Klf4, c-Myc and SOX2 expression levels were also significantly lower. Nanog may regulate stem cell-associated markers and participate in maintaining the MDA-MB-231 breast CSCs undifferentiated phenotype, thus promoting MDA-MB-231 breast CSC tumorigenicity and chemotherapy resistance.

The serum-free culture method yields MDA-MB-231 breast CSCs with high tumorigenicity, strong resistance, high CD44+CD24- expression, few stem cell surface markers, and limited Nanog transcription factors. Nanog may regulate stem cell markers to maintain relevant "stem" characteristics and promote tumorigenicity and resistance in breast CSCs. As such, inhibiting Nanog expression could reduce expression levels of CD44, Oct4, Kif4, c-Myc, and stem cell-related markers. This gene target therapy could reduce the tumorigenicity and drug resistance of breast CSCs. Although serum-free suspension culture can enrich breast CSC populations, further technique refinement is necessary. Another limitation is that we only studied the effect of interfering with Nanog expression. Oct4, Klf4, c-Myc are also involved, and the effects of other factors in on tumorigenicity and resistance remain to be elucidated.

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Disclosure of conflict of interest

None.

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References

[1] DeSantis C, Siegel R, Bandi P and Jemal A. Breast cancer statistics, 2011. CA Cancer J Clin 2011; 61: 409-18.

[2] Costello RT, Mallet F, Gaugler B, Sainty D, Arnoulet C, Gastaut JA and Olive D. Human acute myeloid leukemia CD34+/CD38- progenitor cells have decreased sensitivity to chemotherapy and Fas-induced apoptosis, reduced immunogenicity, and impaired dendritic cell transformation capacities. Cancer Res 2000; 60: 4403-4411.

[3] Rich JN and Bao S. Chemotherapy and cancer stem cells. Cell Stem Cell 2007; 1: 353-355.

[4] Hirschmann-Jax C, Foster A, Wulf G, Nuchtern J, Jax T, Gobel U, Goodell M and Brenner M. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A 2004; 101: 14228-14233.

[5] Reya T, Morrison SJ, Clarke MF and Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414: 105-111.

[6] Zhang M and Rosen JM. Stem cells in the etiology and treatment of cancer. Curr Opin Genet Dev 2006; 16: 60-64.

[7] Odorico JS, Kaufman DS and Thomson JA. Multilineage differentiation from human embryonic stem cell lines. Stem Cells 2001; 19: 193-204.

[8] Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF 3rd, Boiani M and Schöler HR. Derivation of oocytes from mouse embryonic stem cells. Science 2003; 300: 1251-1256.

[9] Ezeh UI, Turek PJ, Reijo RA and Clark AT. Human embryonic stem cell genes OCT4, NANOG, STELLAR, and GDF3 are expressed in both seminoma and breast carcinoma. Cancer 2005; 104: 2255-2265.

[10] Gidekel S, Pizov G, Bergman Y and Pikarsky E. Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 2003; 4: 361-370.
[11] Seigel GM, Hackam AS, Ganguly A, Mandell LM and Gonzalez-Fernandez F. Human embryonic and neuronal stem cell markers in retinoblastoma. Mol Vis 2007; 13: 823-832.
[12] Gu G, Yuan J, Wills M and Kasper S. Prostate cancer cells with stem cell characteristics reconstitute the original human tumor in vivo. Cancer Res 2007; 67: 4807-4815.
[13] Zhang J, Wang X, Li M, Han J, Chen B, Wang B and Dai J. NANOGP8 is a retrogene expressed in cancers. FEBS J 2006; 273: 1723-1730.
[14] Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S and Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003; 113: 643-655.
[15] Hough SR, Clements I, Welch PJ and Wiederholt KA. Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. Stem Cells 2006; 24: 1467-1475.
[16] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003; 100: 3983-3988.
[17] Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA and Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005; 65: 5506-5511.
[18] Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C and Purushotham AD. Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. Int J Cancer 2008; 122: 298-304.
[19] Dontu G and Wicha MS. Survival of mammary stem cells in suspension culture: implications for stem cell biology and neoplasia. J Mammary Gland Biol Neoplasia 2005; 10: 75-86.
[20] Hiraga T, Ito S and Nakamura H. Side population in MDA-MB-231 human breast cancer cells exhibits cancer stem cell-like properties without higher bone-metastatic potential. Oncol Rep 2011; 25: 289-296.
[21] Huang ZJ, You J, Luo WY, Chen BS, Feng QZ, Wu BL, Jiang L and Luo Q. Reduced tumorigenicity and drug resistance through the downregulation of octamer-binding protein 4 and Nanog transcriptional factor expression in human breast stem cells. Mol Med Rep 2015; 11: 1647-1654.
[22] Hamburger AW and Salmon SE. Primary bioassay of human tumor stem cells. Science 1977; 197: 461-463.
[23] Ni C and Huang J. Dynamic regulation of cancer stem cells and clinical challenges. Clinical and Translational Oncology 2013; 15: 253-258.
[24] Wang SH, Tsai MS, Chiang MF, Li H. A novel NK-type homeobox gene, ENK (early embryo specific NK), preferentially expressed in embryonic stem cells. Gene Expr Patterns 2003; 3: 99-103.
[25] Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M and Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003; 113: 631-642.
[26] Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL and Jenner RG. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 2005; 122: 947-956.
[27] Ji J, Werbowetski-Ogilvie TE, Zhong B, Hong SH and Bhatia M. Pluripotent transcription factors possess distinct roles in normal versus transformed human stem cells. PLoS One 2009; 4: e8065.
[28] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K and Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131: 861-872.
[29] Chang CC, Shieh GS, Wu P, Lin CC, Shiau AL, Wu CL. Oct-3/4 expression reflects tumor progression and regulates motility of bladder cancer cells. Cancer Res 2008; 68: 6281-6291.
[30] Jeter CR, Badeaux M, Choy G, Chandra D, Patrawala L, Liu C, Calhoun-Davis T, Zaehres H, Daley GQ and Tang DG. Functional evidence that the self-renewal gene NANOG regulates human tumor development. Stem Cells 2009; 27: 993-1005.
[31] Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A and Weinberg RA. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 2008; 40: 499-507.
[32] Gibbs CP, Kukekov VG, Reith JD, Tchigrinova O, Suslov ON, Scott EW, Ghivizzani SC, Ignatova TN and Steindler DA. Stem-like cells in bone sarcomas: implications for tumorigenesis. Neoplasia 2005; 7: 967.
[33] Wilson H, Huelsmeyer M, Chun R, Young KM, Friedrichs K, Argyle DJ. Isolation and characterisation of cancer stem cells from canine osteosarcoma. Vet J 2008; 175: 69-75.