Expression of the pluripotency markers Oct3/4, Nanog and Sox2 in human breast cancer cell lines

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Abstract. Previous studies have demonstrated that pluripotency-associated transcription factors, such as Oct3/4, Nanog and Sox2, play a crucial role in the development and malignant progression of various types of tumors. Breast cancer is the most frequent cancer among females, being a heterogeneous disease, with distinct morphologies, metastatic behavior and therapeutic responses. The expression of Oct3/4, Nanog and Sox2 in 3 human breast cancer cell lines, MCF7, T-47D and MDA-MB-231, was determined. The expression of Oct3/4, Nanog and Sox2 mRNA was determined by reverse transcription polymerase chain reaction (RT-PCR) and protein expression was detected by immunocytohistochemistry. RT-PCR revealed that all three human breast cancer cell lines tested expressed evident Oct3/4, Nanog and Sox-2 mRNA at various levels. Higher levels of Oct3/4 were identified in MCF7 and MDA-MB-231 cells compared with T-47D cells. Higher levels of Nanog were observed in MCF7 and T-47D cells compared with MDA-MB-231 cells and the highest expression of Sox-2 was detected in MCF7 cells. The nuclear localization of Oct3/4, Nanog and Sox-2 was confirmed by immunostaining. Oct3/4, Nanog and Sox2 were expressed in human breast cancer cell lines. Further studies are required to characterize the role of Oct3/4, Nanog and Sox2 in human breast cancer.

Introduction

Breast cancer is the most frequent cancer among females. It is a heterogeneous disease, with distinct morphologies, metastatic behaviour and therapeutic responses. Breast cancer is now the most common cancer in developed and developing countries, with 690,000 new cases estimated in each region. Incidence rates are higher in the developed regions of the world than in the majority of developing regions (1). Despite advances in early detection, approximately 30% of patients with early-stage breast cancer have recurrent disease. Although the systemic treatment of patients with chemotherapy, hormonal therapy and immunotherapy produces a high response rate initially, progression invariably occurs following a variable time interval (2). It remains the most frequent cause of cancer mortality in females in developing and developed countries. To improve the patient survival rate, elucidation of the underlying molecular mechanisms of the tumorigenesis of breast cancer is required. Previous studies have demonstrated that core transcription factors, such as Oct3/4, Nanog and Sox2, involved in the maintenance of pluripotency and self-renewal in embryonic stem cells (ESCs), have been identified in tumors of various origins (3).

Oct3/4, also known as OCT 3, OCT 4 and POU5F1, is one of the earliest transcription factors expressed in the embryo and is encoded by a homeobox-containing gene named Pou5f1 belonging to the family of Pit Oct Unc (POU) genes and recognized as fundamental in the maintenance of pluripotency and self-renewal in ESCs and in primordial germ cells (4). Nanog is also a homeodomain transcription factor thought to be a key factor in sustaining the pluripotency of ESCs (5). Oct3/4 and Nanog expression have been identified in certain human tumors (6). Studies of Oct3/4 and Nanog have revealed that they are specific markers for seminoma/germinoma and embryonal carcinoma in primary testicular and central nervous system (CNS) tumors (7-10). The importance of Oct3/4 has also been demonstrated in the diagnosis of metastatic seminoma and embryonal carcinoma (11). In addition, the ectopic expression of Oct3/4 and Nanog induced an oncogenic potential in epithelial cells and NIH3T3 cells (12,13). The genetic expression of Oct3/4 and Nanog and the protein expression of Nanog have been identified in breast carcinoma and the MCF7 breast carcinoma cell line (6,14). Sox-2, a member of the SRY-related HMG-box family of transcription factors, regulates ESC pluripotency and is also expressed in many tumors (15-17). Previous studies of Sox-2 in breast tumor samples and cell lines have suggested that Sox-2 has oncogenic potential in breast carcinogenesis (18-20). However, a systematic study of the expression of Oct3/4, Nanog and Sox-2 in human breast cancer cell lines has not been reported.

The present study sought to detect the expression of Oct3/4, Nanog and Sox-2 in human breast cancer cell lines. A heterogeneous population of established human breast cancer cell lines was examined to investigate the diverse expressions of Oct3/4, Nanog and Sox-2 among them.

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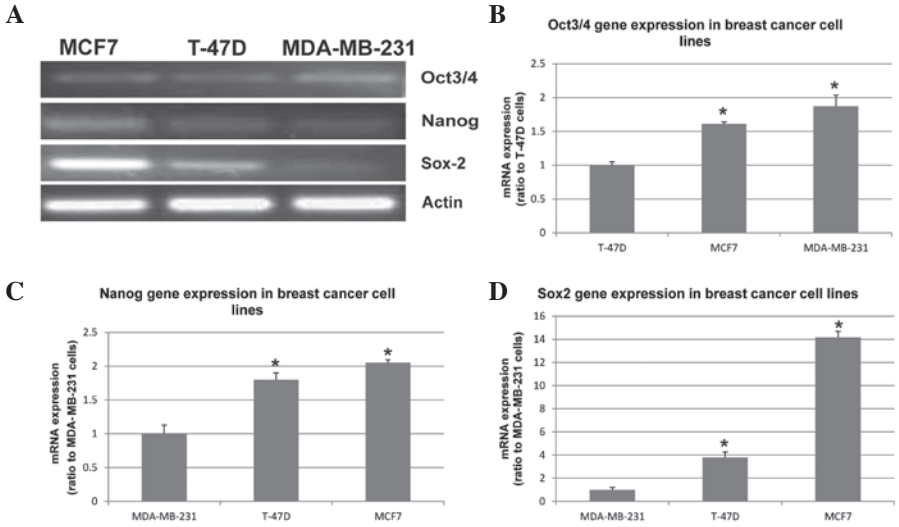

Figure 1. Expression levels of Oct 3/4, Nanog and Sox-2 genes in human breast cancer cell lines were detected by RT-PCR. (A) Oct3/4, Nanog and Sox-2 were expressed in breast cancer cell lines MCF7, T-47D and MDA-MB-231. The quantity of gene expression was normalized to β-actin to determine the quantitative differences among these 3 cell lines. (B) MDA-MD-231 and MCF7 cells expressed significantly higher levels of Oct3/4 than T-47D cells (*P<0.05). (C) MCF7 and T-47D cells expressed significantly higher levels of Nanog compared with MDA-MB-231 cells (*P<0.05). (D) MCF7and T-47D cells expressed significantly higher levels of Sox-2 compared with MDA-MB-231 cells and the highest expression of Sox-2 was detected in MCF7 cells (*P<0.05). RT-PCR, reverse transcription polymerase chain reaction.

Materials and methods

Cell lines and culture conditions. The human breast cancer cell lines MCF7, T-47D and MDA-MB-231 were purchased from the American Type Culture Collection (Shanghai Institute of Cellular Biology, Chinese Academy of Sciences, Shanghai, China). All cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 in air at 37˚C and used when in the log phase of growth.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). To assess the mRNA expression levels of Oct3/4, Nanog and Sox-2, total RNA was extracted using an RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). Reverse transcription was performed using the SuperScript First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA). For semiquantitative PCR, 1 μl target cDNA conversion mixture was amplified using Hotstar *Taq* DNA polymerase (Qiagen, Hilden, Germany) for 35 cycles at 94˚C for 30 sec, at 55˚C for 30 sec and at 72˚C for 1 min. The PCR primers included Oct3/4A (forward, 5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3'; reverse, 5'-GGCAGAGGTCGTTTGGCTGAATAGACC-3', Genbank accession number: NM_002701), Nanog (forward, 5'-TCCTCCTCTTCCTCTATACTAAC-3'; reverse, 5'-CCC ACAATCACAGGCATAG-3', Genbank accession number: NM_024865) and Sox-2 (forward, 5'-GGGAAATGGAGG GGTGCAAAAGAGG-3'; reverse, 5'-TTGCGTGAGTGT GGATGG GATTGGTG-3', Genbank accession number: NM_003106) and β-actin (forward, 5'GCGGGAAATCGT GCGTGACATT-3'; reverse, 5'-GGCAGATGGTCGTTT GGCTGAATA-3', Genbank accession number: NM_001101). PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide.

Immunofluorescent staining. To identify the expression of Oct3/4, Nanog and Sox-2 in breast cancer cell lines, cells were fixed in BD Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA) for 20 min at 4˚C. Following blocking for 20 min with donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) in BD Perm/Wash Buffer (BD Biosciences), cells were incubated with goat anti-Oct3/4 antibody (sc-8628; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti-Sox-2 antibody (MAB-4343; 1:100; Millipore, Billerica, MA, USA) overnight at 4˚C, followed by Rhodamine red conjugated donkey anti-goat antibody (1:200; Jackson Immunoresearch Laboratories, Inc.). For Nanog staining following fixation as described above, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human Nanog antibody (eBioscience, Inc., San Diego, CA, USA). Negative control reactions were included in each experiment and carried out by replacing primary antibodies with PBS. Fluorescent cells were visualized and digital images were captured using an Olympus microscope.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Statistically significant differences were determined using SPSS version 16 software. P<0.05 was considered to indicate a statistically significant result.

Results

Detection of Oct 3/4, Nanog and Sox-2 in human breast cancer cell lines by RT-PCR. Oct3/4, Nanog and Sox-2 are essential transcription factors that regulate and maintain the self-renewal and pluripotency of ESCs (4). To determine whether the genes for these proteins are expressed in three breast cancer cell lines, semiquantitative RT-PCR analyses were first performed. In addition, since the existence of

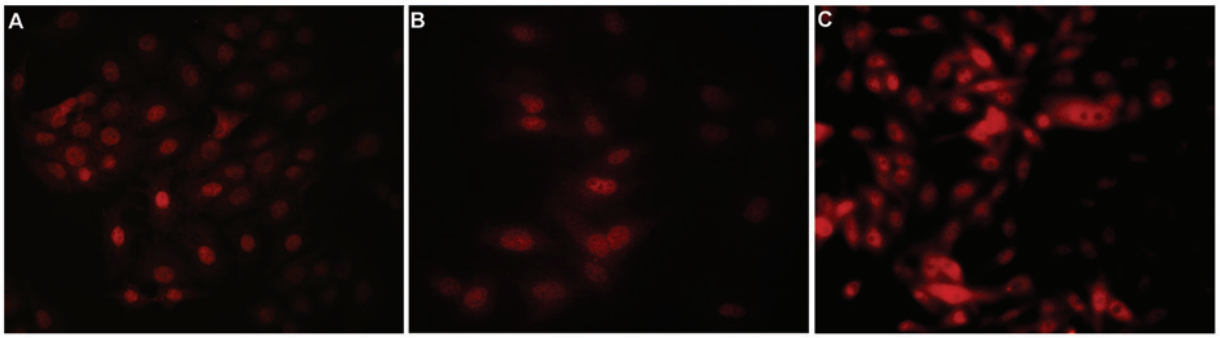

Figure 2. Representative immunofluorescent staining of Oct3/4 protein expression in human breast cancer cell lines (A) MCF7, (B) T-47D, (C) MDA-MB-231. Oct3/4 expression was clearly localized in the nuclei of cells.

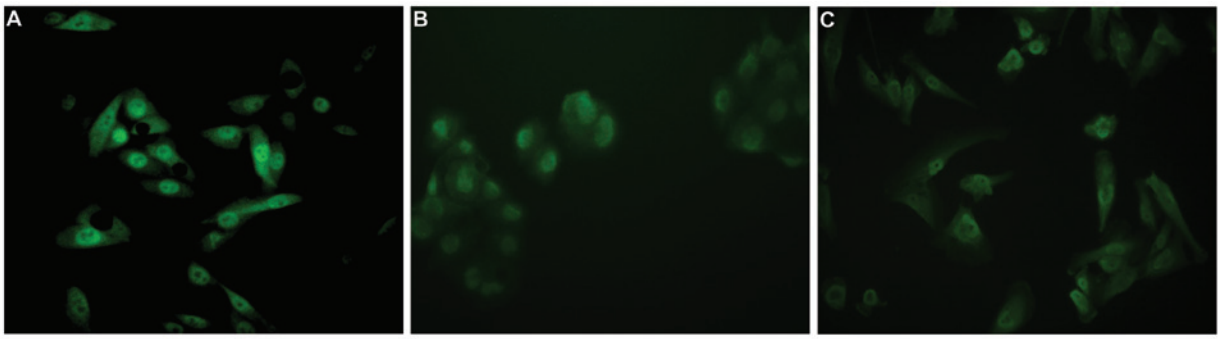

Figure 3. Representative immunofluorescent staining of Nanog protein expression in human breast cancer cell lines (A) MCF7, (B) T-47D, (C) MDA-MB-231. Nanog expression was clearly localized in the nuclei of cells and the Nanog-positive cells were scattered in breast cancer cells.

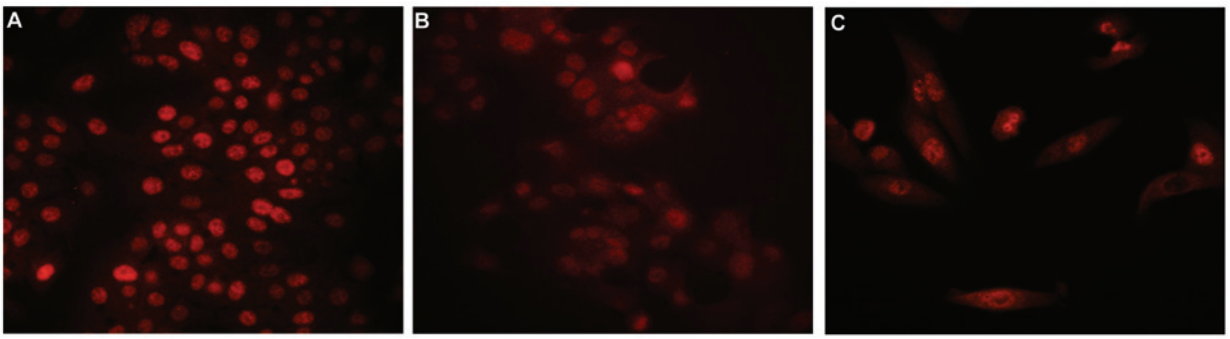

Figure 4. Representative immunofluorescent staining of Sox-2 protein expression in human breast cancer cell lines (A) MCF7, (B) T-47D, (C) MDA-MB-231. Sox-2 expression was clearly localized in the nuclei of cells.

2 mRNA and protein isoforms of Oct3/4 (Oct3/4A and Oct3/4B) has been validated (21), we sought to determine the gene expression of Oct3/4A specifically for the stem cell-like properties. As shown in Fig. 1A, the 3 human breast cancer cell lines tested expressed evident Oct3/4A, Nanog and Sox-2 mRNA at various expression levels. The quantity of gene expression was normalized to β-actin to determine quantitative differences among these 3 cell lines. As shown in Fig. 1B, MDA-MB-231 and MCF7 cells expressed significantly higher levels of Oct3/4A than T-47D cells ($P<0.05$), whereas MCF7 and T-47D cells expressed significantly higher levels of Nanog compared with MDA-MB-231 cells ($P<0.05$; Fig. 1C). MCF7 and T-47D cells expressed significantly higher levels of Sox-2 compared with MDA-MB-231 cells and the highest expression of Sox-2 was detected in MCF7 cells ($P<0.05$; Fig. 1D). The expression of various pluripotency marker genes was noted within the three breast cancer cell lines, suggesting that these pluripotency marker genes are reactivated during the process of breast cancer development.

Localization of Oct3/4, Nanog and Sox-2 in human breast cancer cell lines using immunofluorescence staining. To determine whether these genes were expressed at the protein level, the cells were examined using immunofluorescent staining. As shown in Fig. 2, a punctate nuclear staining of Oct3/4A was observed homogenously in all three cell lines, which is consistent with a previous study which demonstrated the same characteristic in type I breast stem cells (22). As shown in Fig. 3, bright punctate nuclear staining of Nanog was

also displayed in a small population of all 3 breast cancer cell lines tested. The Nanog-positive cells were scattered in breast cancer cells. Fig. 4 confirms nuclear staining of Sox-2 in all three cell lines.

Discussion

In cancer biology, the correlation between embryogenesis and oncogenesis has long been a prevailing theme. Cancer cells and ESCs share numerous key biological properties. A trait possessed by both cell types is extensive proliferative potential for embryogenesis and tumor development. Pluripotency is also fundamental to ESCs and gives rise to the myriad of differentiated daughter cells present in the mature embryo (23). It is important to study the pluripotency-related genes associated with embryogenesis and tumorigenesis. Transcription factors are critical molecular switches regulating ESC fate, which may also operate in renewing cancer cells. Three pluripotency-related transcription factors, Oct 3/4, Nanog and Sox-2, form a core regulatory network that coordinates to determine the self-renewal and differentiation of ESCs. These ESC self-renewal molecules may also contribute to tumorigenesis (24). The dysregulated expression of Oct 3/4, Nanog and Sox-2 has been shown in numerous types of tumors and it is possible that this may contribute to the neoplastic process and play a role in cancer development.

The purpose of the current study was to corroborate the presence of pluripotency-associated markers in human breast cancer cell lines and further investigate whether the diverse expressions of these markers relate to the inherent tumorigenicity among various breast cancer cell lines. Oct3/4 is an embryonic transcription factor highly expressed in ESCs, carcinoma cells and oocytes. Oct3/4 belongs to the POU transcription factor family and plays a critical role in maintaining the pluripotent and self-renewing state of stem cells (25). In humans, two isoforms are encoded, Oct3/4A and Oct3/4B, which share a common C-transactivation domain but have different N-transactivation domains. The existence of 2 isoforms is likely to be functionally significant but only Oct3/4A is responsible for the stem cell-like properties of cancer cells (21,26). It has been previously proposed that Oct3/4 acts as a multifunctional factor in cancer biology based on reports that Oct3/4 increases the malignant potential of ESCs in a dose-dependent manner (12,27). The expression of Oct3/4 has also been shown in some tumors and it is considered to play a critical role in tumorigenesis (6,28-32). However, Oct3/4 expression studies on tumors are generally carried out without considering the two isoforms. The current study sought to examine Oct3/4 A at the mRNA and protein levels in three human breast cancer cell lines. The results revealed that Oct3/4A was expressed in all three human breast cancer cell lines, although various expression patterns were noted. Oct3/4A was detected in the nuclei of breast cancer cells, suggesting a subset of cells with stem cell attributes were present in the breast cancer cell lines. In stem cells, Oct3/4A functions as a master switch during differentiation by regulating the pluripotent potential (33,34). Thus, the overexpression of Oct3/4A in human breast cancer cells implies a link between Oct3/4A and tumorigenesis via the activation of its downstream target genes (35). Furthermore, Oct3/4A mRNA expression was significantly upregulated in the MCF7 and MDA-MB-231 cells compared with the T-47D cells. This suggests that there is higher OCT3/4A expression in the adenocarcinoma cells since MCF7 and MDA-MB-231 cells are derived from an adenocarcinoma. Lower Oct3/4A expression may be seen in the ductal carcinoma cells since T-47D cells are derived from a ductal carcinoma. However, further research using additional cell lines and primary tumors is required to confirm this result.

Nanog is a recently identified transcription factor and is typically expressed in ESCs. The constitutive expression of Nanog maintains the stem cell phenotype allowing for self-renewal and propagation of the cell line, even in the presence of agents that promote differentiation. The overexpression of Nanog in human ECSs promotes pluripotency and is associated with increased self-renewal capacity, whereas the knockdown of Nanog induces differentiation into mature cell types (36,37). Previous studies of Nanog in tumors have suggested its tumorigenic potential and regulation of tumor development (14,38,39). The current study demonstrated that Nanog was detected at the mRNA and protein levels in the three human breast cancer cell lines tested. The scattered Nanog-positive cells were observed in breast cancer cells by immunostaining, suggesting that Nanog is expressed only in a small subset of cancer cells. A small population of cells capable of proliferating extensively and initiating tumors are termed cancer stem cells (40). Higher expression of Nanog has also been shown in the cancer stem cells in human osteosarcoma (41). Jeter *et al* revealed that the downregulation of Nanog inhibited MCF7 breast cancer cell clonal expansion and tumor development (14). Therefore, the expression of Nanog in breast cancer cells may correlate with the tumorigenesis of breast cancer.

Sox-2, a transcription factor located on 3q26.3-q27, is one of the transcription factors expressed by stem cells. There is growing evidence to suggest that this gene is essential for the maintenance of stem cell proliferation and differentiation capabilities (42).

A number of links have been identified between Sox-2 transcription factors and human cancers (43). Sox-2 expression has been observed in embryonal carcinoma, teratoma, lung, pancreactic and gastric adenocarcinoma (44). The present study demonstrated that Sox-2 was expressed in all 3 of the human breast cancer cell lines tested. MCF7 and T-47D cells expressed significantly higher levels of Sox-2 compared with MDA-MB-231 cells. These results are consistent with a previous study by Chen *et al* (20) that identified higher Sox-2 protein expression in MCF7 and T-47D cells than in MDA-MB-231 cells. Chen *et al* further demonstrated that the downregulation of Sox-2 using si-RNA in MCF7 cells has been shown to inhibit cell proliferation and tumor growth (20). In breast cancer, Sox-2 has also been shown to be a possible driver of the basal-like phenotype and play an early role in breast carcinogenesis (18,19).

In conclusion, the present study confirms the expression of the pluripotency-associated markers Oct3/4, Nanog and Sox-2 in 3 separate breast cancer cell lines, as demonstrated by RT-PCR and immnocytohistochemistry. Further experiments are required to explore the complex role of Oct3/4, Nanog and Sox2 in human breast cancer.

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