$CD44^{hi}CD24^{lo}$ mammosphere-forming cells from primary breast cancer display resistance to multiple chemotherapeutic drugs

PING JI[1*], YONG ZHANG[1*], SHU-JUN WANG[1], HAI-LIANG GE[1], GUO-PING ZHAO[2], YING-CHUN XU[3] and YING WANG[1,2]

[1]Shanghai Institute of Immunology, Institute of Medical Sciences, Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025; [2]Shanghai Key Laboratory of Health and Disease Genomics, Chinese National Human Genome Center, Shanghai Academy of Science and Technology, Shanghai 201203; [3]Department of Oncology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, P.R. China

Received December 7, 2015; Accepted December 31, 2015

DOI: 10.3892/or.2016.4739

Abstract. It has been widely suggested that mammosphere-forming cells from tumor cell lines or primary tumors represent the population of cancer stem cells (CSCs), which is supposed to lead to the failure of routine chemotherapy and the recurrence of the disease. However, it is still difficult to obtain CSCs from primary breast cancer for further investigation. We performed a modified culture system to generate mammosphere-forming cells derived from freshly isolated human breast cancer samples and the breast cancer cell line MCF-7. Cancer stem cell-like phenotypes such as CD44 and CD24 were measured by flow cytometry while alkaline phosphatase (AP) and mammaglobin (MGB1) expression was evaluated immunohistochemically. The expression levels of *Klf4, Nanog, Oct4, Sox2* and *mdr1* genes were analyzed by quantitative real-time PCR. Resistance to chemotherapeutic drugs was detected through the apoptosis assay upon drug treatments together with the detection of drug-resistant gene *mdr1*. The results revealed that we successfully obtained mammosphere-forming cells from the primary breast cancer in conditioned medium after 14 days of culture. Mammosphere-forming cells from primary breast cancer displayed a $CD44^{hi}CD24^{lo}$ phenotype as well as positive AP and MGB1 reactivity. Stem cell-related genes such as *Klf4, Nanog* and *Oct4* were detectably expressed in these cells. These cells formed tumor-like structures in the lymph nodes of nude mice, which were morphologically and histologically similar to breast cancer. Compared to the breast cancer cell line MCF-7 or mammosphere-forming cells from MCF-7 cells, the mammosphere-forming cells from the primary breast cancer exhibited resistance to three of four first-line chemotherapeutic drugs investigated through the induction of apoptosis, which was largely associated with the increased expression of drug-resistant gene *mdr1* upon drug treatment. In conclusion, mammosphere-forming cells generated from the primary breast cancer exhibit CSC-like properties together with multiple drug resistance. Determination of the sensitivity of these primary cancer-derived mammosphere-forming cells to chemotherapeutic drugs may thus provide useful instructions for individualized therapy against the recurrence of breast cancer in the future.

Correspondence to: Dr Ying Wang, Shanghai Institute of Immunology, Institute of Medical Sciences, Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, 227 South Chongqing Road, Shanghai 200025, P.R. China
E-mail: ywang@sibs.ac.cn

Dr Yingchun Xu, Department of Oncology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 1630 Dongfang Road, Shanghai 200127, P.R. China
E-mail: xiaoxu2384@163.com

*Contributed equally

Abbreviations: CSC, cancer stem cell; PCR, polymerase chain reaction; ER, estrogen receptor; PR, progesterone receptor; *mdr1*, multi-drug resistance gene 1; AP, alkaline phosphatase; MGB1, mammaglobin; NOD/SCID, non-obese diabetic/severe combined immunodeficiency disease; SP, side population; FBS, fetal bovine serum; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PBS, phosphate buffer saline; BSA, bovine serum albumin; H&E, hematoxylin and eosin; PI, propidium iodide; PBC, primary breast cancer

Key words: primary breast cancer, mammosphere-forming cells, chemotherapy, drug resistance

Introduction

Breast cancer is one of the malignant tumors with the highest incidence in females worldwide (1). In the past decade, the incidence of breast cancer in the Chinese female population has increased by 4%, with more younger and urban citizens affected (2). With the progresses in clinical diagnosis and treatment, the survival rate of patients with breast cancer has dramatically increased in recent years. However, metastasis and recurrence are still refractory to control, which influence the survival time and survival rate of breast cancer patients. Therefore, development of new treatment strategies against

breast cancer as well as identification of the mechanisms of recurrence and metastasis remain urgent.

It is widely supported that there exists a very small population of progenitor cells, termed as cancer stem cells (CSCs), within solid tumors that display self-renewal and differentiation potential (3-5). These cells are able to recapitulate the heterogeneity of solid tumors in immunodeficiency mice (6,7). CSCs are considered to play crucial roles in tumor development, progression, metastasis and recurrence. Cell sorting is the earliest available method for isolating and acquiring stem cells from breast cancer. A group of ESA$^+$CD44$^+$CD24$^{-/low}$ cells from human breast cancer were firstly isolated by Al-Hajj *et al* (8) in 2003 by cell sorting. It was demonstrated that 100-200 of such cells were enough to form tumors in breast tissue of non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice, thereby putting forward the hypothesis of the existence of breast CSCs. As breast CSCs isolated via cell sorting usually display CD44$^+$CD24$^{-/low}$ phenotypic characteristics (9-11), it is well feasible to isolate breast CSCs by using the combination of these surface markers. However, increased studies show that stem cells from primary breast cancer with different histological subtypes vary in regards to surface markers due to their different origins (12), limiting the applicability of one panel of stem cell surface markers to all types of breast cancers. In addition, breast cancers with similar histological subtypes sometimes display heterogeneity in their stem cell populations with diverse ability to form tumors in immune-deficient mice (12) when sorted out using the same markers. The rare numbers of CSCs isolated by surface marker-based cell sorting is another key limitation. The side population (SP) sorting method through flow cytometry with a 355 nm ultraviolet excitation device is an alternative method to isolate CSCs owing to the ability of CSCs to excrete Hoechst 33342 or Rhodamine 123 while differentiated cells do not (13). However, the SP technique may be limited by the biological toxicity of the Hoechst dye as well as the leakage of non-SP cells that exhibit CSC-like properties (14).

The cell suspension culture method used to enrich CSCs relies on their ability to form mammospheres in serum-free culture media with certain growth factors. Reynolds and Weiss (15) used conditioned medium to cultivate nerve cells in which spheres formed containing multi-potential undifferentiated nerve cells; 4-20% of them were stem cells, while others were progenitor cells in various stages of differentiation. By using a similar suspension cell culture method, Dontu *et al* (16) and Ponti *et al* (9) obtained mammospheres from human breast tissues and breast cancer tissues, respectively. Mammospheres from normal tissue form functional ductal alveolar and acinar-like structures in 3D Matrigel culture system *in vitro* resembling the entire ductal-acinar architecture of the mammary tree (17). One thousand cells from mammospheres of tumor tissues formed tumors in the mammary fat pads of mice (18). Grimshaw *et al* successfully carried out mammosphere formation in suspension culture containing CD44$^+$CD24$^+$ cells from the pleural fluid of patients with advanced breast cancer. Implantation of 5,000 or even fewer mammosphere-derived cells formed tumors in NOD/SCID mice whereas the same amount of non-sphere cells could not (19). A serum-free non-adherent suspension culture system thus facilitates the enrichment of stem cell-like cells *in vitro* that maintains the undifferentiated property, making it possible for subsequent research on breast CSCs.

With the feasibility to obtain primary CSCs from primary breast cancer, to determine the drug sensitivity of CSCs from primary breast cancer in advance may become of great value in controlling the recurrence and metastasis of breast cancer. However, to date there are still few clinical studies describing the drug resistance of primary CSCs. This is partially due to the difficulty in isolating CSCs from solid breast cancer. Therefore, to explore new methods for efficient enrichment of breast CSCs is still worthwhile in this research area.

In the present study, we successfully established a modified cell suspension culture system by modifying the combinations of growth factors. The stem cell-like properties of mammosphere-forming cells obtained were further validated *in vivo* by xenograft transplantation. Moreover, drug sensitivity of the mammosphere-forming cells to first-line chemotherapeutic drugs against breast cancer was evaluated, which may provide important clues to the determination of chemotherapy strategy in the clinic.

Materials and methods

MCF-7 cell line and primary breast cancer specimen. Human breast cancer cell line MCF-7 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. A fresh specimen of breast cancer was taken from a 42-year-old patient who had not accepted adjuvant therapy and was delivered to the laboratory for manipulation within 1 h. Invasive ductal carcinoma (IDC), ER$^-$, PR$^-$ and HER2$^+$ were pathologically confirmed. Informed consent was obtained from the patient prior to the specimen acquisition, and this study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Preparation of mammosphere-forming cells from the primary breast cancer. The fresh surgical specimen was rinsed with culture medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and minced with DMEM/F12 medium containing DNase (1 mg/ml), collagenase type IV (1 mg/ml) and 2% fetal bovine serum (FBS) (all from Invitrogen, Carlsbad, CA, USA) followed by digestion at 37˚C overnight. A single-cell suspension was prepared and filtered through a 100-mesh stainless steel sieve to remove incompletely digested tissue debris. Cells were collected by centrifugation at 800 rpm for 5 min and resuspended in DMEM/F12 complete medium supplemented with 2% FBS, B27 and insulin-transferrin-selenium cocktail (both from Invitrogen) and incubated at 37˚C with 5% CO_2. Fibroblasts were removed from the culture suspension by repeated adherence method. EGF (20 ng/ml) and bFGF (10 ng/ml) (Invitrogen) were added to DMEM/F12 complete medium for subsequent culture at 37˚C with 5% CO_2. Mammospheres were collected on the 13th day.

Preparation and passage of MCF-7 mammosphere-forming cells. Mammosphere formation was performed as previously described (20). Briefly, MCF-7 cells were treated using PBS buffer containing 0.25% trypsin and 0.02% EDTA and collected by centrifugation at 1,500 rpm for 5 min. The cells were

resuspended in the DMEM/F12 complete medium containing B27, EGF (20 ng/ml), bFGF (10 ng/ml) and trypsin (5 ng/ml) (Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd, Shanghai, China) at $2x10^5$/ml and incubated at 37˚C with 5% CO_2. After 7 days, the mammospheres were collected by centrifugation at 1,000 rpm for 5 min. To passage the mammosphere cells, cell pellets were treated with 1 ml PBS-0.25% trypsin-0.02% EDTA to obtain single cell suspension for subculture of mammospheres in DMEM/F12 complete medium.

Detection of CD44 and CD24 expression on mammosphere-forming cells. Mammospheres were digested using PBS containing 0.25% trypsin-0.02% EDTA and washed twice using PBS containing 1% BSA. A single-cell suspension was incubated with FITC-mouse anti-human CD44 and PE-mouse anti-human CD24 antibodies (BD Biosciences, USA) at 4˚C for 30 min. After washing twice with PBS containing 1% BSA, the cells were resuspended in PBS containing 1% paraformaldehyde solution and acquired through a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed by using CellQuest software (BD Biosciences).

Quantitative real-time PCR. Expression of genes including *Nanog, Sox2, Klf4, Oct4* and *mdr1* was detected by semi-quantitative real-time PCR. Briefly, total RNA was extracted from the mammosphere cells and subjected to reverse transcription using a reverse-transcription kit (Ferment, China) for first-strain cDNA synthesis. Quantitative real-time PCR was performed under conditions recommended by commercial kits (Takara, Japan). Primers were designed using Primer Express Software (version 2.0) as shown in Table I. *GAPDH* gene was used as the endogenous control. The reaction was initiated at 50˚C for 2 min and 95˚C for 10 min. Forty cycles of two-step PCR (95˚C for 15 sec and 60˚C for 60 sec) were performed. Data were collected and analyzed by using ABI Prism 7500 serial detection system (ABI, USA). The gene expression levels of each sample were calculated according to the cycle threshold (CT) value. The relative expression of target genes was calculated by $2^{-\Delta Ct}$ (ΔCt is the difference of Ct value between the target gene and the endogenous control).

Histochemistry and cytochemistry. Mammosphere-forming cells from the primary breast cancer were subjected to paraffin embedding. Hematoxylin and eosin (H&E) staining was performed as usual. For alkaline phosphatase (AP) staining, mammosphere-forming cells were fixed with 4% PFA for 2 min and washed twice with PBS. Cells were incubated with PBS-2% cobalt nitrate for 5 min and PBS-2% ammonium sulfate for 1 min, followed by rinsing with distilled water. The staining results were observed under a light microscope. Cells with dark brown staining were deemed to be undifferentiated cells whereas colorless cells were differentiated cells.

To determine the breast cancer origin of the mammosphere-forming cells, breast-specific mammaglobin (MGB1) expression was evaluated by immunohistochemistry (IHC) assay using the mouse anti-MGB1 (Dako, Denmark) antibody as the primary antibody. After incubation with the primary antibody, the slides were sequentially incubated with biotinylated goat anti-mouse IgG and ExtrAvidin®-conjugated horseradish peroxidase (Sigma) at dilutions of 1:200 and 1:30, respectively. The slides were developed with diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) substrate and counterstained with hematoxylin, dehydrated and mounted. For the negative control in the IHC procedures, PBS with 10% normal mouse serum was used for substitution of primary antibody.

Table I. Primer sequences used in real-time PCR.

Genes	Sequences
Nanog	F: AGAATAGCAATGGTGTGACGCAGAAGG
	R: TCACACGTCTTCAGGTTGCATGTTCAT
Oct4	F: GACAACAATGAGAACCTTCAGGAGA
	R: CTGGCGCCGGTTACAGAACCA
Klf4	F: GACGCGCTGCTCCCATCTTT
	R: TGACTCCGGAGGATGGGTCA
Sox2	F: ACAACTCGGAGATCAGCA
	R: GCAGCGTGTACTTATCCTTC
mdr1	F: TGCGACAGGAGATAGGCTG
	R: GCCAAAATCACAAGGGTTAGCTT
GAPDH	F: GAAGGTCGGAGTCAACGGAT
	R: CCTGGAAGATGGTGATGGG

F, forward; R, reverse.

Xenograft formation of mammosphere-forming cells in nude mice. Mammosphere-forming cells (10^3, 10^4 and 10^6) from the primary breast cancer were subcutaneously inoculated in 6-week-old female nude mice. Sixty days after inoculation, auxiliary lymph nodes were extracted to prepare paraffin sections for H&E and MGB1 staining. All animal experiments were approved by the Committee on the Use of Live Animals of Shanghai Jiao Tong University School of Medicine.

Apoptosis detection. Mammosphere-forming cells ($5x10^4$) from the primary breast cancer, and MCF-7 cells were incubated with chemotherapeutic drugs including paclitaxel, doxorubicin, 5-fluorouracil and cisplatin at different concentrations for 24 h at 37˚C with 5% CO_2. Annexin V-propidium iodide (PI) staining (BD Pharmingen, USA) was performed for the detection of apoptosis after the treatment of the chemotherapeutic drugs according to the manufacturer's instructions. Briefly, the cells were collected after drug treatment and resuspended in 500 μl of binding buffer. After addition of 5 μl of Annexin V-FITC and 5 μl of PI solution, the cells were incubated at room temperature for 5 min in the dark and acquired through a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed by using CellQuest software (BD Biosciences).

Statistical analyses. Statistical analysis was performed with SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). The inter-group differences were evaluated by one-way ANOVA analysis. $p<0.05$ was considered statistically significant.

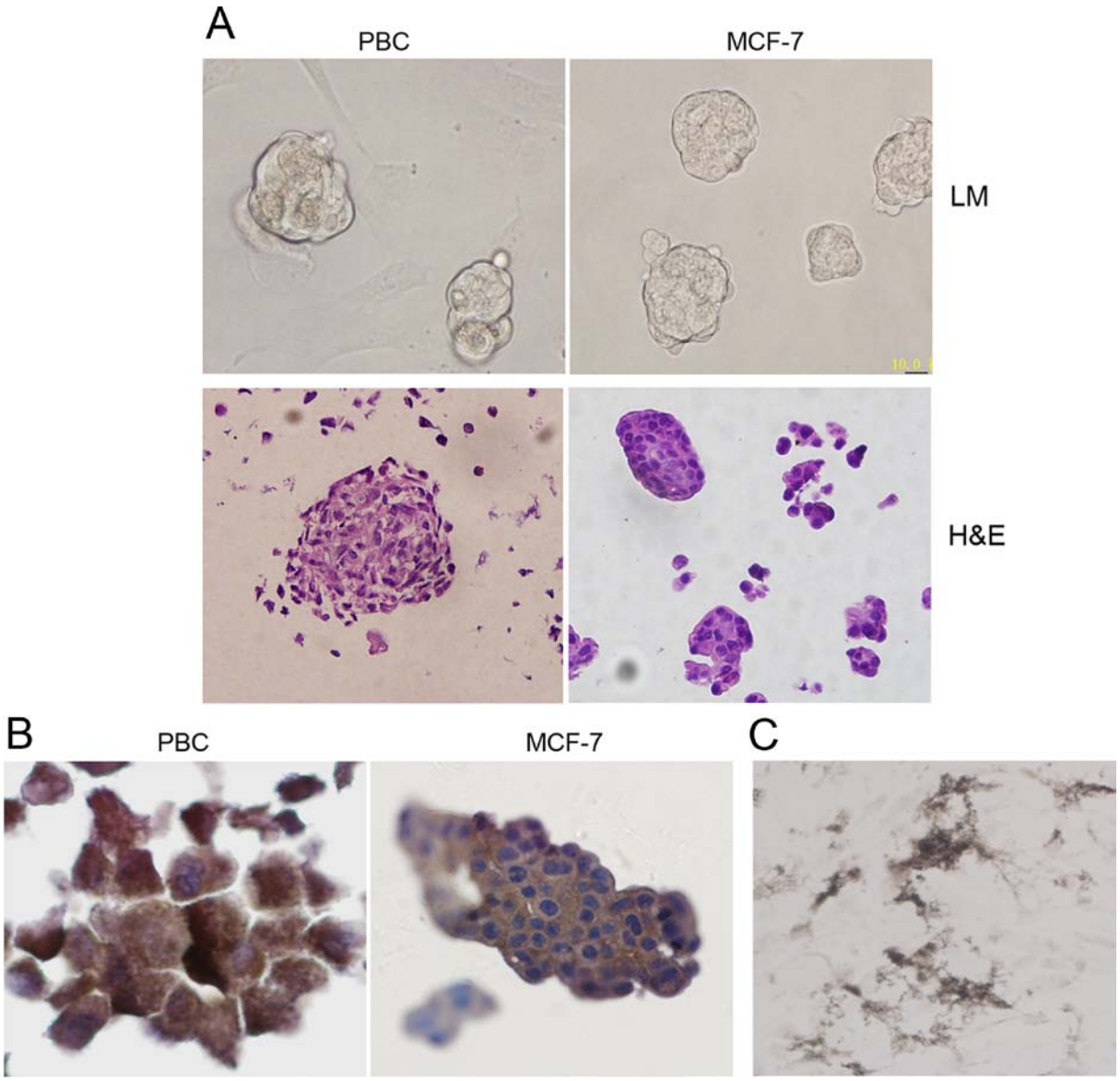

Figure 1. Preparation and characterization of the mammosphere-forming cells from a primary breast cancer. Mammosphere-forming cells were obtained through conditioned culture for 13 days. (A) Mammospheres were observed under light microcrope (upper panels) as well as H&E staining (lower panels). (B) MGB1 staining was performed to determine the properties of the breast cancer. (C) Breast origin was determined by AP staining. Magnification, x400. PBC, primary breast cancer.

Results

Preparation and characterization of mammosphere-forming cells from the primary breast cancer. After the removal of fibroblasts by differential trypsinization and repetitive adherence, a modified suspension cell method was carried out to obtain mammosphere-forming cells from a patient with breast cancer. Freshly isolated cells were cultured in conditioned medium with a low concentration of serum and growth factor cocktail. Mammospheres were formed after 7 days and collected on day 14 (Fig. 1A, upper panel). The ability to form mammospheres remained even after the passage of mammosphere-forming cells. Histological analysis of these mammospheres showed that they resembled the mammospheres derived from breast cancer cell line MCF-7 in conditioned medium in cell density and size (Fig. 1A, lower panel). To determine the origin of the mammosphere-forming cells obtained, we performed labeling for breast-specific markers. Our results showed that the breast cancer-specific MGB1 staining of the cells was apparent (Fig. 1B). In addition, all mammosphere-forming cells exhibited strong AP-positive staining (Fig. 1C), which indicated that these cells still retained the tumorigenic potential of breast cancer. The above results indicated that we successfully established mammospheres from primary breast cancer using the modified cell suspension method in the conditioned medium.

Mammosphere-forming cells from the primary breast cancer exhibit cancer stem cell-like properties. It is widely suggested that mammosphere-forming cells generated from conditioned medium possess the CSC-like properties (21). We next determined whether the mammosphere-forming cells established from primary breast cancer displayed CSC-like phenotypes. We first analyzed the expression of CD44 and

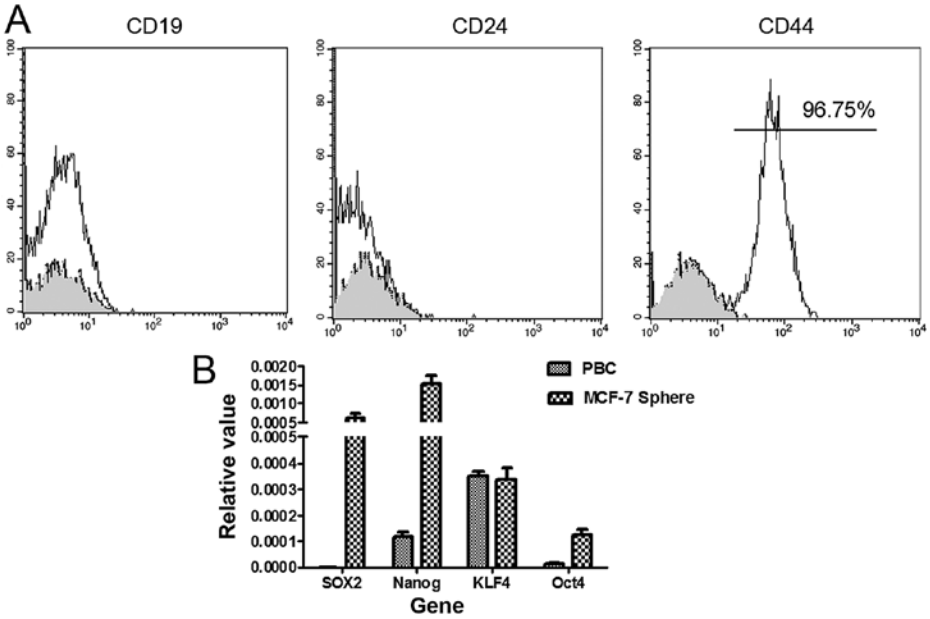

Figure 2. Determination of the cancer stem cell-like properties in mammosphere-forming cells from a primary breast cancer (A). Surface CD44 and CD24 phenotypes were analyzed on mammosphere-forming cells from a primary breast cancer by flow cytometry. (B) Four stem cell-related genes were detected in mammosphere-forming cells by real-time PCR. PBC, primary breast cancer.

CD24, the representative phenotypes of CSCs, in mammosphere cells by flow cytometry. Mammosphere-forming cells from the primary breast cancer exhibited $CD44^{+}CD24^{-}$ characteristics (Fig. 2A), which differed from the MCF-7-derived mammosphere-forming cells ($CD44^{lo}CD24^{lo}$).

Sox2, Nanog, Klf4 and *Oct4* are the most commonly used gene markers that are related to stem cell differentiation (22). We further analyzed the expression of these four genes in mammosphere-forming cells from the primary breast cancer. As indicated in Fig. 2B, mammosphere-forming cells expressed *Nanog, Klf4* and *Oct4* whereas the expression level of *Sox2* was relatively low (Fig. 2B). The expression level of *Klf4* in the mammosphere-forming cells from primary breast cancer was comparable to that of the MCF-7-derived mammosphere-forming cells, while *Nanog* and *Oct4* expression was lower.

According to the above results we deduced that the mammosphere-forming cells obtained from primary breast cancer through conditioned culture belonged to a cell population with CSC-like properties.

Mammosphere-forming cells from the primary breast cancer maintain proliferative capacity in vitro and in vivo. With the determination of CSCs-like properties of the mammosphere-forming cells from the primary breast cancer, we further evaluated the proliferative capacity of these cells *in vitro* and *in vivo*. When compared with widely used breast cancer cell line MCF-7, the proliferative rate of cells from the mammospheres was comparable on day 1, but proliferated rapidly afterwards. On days 4 and 7, the cellularity of the mammosphere-forming cell group was significantly higher than that of the MCF-7 cells (day 7, $p=0.0134$) (Fig. 3A), which is largely due to the fact that the mammosphere-forming cells obtained from the primary breast cancer retained the capacity of long-term survival and proliferation.

Mammosphere-forming cells (10^3, 10^4 and 10^6) from the primary breast cancer were inoculated into sub-lethal irradiated nude mice to determine their tumorigenicity *in vivo*. Although no apparent neoplasia was formed after 60 days, axillary lymph nodes were dramatically enlarged in all mice even when the inoculated cell number was as low as 10^3. Histological analysis of the axillary lymph nodes showed that the structures of the lymph nodes were destroyed by the compartmentalization of lymphocytes and the extensive infiltration of tumor cells (Fig. 3B). Consistent with the histological observations, tumor cells infiltrating into lymph nodes were mostly MGB1-positive, indicating that these cells were derived from breast cancer (Fig. 3C).

These results indicated that the mammosphere-forming cells that we obtained from a primary breast cancer maintained the proliferative capacity *in vitro* and *in vivo*, further verifying their CSC-like characteristics.

Mammosphere-forming cells from the primary breast cancer are resistant to multiple chemotherapeutic drugs. Preparation of primary tumor cells largely facilitates the determination of the chemotherapy strategy through drug sensitivity assays in the clinic. Considering the potential roles of CSCs in the recurrence of tumors, drug sensitivity screening of CSCs from primary breast cancer to chemotherapeutic drugs may be of great value to control the recurrence of breast cancers. Benefiting from the expansion of CSC-like mammosphere-forming cells from the primary breast cancer in our study, we further performed the drug sensitivity assay by drug-induced apoptosis detection with four widely used first-line chemotherapeutic drugs. As shown in Fig. 4, the mammosphere-forming cells from the primary breast cancer exhibited resistance to apoptosis upon the treatment of three of the four chemotherapeutic drugs tested (5-fluorouracil, paclitaxel and cisplatin) (Fig. 4) while they were more sensitive to doxorubicin. However, MCF-7 or

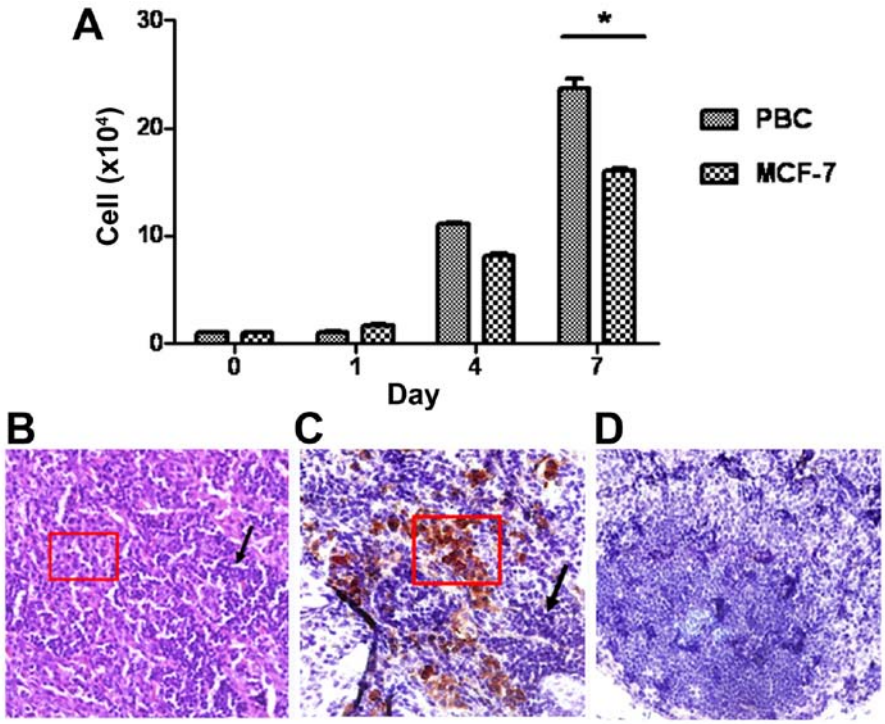

Figure 3. *In vitro* and *in vivo* proliferation of mammosphere-forming cells from a primary breast cancer. (A) Proliferation of mammosphere-forming cells *in vitro* was evaluated by CCK-8 assay at different time points. Mammosphere-forming cells (10^3) were inoculated into nude mice and lymph node tissue was subjected to histological analysis. (B) H&E staining and (C) MGB1 immunohistochemistry analysis were performed. (D) Negative control. Magnification, x400. PBC, primary breast cancer; arrow, lymphnoid cells; square, inoculated tumor cells. $^*p<0.05$.

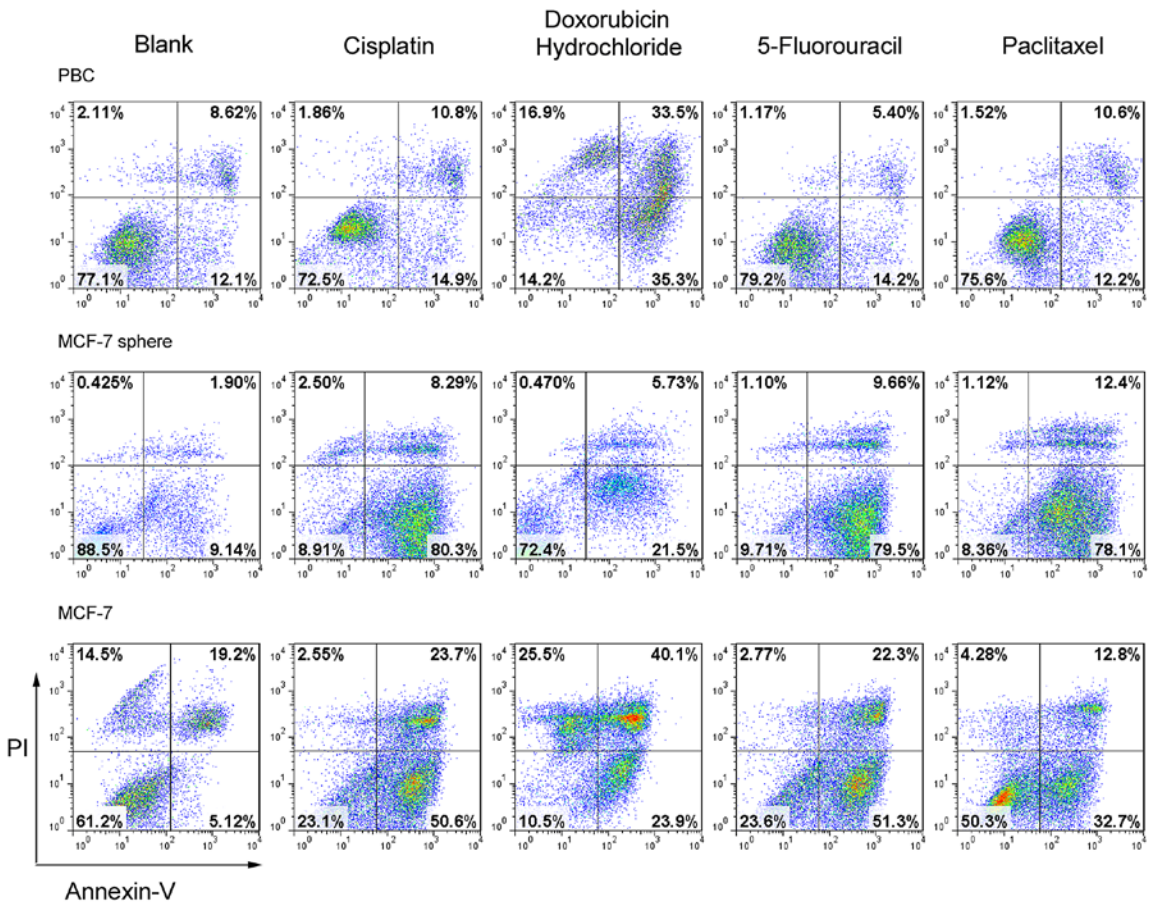

Figure 4. Apoptosis upon the treatment of chemotherapeutic agents. Apoptosis was analyzed in the mammosphere-forming cells from a primary breast cancer upon the treatment of four chemotherapeutic agents. For apoptosis analysis, cells were treated with the agents for 72 h at 5 μg/ml and labeled with Annexin V-PI. MCF-7 and MCF-7 mammosphere cells were simultaneously subjected to the assay. PBC, primary breast cancer.

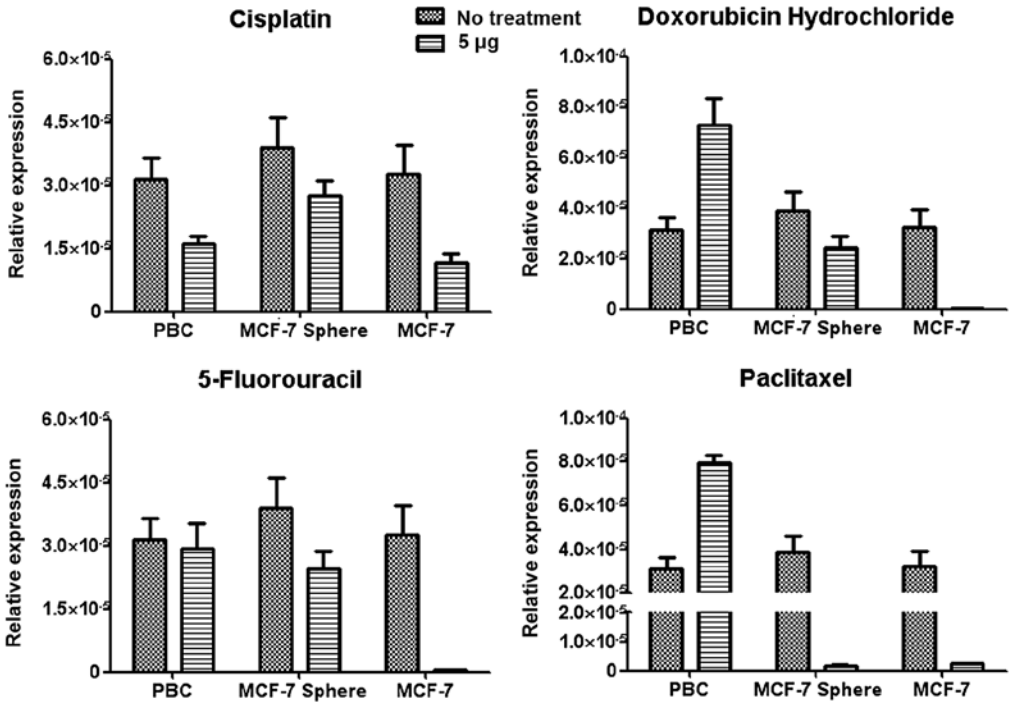

Figure 5. *Mdr1* expression upon the treatment of chemotherapeutic agents. *Mdr1* expression was analyzed in mammosphere-forming cells from a primary breast cancer by real-time PCR after 72 h treatment of four chemotherapeutic agents at a concentration of 5 μg/ml. MCF-7 and MCF-7 mammosphere cells were simultaneously subjected to the assay. PBC, primary breast cancer.

MCF-7 mammosphere cells underwent apoptosis at a much higher frequency upon treatment of these four drugs at the same concentration. Together with the data from the proliferation assay, it was deduced that the mammosphere-forming cells from the primary breast cancer exhibited more resistance to multiple chemotherapeutic drugs, which may provide useful information for breast CSC targeted therapy.

Mdr1 expression level in mammosphere-forming cells from the primary breast cancer is altered upon chemotherapeutic drug treatment. Increased expression of drug-resistant genes is one of the important factors contributing to the resistance to chemotherapeutic agents. In our study, we also analyzed the expression of the drug-resistant *mdr1* gene in three groups of cells after drug treatment. The *mdr1* expression of mammosphere-forming cells from the primary breast cancer increased upon 5-fluorouracil and paclitaxel treatment, whereas maintained upon doxorubicin treatment and decreased upon cisplatin treatment. On the contrary, the expression of the *mdr1* gene in the MCF-7 or MCF-7-derived mammosphere cells decreased after the treatment of all four drugs (Fig. 5). As *mdr1* is responsible for the efflux of drugs from inside of the cells, the decrease in *mdr1* expression in the MCF-7 or MCF-7-derived mammosphere-forming cells largely leads to either increased apoptosis or lower proliferative capacity upon drug treatment. The increased resistance of mammosphere-forming cells from the primary breast cancer to the drugs may be partially attributed to the higher expression of *mdr1* expression on the cell surface, which will shorten the detaining time of the chemotherapeutic drugs inside the cells and reduce the sensitivity to these drugs.

Discussion

In the present study, by using a modified cell suspension culture method, we successfully established mammosphere-forming cells from a primary breast cancer. These cells displayed CSC-like phenotypes as well as the resistance to the efficacy of multiple chemotherapeutic drugs widely used in the clinic, which may provide important insight into the determination of chemotherapeutic drugs for patients.

Breast CSCs are reported to be identified by their capacity to grow in serum-free suspension cultures similar to neural stem cell-forming aggregates. Enrichment of breast CSCs through mammosphere culture thus has provided a facilitative *in vitro* experimental model for study on breast CSCs. Rappa and Lorico (23) reported that mammospheres formed from a breast cancer cell line MA-11 had higher oncogenic ability than the parent cells. Grimshaw *et al* (19) performed suspension culture with patient pleural fluid and found that, of 27 specimens investigated, 20 formed mammospheres and had the ability to further expand and differentiate. However, the difficulty in obtaining a sufficient quantity of CSCs as well as maintaining the undifferentiated state of CSCs in an *in vitro* culture environment still greatly limits the application of these cells. Along with the passage of primary breast cancer-derived mammosphere-forming cells, the sizes of the mammospheres gradually decreased and so did their reproductive capacity and the formation rate. In addition, the currently used origins in mammosphere studies are mostly breast cancer cell lines (24) or metastatic malignant cells from pleural or ascitic fluid (25,26). The establishment of mammospheres originating from primary breast cancer tissues requires further investigation.

In this study, we partially modified the *in vitro* culture conditions of embryonic stem cells (27) by adding growth factor cocktails such as insulin-transferrin-selenium cocktail rather than insulin alone together with a low concentration of serum. Insulin can improve the intake of glucose and amino acids and synthesis of fat. Addition of transferrin and selenium can reduce the toxicity of oxygen free radicals and peroxides (28). In addition, bFGF and EGF are both members of the growth factor family with properties of mitogens, which can promote cell division and proliferation (29). In conventional culture system, the presence of serum promotes cell differentiation while the cell proliferative rate is relatively low in serum-free medium which becomes the bottleneck in CSC research. We tried the addition of serum at a low concentration (0.5-2%) with the significant acceleration of cell proliferation without influencing the formation of mammospheres. With the modification of the culture medium, we established and expanded the mammosphere-forming cells from the primary breast cancer for further investigation. In fact, it has been previously reported that the addition of low level serum maintained the undifferentiated state of stem cells in *in vitro* cultivation. Wang *et al* induced mouse spermatogonial stem cells to dedifferentiate to oocyte-like cells under a certain culture condition containing 1% FBS (30).

Previous studies also indicate that the size of the mammospheres reflects the self-renewal capacity of cancer stem cells (9,31) and is one of the indicators for the determination of CSCs. For mammosphere passage in our study, mammospheres were digested into single cells using trypsin and subcultured at a concentration of 1,000 cells/ml. Formation of the 2nd-generation mammospheres was observed to be formed with less time than the 1st generation. Second-generation mammospheres were observed after 3 days and the mammospheres became stable at day 7-10 with similar morphology to the first generation. From day 10, the mammosphere centers gradually darkened with decreased refraction and aging signs without the passage. Therefore, the ideal passage time was between day 7 and 10 and mammospheres of primary culture could be subcultured for at least 5 generations.

Cell surface proteins such as CD44 and CD24 are the most commonly used markers of breast CSCs. In addition, CD20 (32), CD117 (33), CD133 (34-36) and ALDH1 (37,38) are also prominent markers of CSCs. We identified the CSC properties of the mammosphere-forming cells with high CD44 expression and low CD24 expression together with the breast-origin phenotype such as AP and MGB1-positive staining. CSC-like mammosphere cells from the primary breast cancer could be subcultured for at least 5 generations. Single-cell culture by limited dilution revealed that the mammospheres were gradually formed by single cells. Additionally, no significant change was detectable in the mammosphere formation rate along with passage progression (data not shown). As a control, we also generated mammospheres originating from the MCF-7 cell line. They displayed a phenotype such as CD44^{low} and CD24^{low} similar to previous studies (8,9). Concerning the biological significance, it has been reported that CD24^{-} cells have higher oncogenicity than CD24^{+} cells with increased progenitor properties (8). Our results, to some extent, imply that mammospheres obtained from primary breast cancers may have even higher malignancy, which also corresponds to the expression of ER/PR/HER2.

In additon to surface markers, mammosphere-forming cells from the primary breast cancer also exhibited intrinsic stem cell characteristics with the expression of relevant genes. *Nanog, Oct4, Sox2* and *Klf4* are the most widely studied transcription factors in maintaining self-renewal and pluripotency of embryonic stem cells with transcriptional regulation network (39-42). The stem cell phenotypes of human embryonic cells are maintained by a self-stabilizing network of transcription factors, such as *Nanog, Oct4* and *Sox2* (43). These factors positively regulate genes responsible for the ES cell phenotype while repressing transcription of genes required for inducing differentiation. *Klf4* has been demonstrated to be expressed in adult somatic tissues with a higher rate of cell proliferation (44) and is an upstream regulator of a feed-forward loop that contains *Nanog, Oct4* and *Sox2* (45,46). These factors are also demonstrated to play important roles in the tumorigenesis of prostate cancer (47), colorectal cancer (48) and bladder carcinomas (49) and correlate with poor prognosis (39-42). In mammosphere-forming cells from the breast cancer, the expression level of *Sox2* was low whereas the expression levels of *Klf4*, *Nanog* and *Oct4* were detectable. On the contrary, four genes were all highly expressed in the MCF-7-derived mammospheres.

Drug resistance is an important characteristic of CSCs (50,51) and is also one of the important reasons for chemotherapy resistance and the recurrence of tumors. It has been demonstrated that the key drug-resistance gene, *mdr1*, is highly expressed in many CSCs (52). The product of the *mdr1* gene is P-glycoprotein (P-gp) with 1,280 amino acid residues. It was first isolated from drug-resistant tumor cells (53) and induces multi-drug resistance depending on its capacity for ATP-dependent transmembrane transport to carry drugs out of cells. High expression of the *mdr1* gene in CSCs decreases the sensitivity of cells to chemotherapeutic agents which in turn leads to the failure of CSC elimination, while terminal differentiated tumor cells are cleared due to the low expression of *mdr1*. In our study, mammosphere-forming cells from the primary breast cancer inducibly expressed *mdr1* upon drug treatment (Fig. 5). This finding may provide useful hints for the selection of chemotherapeutic drugs.

The significance of our study covers two aspects: the relationship between mammosphere formation rate with the malignancy and the drug resistance of mammosphere-forming cells from primary breast cancer. Concerning the relationship between the malignant degree of tumors and the formation of mammospheres, previous studies have indicated that ER^{+} MCF-7 cells, whose growth relies on estrogen, have the lowest malignant potential and mammosphere formation rate; SKBR3 cells with HER2 overexpression have the highest malignant potential and the highest mammosphere formation rate within the same time period; MDA-MB-231 cells exhibit highest malignant potential with triple-negative phenotype and require complicated conditions for mammosphere formation (54). The mammosphere formation rate of tumor cells may be due to the malignancy of tumors while the formation rate of *in vitro* culture mammospheres of primary tumors may be an important indicator for prognosis. In this study, the mammosphere formation rate of the primary breast cancer was high due to optimization of the culture conditions and possibly also the apparent stem cell-like characteristics.

Such tumors may have even poorer clinical prognosis. Future studies will assess long-term patient outcome and investigate the correlation between mammosphere formation with the cellular characteristics and disease prognosis.

Due to the potential roles of CSCs in the recurrence of tumors, the establishment of CSCs from primary cancers may facilitate the determination of the efficacy of chemotherapy against recurrent tumors. However, to date there are few studies concerning the significance of drug sensitivity of primary CSCs to the treatment of recurrent cancers. We detected the drug sensitivity of the mammosphere-forming cells but still require the supportive evidence from the clinic. Nevertheless, the present results obtained may be useful for further treatment if needed.

In conclusion, we successfully obtained mammosphere-forming cells from a primary breast cancer under a modified conditioned culture system. The mammosphere-forming cells displayed CSC-like phenotypes as well as expressed stem cell-related genes. These cells proliferated *in vitro* and displayed lymph node metastasis potential. With regard to the resistance of mammosphere-forming cells to chemotherapeutic drugs, the results from this study may provide important clues for the determination of the most effective individualized chemotherapy for patients with recurrent tumors.

Acknowledgements

We appreciated Professor Feng-Chun Zhang for the kind comments on the manuscript. This study was supported by grants from the China National Basic Research Program (2014CB964704) and the National Natural Science Foundation of China (nos. 81272328 and 81301858).

References

1. World Health Organization: Breast Cancer: Prevention and Control. http://wwwwhoint/cancer/detection/breast-cancer/en/indexhtml. 2013.
2. Huang ZZ, Chen WQ, Wu CX, Zheng RS, Chen JG, Yang NN, Wang N, Zhang SW and Zheng Y: Incidence and mortality of female breast cancer in China - a report from 32 Chinese cancer registries, 2003-2007. Tumor 32: 435-439, 2012.
3. Visvader JE and Lindeman GJ: Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768, 2008.
4. Polyak K and Hahn WC: Roots and stems: stem cells in cancer. Nat Med 12: 296-300, 2006.
5. Dalerba P, Cho RW and Clarke MF: Cancer stem cells: models and concepts. Annu Rev Med 58: 267-284, 2007.
6. Ginestier C, Liu S, Diebel ME, Korkaya H, Luo M, Brown M, Wicinski J, Cabaud O, Charafe-Jauffret E, Birnbaum D, *et al*: CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. J Clin Invest 120: 485-497, 2010.
7. Sarry JE, Murphy K, Perry R, Sanchez PV, Secreto A, Keefer C, Swider CR, Strzelecki AC, Cavelier C, Récher C, *et al*: Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD/SCID/IL2Rγc-deficient mice. J Clin Invest 121: 384-395, 2011.
8. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983-3988, 2003.
9. 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 65: 5506-5511, 2005.
10. Idowu MO, Kmieciak M, Dumur C, Burton RS, Grimes MM, Powers CN and Manjili MH: $CD44^{+}/CD24^{-/low}$ cancer stem/progenitor cells are more abundant in triple-negative invasive breast carcinoma phenotype and are associated with poor outcome. Hum Pathol 43: 364-373, 2012.
11. Bauerschmitz GJ, Ranki T, Kangasniemi L, Ribacka C, Eriksson M, Porten M, Herrmann I, Ristimäki A, Virkkunen P, Tarkkanen M, *et al*: Tissue-specific promoters active in $CD44^{+}CD24^{-/low}$ breast cancer cells. Cancer Res 68: 5533-5539, 2008.
12. Hwang-Verslues WW, Kuo WH, Chang PH, Pan CC, Wang HH, Tsai ST, Jeng YM, Shew JY, Kung JT, Chen CH, *et al*: Multiple lineages of human breast cancer stem/progenitor cells identified by profiling with stem cell markers. PLoS One 4: e8377, 2009.
13. Goodell MA, Brose K, Paradis G, Conner AS and Mulligan RC: Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183: 1797-1806, 1996.
14. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, Laino L, De Francesco F and Papaccio G: Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J 27: 13-24, 2013.
15. Reynolds BA and Weiss S: Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175: 1-13, 1996.
16. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ and Wicha MS: In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17: 1253-1270, 2003.
17. Krause S, Maffini MV, Soto AM and Sonnenschein C: A novel 3D in vitro culture model to study stromal-epithelial interactions in the mammary gland. Tissue Eng Part C Methods 14: 261-271, 2008.
18. Phuc PVKT, Dong LV, Kiet TD, Giang TT and Ngoc PK: Isolation and characterization of breast cancer stem cells from malignant tumours in Vietnamese women. J Cell Anim Biol 4: 163-169, 2010.
19. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, Taylor-Papadimitriou J and Burchell JM: Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res 10: R52, 2008.
20. Shaw FL, Harrison H, Spence K, Ablett MP, Simões BM, Farnie G and Clarke RB: A detailed mammosphere assay protocol for the quantification of breast stem cell activity. J Mammary Gland Biol Neoplasia 17: 111-117, 2012.
21. Dontu G, Al-Hajj M, Abdallah WM, Clarke MF and Wicha MS: Stem cells in normal breast development and breast cancer. Cell Prolif 36 (Suppl 1): 59-72, 2003.
22. Ralston A and Rossant J: The genetics of induced pluripotency. Reproduction 139: 35-44, 2010.
23. Rappa G and Lorico A: Phenotypic characterization of mammosphere-forming cells from the human MA-11 breast carcinoma cell line. Exp Cell Res 316: 1576-1586, 2010.
24. Holliday DL and Speirs V: Choosing the right cell line for breast cancer research. Breast Cancer Res 13: 215, 2011.
25. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, *et al*: let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131: 1109-1123, 2007.
26. Singh JK, Farnie G, Bundred NJ, Simões BM, Shergill A, Landberg G, Howell SJ and Clarke RB: Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and-independent mechanisms. Clin Cancer Res 19: 643-656, 2013.
27. Yu Z, Ji P, Cao J, Zhu S, Li Y, Zheng L, Chen X and Feng L: Dazl promotes germ cell differentiation from embryonic stem cells. J Mol Cell Biol 1: 93-103, 2009.
28. Raghu HM, Nandi S and Reddy SM: Effect of insulin, transferrin and selenium and epidermal growth factor on development of buffalo oocytes to the blastocyst stage in vitro in serum-free, semidefined media. Vet Rec 151: 260-265, 2002.
29. Sakaguchi M, Dominko T, Yamauchi N, Leibfried-Rutledge ML, Nagai T and First NL: Possible mechanism for acceleration of meiotic progression of bovine follicular oocytes by growth factors in vitro. Reproduction 123: 135-142, 2002.
30. Wang L, Cao J, Ji P, Zhang D, Ma L, Dym M, Yu Z and Feng L: Oocyte-like cells induced from mouse spermatogonial stem cells. Cell Biosci 2: 27, 2012.
31. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P and Wicha MS: Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 66: 6063-6071, 2006.
32. Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE and Herlyn M: A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 65: 9328-9337, 2005.

33. Adhikari AS, Agarwal N, Wood BM, Porretta C, Ruiz B, Pochampally RR and Iwakuma T: CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. Cancer Res 70: 4602-4612, 2010.
34. Tirino V, Desiderio V, d'Aquino R, De Francesco F, Pirozzi G, Graziano A, Galderisi U, Cavaliere C, De Rosa A, Papaccio G, *et al:* Detection and characterization of CD133$^+$ cancer stem cells in human solid tumours. PLoS One 3: e3469, 2008.
35. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, Fazioli F, Pirozzi G and Papaccio G: Human primary bone sarcomas contain CD133$^+$ cancer stem cells displaying high tumorigenicity in vivo. FASEB J 25: 2022-2030, 2011.
36. Tirino V, Camerlingo R, Franco R, Malanga D, La Rocca A, Viglietto G, Rocco G and Pirozzi G: The role of CD133 in the identification and characterisation of tumour-initiating cells in non-small-cell lung cancer. Eur J Cardiothorac Surg 36: 446-453, 2009.
37. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, *et al*: ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1: 555-567, 2007.
38. Ohi Y, Umekita Y, Yoshioka T, Souda M, Rai Y, Sagara Y, Sagara Y, Sagara Y and Tanimoto A: Aldehyde dehydrogenase 1 expression predicts poor prognosis in triple-negative breast cancer. Histopathology 59: 776-780, 2011.
39. Jung M, Peterson H, Chavez L, Kahlem P, Lehrach H, Vilo J and Adjaye J: A data integration approach to mapping OCT4 gene regulatory networks operative in embryonic stem cells and embryonal carcinoma cells. PLoS One 5: e10709, 2010.
40. Lengerke C, Fehm T, Kurth R, Neubauer H, Scheble V, Müller F, Schneider F, Petersen K, Wallwiener D, Kanz L, *et al*: Expression of the embryonic stem cell marker SOX2 in early-stage breast carcinoma. BMC Cancer 11: 42, 2011.
41. Zhang P, Andrianakos R, Yang Y, Liu C and Lu W: Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. J Biol Chem 285: 9180-9189, 2010.
42. Chiou SH, Wang ML, Chou YT, Chen CJ, Hong CF, Hsieh WJ, Chang HT, Chen YS, Lin TW, Hsu HS, *et al*: Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. Cancer Res 70: 10433-10444, 2010.
43. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, e*t al*: Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122: 947-956, 2005.
44. Adachi K and Schöler HR: Directing reprogramming to pluripotency by transcription factors. Curr Opin Genet Dev 22: 416-422, 2012.
45. Chan KK-K, Zhang J, Chia N-Y, Chan Y-S, Sim HS, Tan KS, Oh SK, Ng HH and Choo AB: KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. Stem Cells 27: 2114-2125, 2009.
46. Wei Z, Yang Y, Zhang P, Andrianakos R, Hasegawa K, Lyu J, Chen X, Bai G, Liu C, Pera M, *et al*: Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. Stem Cells 27: 2969-2978, 2009.
47. Bae KM, Su Z, Frye C, McClellan S, Allan RW, Andrejewski JT, Kelley V, Jorgensen M, Steindler DA, Vieweg J, *et al*: Expression of pluripotent stem cell reprogramming factors by prostate tumor initiating cells. J Urol 183: 2045-2053, 2010.
48. Lin CW, Liao MY, Lin WW, Wang YP, Lu TY and Wu HC: Epithelial cell adhesion molecule regulates tumor initiation and tumorigenesis via activating reprogramming factors and epithelial-mesenchymal transition gene expression in colon cancer. J Biol Chem 287: 39449-39459, 2012.
49. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F and Cordon-Cardo C: Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J Clin Oncol 24: 778-789, 2006.
50. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JI, *et al*: Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci USA 106: 13820-13825, 2009.
51. Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, Qian D, Lam JS, Ailles LE, Wong M, *et al*: Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature 458: 780-783, 2009.
52. Donnenberg VS, Meyer EM and Donnenberg AD: Measurement of multiple drug resistance transporter activity in putative cancer stem/progenitor cells. Methods Mol Biol 568: 261-279, 2009.
53. Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM and Roninson IB: Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47: 381-389, 1986.
54. Huang M-z, Zhang F-c and Zhang Y-y: Influence factors on the formation of mammospheres from breast cancer stem cells. Beijing Da Xue Xue Bao 40: 500-504, 2008 (In Chinese).