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miR-125b Regulates Side Population in Breast Cancer and Confers a Chemoresistant Phenotype

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ABSTRACT

Resistance to chemotherapy is a major obstacle for the effective treatment of breast cancer and is partially due to the presence of drug resistant stem cell-like side population (SP). Previous studies have shown elevated miR-125b is associated with chemoresistance and metastasis; however, the relationship between miR-125b and SP cells remains unknown. In this study, we isolated and characterized SP cells in a panel of breast cancer cell lines and primary cancer cells from breast cancer patients. SP cells showed cancer stem cells (CSCs) properties, including self-renewal, resistance to chemotherapy and high expression of stem cell markers. The percentage of SP cells was higher in chemotherapy resistant patients compared to that in chemotherapy responsive patients ($5.8 \pm 2.4\%$ in non-responsive patients vs. $1.2 \pm 0.5\%$ in responsive patients, $P = 0.012$). Importantly, SP cells had higher level of miR-125b than NSP cells and the elevated miR-125b expression in chemoresistant cancer cells were due to high percentage of SP cells. Overexpression of miR-125b correlated with an increase in tumor SP and CSC property, whereas knockdown of miR-125b correlated with decreased incidence of SP. In addition, miR-125b overexpression in breast cancer cells induced epithelial–mesenchymal transition (EMT)-like cellular marker alteration, suggesting a potential mechanism of miR-125b in the regulation of cancer stem-like SP cells. Taken together, these results suggest an important role for miR-125b in breast cancer chemoresistance by maintaining cancer stem-like SP fraction, and raise the possibility that miR-125b may be a significant prognostic response marker for cancer therapy. J. Cell. Biochem. 114: 2248–2257, 2013.

KEY WORDS: miR-125B; SIDE POPULATION; CHEMORESISTANCE; BREAST CANCER; CANCER STEM CELLS

Breast cancer is the leading cause of cancer and the second leading cause of cancer death in American women [Jemal et al., 2009], and its incidence is increasing in many countries including China [He et al., 2011]. Chemotherapy is an important component in the treatment paradigm for breast cancers. However, despite a rapid shrinkage in tumor mass following chemotherapeutic cycles, the resistance of cancer cells to chemotherapeutic agents frequently results in the subsequent recurrence and metastasis of cancer [Chen et al., 1995].

The molecular mechanisms that may contribute to chemotherapeutic resistance in breast cancers include overexpression of ATP-binding cassette (ABC) transporters, anti-apoptotic factors and cell cycle deregulation [Tan et al., 2002; Pattingre et al., 2005; Lange and Yee, 2011]. One of the well-known mechanisms of chemoresistance in cancers is the enhanced efflux of a broad spectrum of hydrophobic cytotoxic drugs. Recently, the ability to exclude the fluorescent Hoechst 33342 dye has been employed to isolate a side population (SP) from hematopoietic cells [Goodell et al., 1996]. These SP cells have stem cell-like properties and are capable of self-renewal and differentiation [Dean, 2009]. These characteristics of SP cells have been found in various types of tumors, and SP cells with a high drug efflux capacity are identified as the potential cause of the chemoresistant phenotypes of cancer stem cells (CSCs) in human tumors [Britton et al., 2012; Lu et al., 2012; Luo et al., 2012; Van den Broeck et al., 2012]. Chemoresistant SP cells will survive and sustain their clonogenicity during initial exposure to cytostatic drugs, thereby allowing disease recurrence when therapy is withdrawn. These subsets of CSCs are thus considered a viable target for improved therapeutic intervention and preventing chemoresistance and cancer relapse.

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The development of chemoresistance through an increase in the number of cancer stem like cells, including SP fractions has been attributed to alterations at the level of microRNAs (miRNAs) in various cancer types [Misawa et al., 2010; Singh et al., 2012]. These non-coding RNA molecules can act as oncogenes or tumor suppressing genes. Dysregulation of miRNAs has been implicated in tumorigenesis and drug resistance as well. Among them, miR-125b has been reported to be up-regulated in pancreatic cancer [Bloomston et al., 2007], prostate cancer [Shi et al., 2007], and acute myeloid leukemia [Tian et al., 2010], and promote cancer cell proliferation and suppress p53-dependent apoptosis in human neuroblastoma cells [Xia et al., 2009]. We and others have previously shown that elevated miR-125b expression was related to resistance of breast cancer cells to chemotherapy [Zhou et al., 2010; Wang et al., 2012]. In addition, a recent study demonstrated miR-125b induced metastasis by targeting STARD13 in breast cancer cells [Tang et al., 2012]. While chemoresistance and metastasis are characteristics of CSCs, the role of miR-125b in CSC-rich SP remains elusive.

In this study, we analyzed the percentage of SP and non-side population (NSP) cells in several breast cancer cell lines including primary cultures from breast cancer patients and correlated with patients' responses to chemotherapy and miR-125b expression. Elevated miR-125b levels were observed in SP compared to NSP. In addition, the effects of overexpression and knockdown of miR-125b on SP fraction in breast cancer cells and stem cell property was investigated by in vitro assays.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENT

Human breast cancer cell lines, MCF-7, T47D, and MDA-MB-231, were purchased from American Type Culture Collection (ATCC) and grown according to standard protocols. The different inhibitory concentrations of paclitaxel were determined by generating dose–response curves after treating the cells with increasing concentrations of paclitaxel and analyzing the cell sensitivity using trypan blue dye exclusion assays as previously described [Wang et al., 2012]. Briefly, the cell suspension was appropriately diluted with 0.4% trypan blue dye (Sigma-Aldrich) and hematocytometer was used to estimate the percentage of unstained treated cells compared to the control cells. The inhibition rate was calculated as follows: Inhibition rate = (1 − unstained treated cells/control cells) × 100. The IC50 (concentration of 5-FU to produce 50% cell inhibition) was determined and used for further analysis. All experiments were carried in triplicate.

Fluorouracil (5-FU) resistant MCF-7 (FuR/MCF-7) cells were selected in stepwise increasing concentrations beginning at 0.1 μM and ending at 20 μM of 5-FU (Sigma-Aldrich) as previously described [Wang et al., 2012]. Cells did not receive next treatment until they had got an 80% confluence after each treatment. Despite massive cell deaths among the sensitive MCF-7 cells under treatment, the cultures were maintained by regular changes of medium intermittently increasing the 5-FU concentration until the surviving cells recovered a normal growth pattern in the medium with 1 μM 5-FU, which was included in the culture medium for MCF-7/5-FU to maintain the drug resistance. The cells were maintained in 5-FU free medium at least 2 weeks before each experiment.

Primary breast cancer cells were isolated from fresh surgical specimens using the Cancer Cell Isolation kit (Panomics) following the manufacturer's protocol. Briefly, tumor pieces of <2 mm size were seeded into six-well culture plates and cultured in RPMI 1640 supplemented with 20% fetal bovine serum, 1% L-glutamine, 1% MEM non-essential amino acid, and 1% antibiotic/penicillin–streptomycin solution. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested with 0.1% trypsin/0.05% EDTA when the cultures had reached ~80% confluence. These cells were expanded for three to four subsequent passages and thereafter used for the studies.

PATIENTS

The study obtained the permission of the ethics committee of Dalian medical university and all patients provided written informed consent. Patients receiving chemotherapy treatment for primary invasive ductal carcinoma of the breast between 01/2010 and 12/2011 at the Department of General Surgery were reviewed. A total of 19 patients (median age 56, range 38–72), from whom fresh surgical specimens after chemotherapy were available for cell culture, were included in the study. All the patients underwent pre-operative neoadjuvant chemotherapy with an association of 5-FU, Epirubucin and cyclophosphamide for 4–6 cycles. Tumor assessment was performed every 3 months by CT-scan and/or chest X-ray coupled with abdomen ultrasound depending on those used at baseline. Subsequently, the patients underwent total mastectomy or breast reservation. The patients with breast reservation received post-operative radiotherapy. Treatment response was assessed by the radiologic Response Evaluation Criteria in Solid Tumors (RECIST) criteria [Therasse et al., 2000]. Patients achieving complete (CR) or partial (PR) response were considered as responder; disease stabilization (SD) or disease progression (PD) considered as non-responder.

SP AND FLUORESCENCE-ACTIVATED CELL SORTING ANALYSES

The SP protocol was essentially performed as described by Goodell [Goodell et al., 1996]. Cells (1×10^6 cells/ml) were incubated in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 10 mM HEPES, and 5 μg/ml Hoechst 33342 (H33342, Sigma-Aldrich) for 90 min at 37°C with or without ABCG2 transporter inhibitor, Fumitremorgin C (10 μm FTC, EMD Chemicals). Prior to cell analysis, non-viable cells were excluded by the addition of 2 μg/ml propidium iodide. For all analyses, cell analysis and sorting were performed on a Becton Dickinson FACS Digital Vantage cell sorter (BD Biosciences). At least 10^7 cells were subjected to fluorescence-activated cell sorting to yield sufficient SP cells for in vitro experiments.

IMMUNOHISTOCHEMISTRY AND TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT)-MEDIATED DUTP LABELING (TUNEL)

The level of proliferating cell nuclear antigen (PCNA) was detected by immunohistochemistry in paraffin-embedded tissue sections. Mouse monoclonal anti-PCNA antibody (ab29, Abcam) (1:6,000 dilutions) were used as primary antibody. The mouse IgG was added as the negative control.

TUNEL assay was done using an In situ Apoptosis Detection Kit (R&D Systems). Briefly, after digesting with Protease K, TdT, reaction mix was applied to the cells for incubation at 37°C for 60 min, followed by incubation with streptavidin horseradish peroxidase for 10 min. The final reaction of the product was visualized by 3,39-diaminobenzidine. Approximately 1,000 tumor cells were counted (400×) in each section, and apoptotic index (AI) was expressed as the percentage of TUNEL-positive tumor cells.

The proportion of positive tumor cells was evaluated by two pathologists who were blinded to the study endpoints. For the statistical purpose, we divided cases into two groups: low expression (PCNA $\leq$ 25% or AI $\leq$ 1%) and high expression (PCNA $>$ 25% or AI $>$ 1%) [Ma, 2010].

EXTRACTION OF TOTAL RNA AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from cultured or sorted cells using TRIzol (Invitrogen) according to manufacturer's instructions. RNA concentration and integrity were determined using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The RNA samples were immediately stored at −80°C until use.

The miR-125b level and mRNA expression of NANOG, SOX2, OCT4, ABCG2, MDR1, E-cadherin, N-cadherin, vimentin, and SNAIL were evaluated via quantitative real-time PCR using the ABI 7900HT system (Applied Biosystems) as previously described [Guo et al., 2012]. Specific primers of mRNA (Supplementary Table I) were designed based on published gene sequences (NCBI Entrez search system). The expression of miR-125b was determined using the Taqman Universal PCR Master Mix and Taqman MicroRNA Assays (Applied Biosystems) as described by the manufacturer. The comparative Ct method was used to calculate the relative gene expression. Expression of the amplified products for mRNAs and miRNAs was calculated relative to β-actin and RNU6B, respectively.

miRNA MIMIC AND ANTISENSE TRANSFECTION

miR-125b overexpression and inhibition were achieved by transfection of miRIDIAN miRNA mimic (Thermo Scientific) and LNA antisense oligonucleotides (Exiqon), respectively. Transfection was performed in 6-well tissue culture plates using Lipofectamine RNAiMAX reagent (Invitrogen) and reverse transfection protocol according to the manufacture's instructions. To transfect, miRNA mimic/inhibitor was diluted into 500 μl Opti-MEM I Medium without serum (Invitrogen) per well. After gentle mixing, Lipofectamine RNAiMAX was added to each well containing the diluted mimic/inhibitor molecules and incubated for 15 min at room temperature. Breast cancer cells were diluted in complete growth medium without antibiotics and 2 ml containing 6×10^4 cells per well was added on top of the transfection complex. As a negative control, a random sequence that doesn't inhibit any known gene (mimic scramble) or miRNA (antisense scramble) was also transfected into cells.

SPHERE FORMATION ASSAYS

To test sphere formation in suspension, sorted SP and NSP cells were passed through a 40-μm filter to provide a single cell suspension. Next, 4 ml medium containing 100 cells were added to the 6-well plates with serum-free media including F12 with EGF (10 μg/L), insulin (20 mg/L), and basic fibroblast growth factor (bFGF) (10 μg/L). After 10–14 days, plates were visually assayed for the formation of floating spheres. To assess the ability of primary spheres to form secondary spheres, colonies containing more than 30 cells were collected by centrifugation and trypsinized to yield single cells. After passing through a 40-μm filter, 4 ml serum-free media containing 100 cells were added to each 6-well plate and were cultured 10–14 days for secondary sphere formation. Spheres were counted under a dissecting microscope.

CLONOGENIC ASSAYS

Cells were mixed in 0.3% Noble agar (in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) and plated at 1,000 cells/well onto 6-well plates containing a solidified bottom layer (0.6% Noble agar in the same growth medium). After reaching a diameter between 50 and 200 μm, colonies were either unstained or stained with 5 mg/ml MTT (Sigma-Aldrich), and photographed. Plating efficiency was determined as % of cells forming a colony per number of cells seeded.

STATISTICAL ANALYSIS

The statistical analyses were performed using the SPSS software package, version 18.0. Wilcoxon or Student's *t*-tests were used to assess the significance of the difference between the two means of data resulting from in vitro assays. Data were presented as mean ± standard deviation (SD). A value of $P < 0.05$ in all cases was considered statistically significant.

RESULTS

ASSESSMENT OF SP CELLS IN BREAST CANCER AND CORRELATION WITH CHEMORESISTANCE

SP were identified using dual-wavelength flow cytometry combined with Hoechst 33342 dye efflux and were a subpopulation of cells that have an elevated rate of Hoechst efflux [Goodell et al., 1996]. The SP phenotype was confirmed with decreased number following addition of ABCG2 specific inhibitor FTC (Fig. 1A). The mean percentage of SP cells in human breast cancer cell lines was 0.9% in MCF-7, 0.7% in T47D, and 1.5% in MDA-MB-231 (Fig. 1B). In addition, we measured SP fractions in FuR/MCF-7, a MCF-7 breast cancer cell line that was induced to be resistant to 5-FU [Wang et al., 2012] and also exhibited resistance to doxorubicin and paclitaxel (Supplementary Fig. 1). Interestingly, the percentage of SP was 8-times higher in FuR/MCF-7 compared to those in parental MCF-7 (7.2 ± 1.8% in FuR/MCF-7 vs. 0.9 ± 0.3% in MCF-7, $P = 0.012$) (Fig. 1B), suggesting a positive relationship between the occurrence of SP cells and chemoresistance.

To test this hypothesis, we further investigated the existence of SP cells in primary cancer cells isolated from 19 breast cancer patients, who had advanced ductal breast cancer and received pre-operative neoadjuvant chemotherapy. To determine whether the percentage of SP cells was associated with chemotherapeutic efficacy, therapeutic response was evaluated by RECIST as well as PCNA immunostaining for cell proliferation and TUNEL assay for AI in surgical specimens after chemotherapy. According to RECIST, 10 patients (53%) responded to chemotherapy with PR or CR; 9 patients (47%) were not responsive with SD or PD. The existence of SP cells had a negative association with therapeutic response, exhibiting higher percentage in non-responsive patients (5.8 ± 2.4% in non-responsive patients vs.

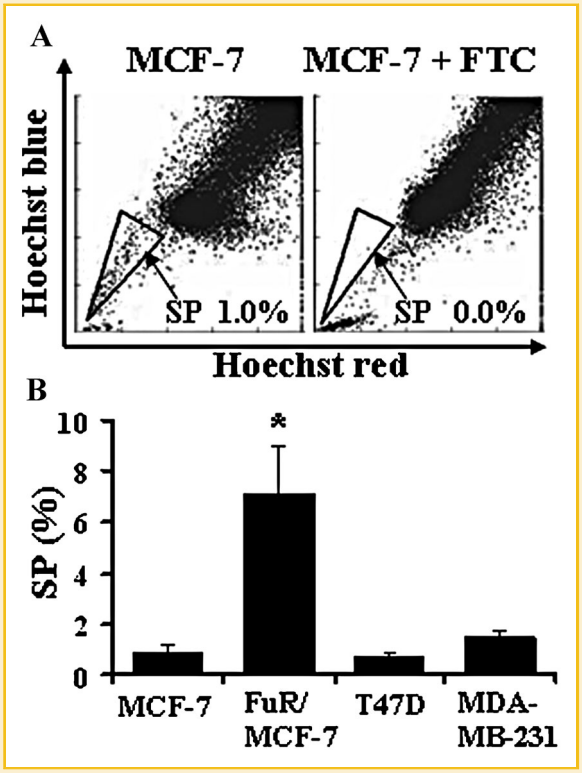

Fig. 1. Characterization of side population (SP) in breast cancer cell line. A: SP cells were identified in MCF-7 through uptake of the DNA binding dye Hoechst33342 with or without the presence of FTC. B: Percentage of SP in each breast cancer cells line. Data represent the mean ± SD of triplicate independent experiments. *$P = 0.012$ compared to MCF-7.

$1.2 \pm 0.5\%$ in responsive patients, $P = 0.012$, Table I). In addition, the percentage of SP cells was positively correlated with breast cancer cell proliferation, but reversely correlated with apoptosis in the corresponding surgical specimens obtained after neoadjuvant chemotherapy (Table I).

SP CELLS DISPLAY CANCER STEM CELL PHENOTYPE AND CHEMORESISTANCE

To evaluate whether SP cells show features of stem cell, SP, and NSP cells isolated from MCF-7, T47D, and MDA-MB-231 were examined for the levels of stem cell markers NANOG, SOX2, and OCT4. As shown in Figure 2, real-time PCR analysis revealed that NANOG, SOX2, and OCT4 transcription factors were expressed at higher levels in SP cells in all the three breast cancer cell lines. In addition, ABC transporters, which contribute to drug resistance in SP cells including *ABCG2* and *MDR1* [Duan et al., 2004; Dean, 2009], were significantly higher in the SP fraction in breast cancer cells (Fig. 2).

TABLE I. Correlation of SP Cells and Response to Chemotherapy in Breast Cancer Patients

	Total (n = 19)	SP (%), mean ± SD	*P*-value*
Treatment response			
CR + PR	10 (57%)	5.8 ± 2.4	0.012
SD + PD	9 (43%)	1.2 ± 0.5	
PCNA			
Low	12 (63%)	1.8 ± 0.6	0.019
High	7 (37%)	6.2 ± 1.8	
Apoptotic index			
Low	8 (42%)	6.5 ± 1.1	0.005
High	11 (58%)	1.5 ± 0.5	

CR, complete response; PR, partial response; SD, stable disease; PD, disease progression.
*Student's *t*-tests.

The ability of SP and NSP cells to generate spherical clones and self-renewal was evaluated by sphere formation assays. It was difficult to observe floating spheres from NSP cells. SP cells started to form floating spheres after 2–3 days of seeding, and became primary spheres of more than 30 cells after 10–14 days (Fig. 3A). The secondary sphere occurred slightly more quickly and frequently than the primary spheres, suggesting that SP cells have self-renewal ability. Similar results were obtained in SP and NSP cells from all the three breast cancer cell lines (Fig. 3B).

SP cells expressing ABC transporters have been shown to be resistant to multiple drugs [Duan et al., 2004; Dean, 2009]. Paclitaxel, a substrate of MDR1 transporter [Duan et al., 2004], has been recommended as first line chemotherapy in patients with advanced and metastatic breast cancer [Gradishar et al., 2012]. Therefore, we treated breast cancer cells with paclitaxel to examine the drug sensitivity of SP cells. The IC50 values of breast cancer cell lines to paclitaxel treatment were 2.2 nM for MCF-7, 6.5 nM for T47D, and 3.8 nM for MDA-MB-231, respectively. This treatment resulted in 7.5- to 11.5-fold increases in SP cells compared with vehicle control (Fig. 4A), which was accompanied by 7.8- to 13.1-fold increases in colony-forming capacities as determined by colonogenic assays (Fig. 4B). The increase in SP cells and colony-forming capacities after drug treatment despite the absence of overall cell increase indicates that SP cells are more resistant to the drugs than NSP cells. This relative resistance of SP cells to drugs was further investigated by treating SP and NSP cells with paclitaxel separately. When SP cells and NSP cells from MCF-7 were treated with different concentration of paclitaxel, SP cells showed less sensitivity than NSP cells (Fig. 4C), confirming the drug resistance of SP cells compared with NSP cells.

miR-125B EXPRESSION IN SP ISOLATED FROM BREAST CANCER CELLS

Our and other recent studies have shown that miR-125b plays a critical role in chemoresistance and metastasis in breast cancer [Tang et al., 2012; Wang et al., 2012], which prompted us to examine the role of miR-125b in regulating breast CSCs. First, real-time PCR was used to determine the expression of miR-125b in a panel of breast cancer cells, including MCF-7, T47D, MDA-MB-231 and recently established primary cancer cell lines derived from patients who were resistant to chemotherapy (BC-R1 and BC-R2) or responded to chemotherapy with PR/CR (BC1 and BC2).

In the whole cell population, MCF-7 and T47D exhibited similar miR-125b expression, whereas MDA-MB-231 had approximately twofold higher miR-125b expression than MCF-7 and T47D (Fig. 5A). However, all SP cells had increased miR-125b expression compared to their whole cell population and corresponding NSP cells in the three breast cancer cell lines (Fig. 5A). The whole cell population also had higher miR-125b expression than NSP cells. Similarly, SP had higher miR-125b expression in primary cancer cell lines isolated from both

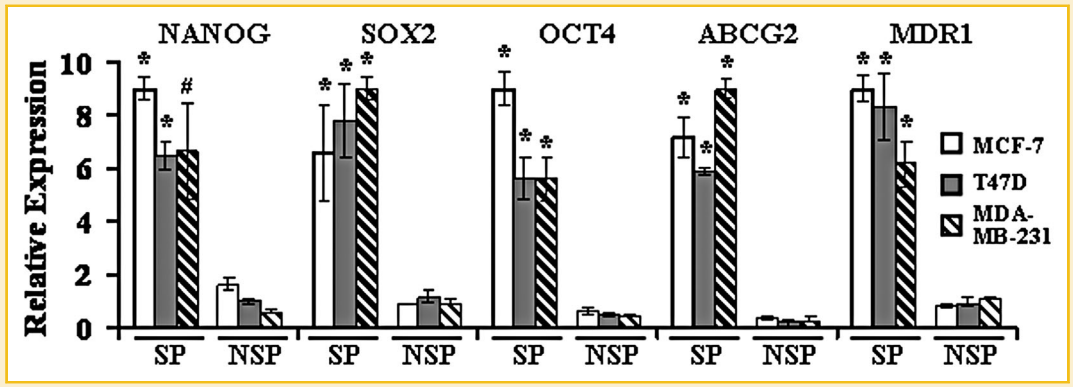

Fig. 2. Expression of stem cell markers in side population (SP) and non-side population (NSP) isolated from breast cancer cell lines. Gene expression was determined by quantitative real-time PCR. Data represent the mean ± SD of three different SP or NSP cells isolated from each breast cancer cell line, $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to corresponding NSP.

chemotherapy resistant patients (BC-R1 and BC-R2) and chemotherapy responsive patients (BC1 and BC2) compared to their corresponding NSP (Fig. 5B), although BC-R1 and BC-R2 had higher miR-125b in the whole cell population than BC1 and BC2 (average 3.2-fold, $P = 0.001$). There were no differences in miR-125b expression among SP or NSP cells in all tested breast cancer cell lines. These data indicate that SP cells overexpress miR-125b and the high miR-125b expression in chemoresistant cancer cells is due to high percentage of SP cells.

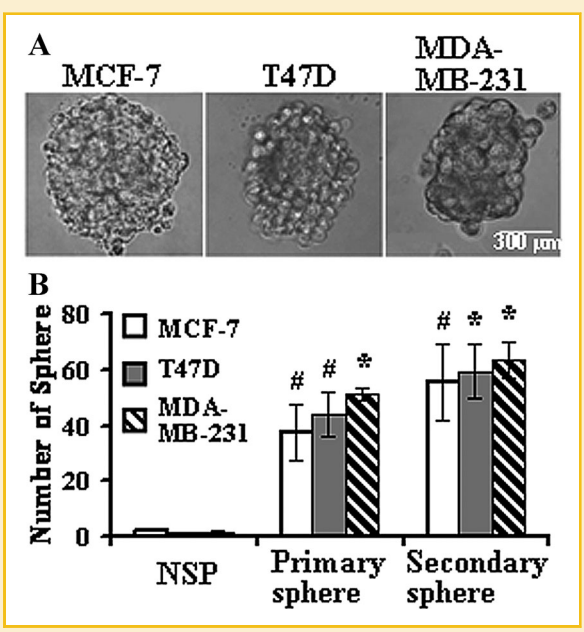

Fig. 3. Serial sphere formation assays. A: The side population (SP) from breast cancer cells were cultured in serum-free sphere media for 10–14 days. B: Spheres were counted under a dissecting microscope. Data represent the mean ± SD of triplicate independent experiments. $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to corresponding non-side population (NSP).

miR-125B REGULATES BREAST CANCER SP CELLS

To determine if miR-125b regulates breast cancer SP cells, we further conducted Hoechst 33342 exclusion technique to identify SP in different breast cancer cell lines following miR-125b overexpression or knockdown. Because MCF-7 and primary cancer cell line BC1 have low endogenous miR-125b, they were used for miR-125b overexpression experiments. miR-125b mimics resulted in a dose-dependent overexpression of miR-125b (Fig. 6A). A dramatic increase in SP cells was observed following miR-125b overexpression in both cell lines, which was dose-dependent according to relative miR-125 expression (Fig. 6B). Because FuR/MCF-7 and primary cancer cell line BC-R1 have high percentage of SP cells and endogenous miR-125b, they were used for miR-125b knockdown experiments. miR-125b antisense oligonucleotides significantly inhibited ($P < 0.001$) miR-125b expression compared to a scramble sequence used as a control (Fig. 6C). Decreased miR-125b expression resulted in 8.5- and 5.3-fold decrease in the percentage of SP cells in FuR/MCF-7 and BC-R1, respectively (Fig. 6D). Together, these data indicate a strong positive correlation between the expression of miR-125b and the relative proportion of SP, raising the interesting notion that miR-125b may promote SP population in breast cancer, and thereby enhance chemoresistance.

In addition, we performed clonogenic assays as in vitro tests for self-renewal [Fiebig et al., 2004] in a panel of breast cancer cell lines. There were no differences in colony formation between breast cancer cells without treatment and those transfected with miRNA mimic/antisense scramble control (data not shown). The percentage plating efficiency representing colony-forming units in the whole cell population was highest in cell lines with a large SP and high miR-125b expression, including FuR/MCF-7 and BC-R1, and lowest in those cells lacking an SP and miR-125b, including MCF-7 and BC1 (Fig. 7). Knockdown of miR-125b in FuR/MCF-7 and BC-R1 decreased whereas ectopic expression of miR-125b in MCF-7 and BC1 enhanced colony formation, respectively (Fig. 7). More importantly, miR-125b antisense decreased colony formation of FuR/MCF-7 to the similar level of parental MCF-7 (Fig. 7). Overall, these data are in support of a regulatory role of miR-125b in expansion of cells with stem cell properties.

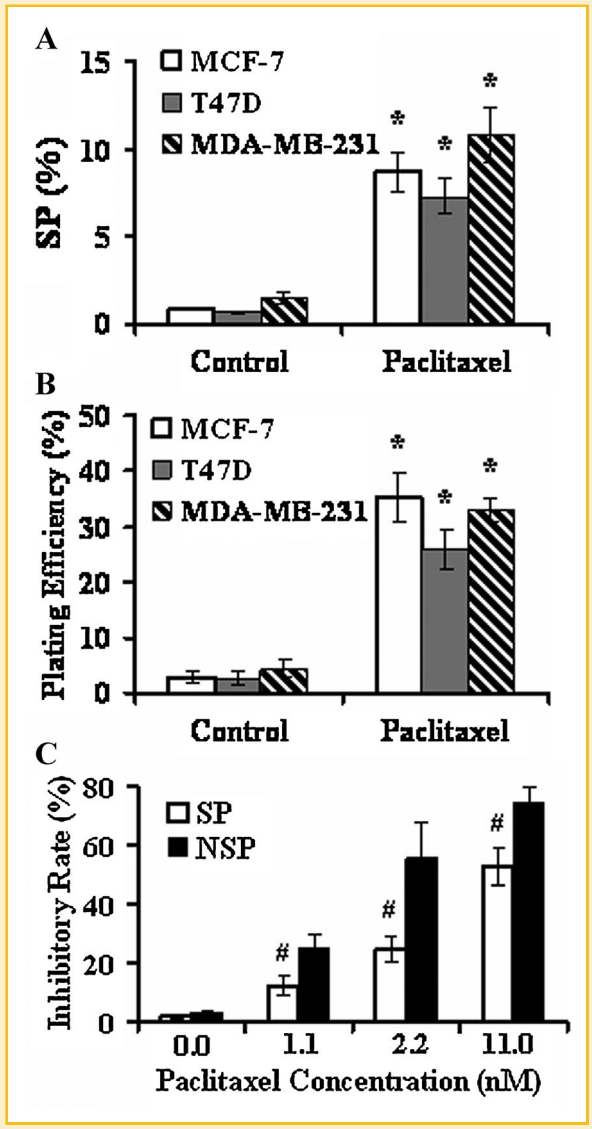

Fig. 4. Drug resistance of side population (SP) cells. A: After treatment of breast cancer cells with paclitaxel or vehicle solution for 48 h, the percentage of SP cells was determined by flow cytometry through uptake of the DNA binding dye Hoechst33342. The paclitaxel concentrations used in each cell line were 2.2 nM for MCF-7, 6.5 nM for T47D, and 3.8 nM for MDA-MB-231. $^{*}P \leq 0.01$ compared to vehicle control. B: The colony forming capacity of breast cancer cells was measured by clonogenic assays after treatment of paclitaxel or vehicle control. $^{*}P \leq 0.01$ compared to vehicle control. C: SP and non-SP (NSP) cells from MCF-7 were treated with 0, 1.1, 2.2, and 11 nM paclitaxel for 48 h. Cell variability was determined by trypan blue dye exclusion assays and an inhibition rate was calculated as (1 − unstained treated cells/control cells) × 100. Data represented mean ± SD of triplicate independent experiments. $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to NSP.

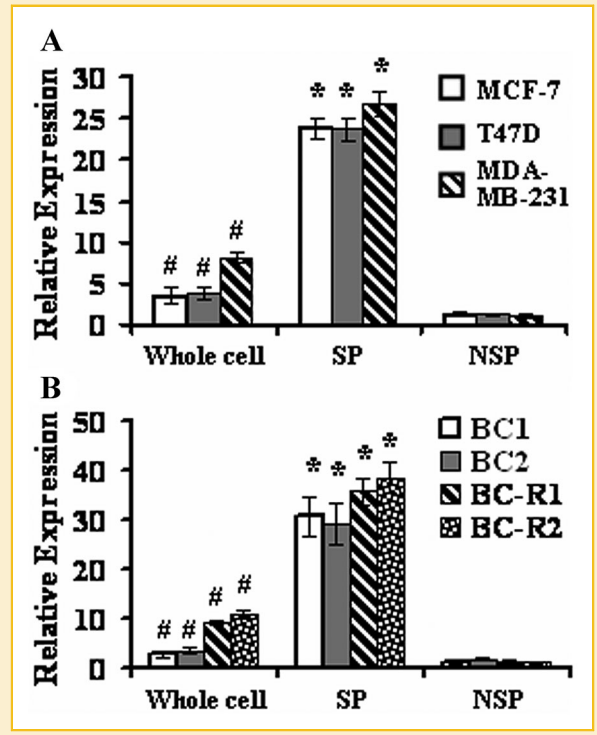

Fig. 5. miR-125b expression in human breast cancer cell lines (A) and primary cancer cells isolated from breast cancer patients (B). miRNA expression in whole cell population, side population (SP), or non-side population (NSP) was determined by quantitative real-time PCR. $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to NSP.

miR-125B INDUCES EPITHELIAL–MESENCHYMAL TRANSITION (EMT)-LIKE CELLULAR MARKER ALTERATION IN BREAST CANCER CELLS

EMT is considered to play an important role in chemoresistance [Zhang et al., 2012] and generates cells with stem-cell like properties [Mani et al., 2008]. Real-time PCR results showed that SP isolated from MCF-7, T47D, and MDA-MB-231 have an EMT-like marker shift (Fig. 8A), including a dramatic downregulation of the epithelial marker E-cadherin, and up-regulation of the mesenchymal markers N-cadherin, vimentin, and EMT-associated transcription factor SNAIL.

miR-125b has been demonstrated to function as a key mediator for SNAIL-induced stem cell propagation and chemoresistance [Liu et al., 2013]. To determine the potential role of miR-125b in EMT, we analyzed a panel of representative epithelial and mesenchymal markers by real-time PCR in breast cancer cells following miR-125b overexpression. As shown in Figure 8B, ectopic miR-125b caused a marked downregulation of the epithelial marker E-cadherin, and up-regulation of the mesenchymal markers N-cadherin, vimentin, and SNAIL in both MCF7 and BC1 cells. These results indicate that miR-125b induces EMT-like molecular alterations in breast cancer cells. However, overexpression of miR-125b did not induce any obvious morphological changes in breast cancer cells in monolayer cultures (data not shown).

DISCUSSION

Recent studies have shown that SP cells exist in various types of tumors, including the breast cancer [Britton et al., 2012; Lu et al., 2012]. However, the stringent functional characterization of

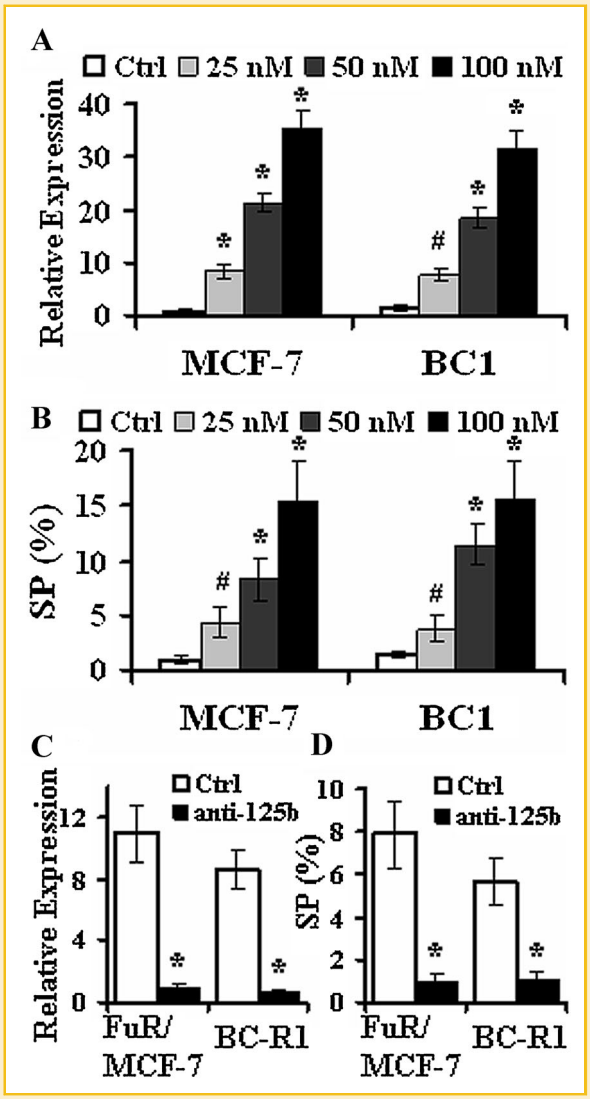

Fig. 6. miR-125b regulated breast cancer SP cells. A: MCF-7 and BC1 cells were transfected with 25, 50, or 100 nM miR-125b mimics and miR-125b expression levels were measured by quantitative real-time PCR. A scramble pre-miRNA sequence (100 nM) served as a negative control (Ctrl). B: The percentage of SP cells was determined by flow cytometry through uptake of the DNA binding dye Hoechst33342 after miR-125b mimic or scramble control transfection. C: FuR/MCF-7 and BC-R1 cells were transfected with 100 nM miR-125b antisense or scramble control sequence (Ctrl) and miR-125b expression levels were measured by quantitative real-time PCR. D: The percentage of SP cells was determined by flow cytometry. Data represented mean ± SD of triplicate independent experiments. $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to Ctrl.

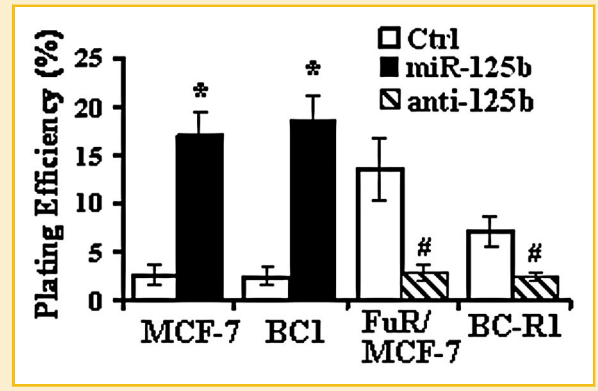

Fig. 7. Clonogenic assays for breast cancer cells. MCF-7 and BC1 cells were transfected with 100 nM miR-125b mimics or scramble control (Ctrl). FuR/MCF-7 and BC-R1 cells were transfected with 100 nM miR-125b antisense or scramble control (Ctrl). The cells were cultured for 10 days after transfection and the plating efficiency was determined as % of cells forming a colony per number of cells seeded. Data represented mean ± SD of triplicate independent experiments. $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to Ctrl.

SP cells in breast cancer is unclear. In the current study, we demonstrated that breast cancer cell lines and primary tissues contained cancer stem-like SP cells. Further characterization of SP cells demonstrated a significant correlation of SP fraction with the expression of miR-125b and patients' responses to chemotherapy. Our previous studies have shown that miR-125b was significantly up-regulated in breast cancer patients with poor response to chemotherapy and induced chemoresistance in breast cancer cells [Wang et al., 2012]. When we directly tested the function of miR-125b in breast cancer SP, we found that overexpression of miR-125b correlated with an increase in tumor SP and CSC property, whereas knockdown of miR-125b correlated with decreased incidence of SP. Taken together, these results suggest an important role for miR-125b in breast cancer chemoresistance by maintaining cancer stem-like SP fraction, and raise the possibility that miR-125b may be a significant prognostic response marker for cancer therapy.

Consistent with previous studies, our current data showed that SP cells isolated from breast cancer cell lines have many CSC properties, including self-renewal, resistance to chemotherapy and high expression of stem cell markers (Figs. 2–4). Some recent studies revealed that cellular stresses like drug exposure [Calcagno et al., 2010; Fong et al., 2010], hypoxia [Bar et al., 2010], and UV lighting [Liang et al., 2010] enriched for a CSC phenotype in cancer cell lines. In this study, we detected a higher percentage of SP in a 5-FU resistant cell line (FuR/MCF-7) compared to the parental MCF-7. More interestingly, the occurrence of SP in primary cultures was significantly associated with therapeutic response in breast cancer patients, exhibiting higher percentage in non-responsive patients. In addition, breast cancers with high SP percentage had higher proliferation rate and lower apoptotic activity in the corresponding surgical specimens obtained after neoadjuvant chemotherapy. To the best of our knowledge, this is the first study investigating the association of SP cells and chemotherapeutic response in breast cancer patients.

The mechanisms contributed to SP chemoresistance include relative quiescence, expression of ABC transporters and/or multidrug resistance transporter 1 (MDR1), a more robust DNA repair capability, and the elevated expression of antiapoptotic proteins [Dean, 2009]. Emerging evidence suggests that miRNA has important roles in physiologic processes and aberrant expression could contribute to the

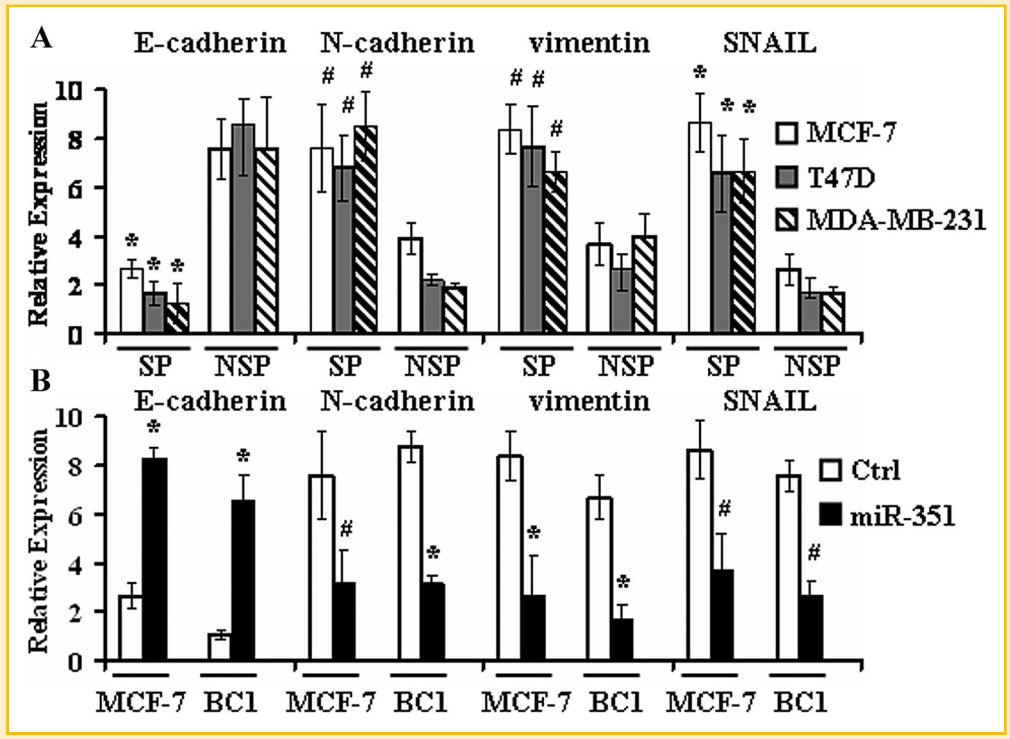

Fig. 8. Expression of epithelial and mesenchymal markers. A: Side population (SP) and non-side population (NSP) were isolated from breast cancer cell lines. Gene expression was determined by quantitative real-time PCR. Data represent the mean ± SD of three different SP or NSP cells isolated from each breast cancer cell line, $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to corresponding NSP. B: MCF-7 and BC1 cells were transfected with 100 nM miR-125b mimics (miR-125b) or scramble control (Ctrl). Expression of epithelial and mesenchymal markers was determined by quantitative real-time PCR. Data represent the mean ± SD of triplicate independent experiments, $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to corresponding Ctrl.

initiation and progression of cancer [Calin and Croce, 2006]. The present study demonstrated a high SP fraction in breast cancer cells was associated with increased miR-125b expression. In addition, aberrant expression of miR-125b increased the percentage of SP and contributed to the cancer stem-like phenotypes of SP cells. miR-125b was reported to have complicated functions as either oncogene-like or tumor suppressor-like in different cancer types or cell lines. Up-regulation of miR-125b was observed in various human malignancies, including pancreatic cancer [Bloomston et al., 2007], prostate cancer [Shi et al., 2007], and acute myeloid leukemia [Tian et al., 2010]. For a long time, miR-125b was considered as a tumor suppressor in breast cancer because of downregulation in breast cancer tissue [Iorio et al., 2005; Ozek et al., 2010]. However, a precise study using microdissection demonstrated that miR-125 was expressed in basal myoepithelial cells but not in luminal ductal cells [Bockmeyer et al., 2011]. The under-expression of miR-125b in breast cancer tissue may be due to loss of myoepithelial cells in cancer tissue instead of downregulation of miR-125b in cancer cells. Moreover, recent studies revealed that miR-125b was up-regulated in breast CSCs [Shimono et al., 2009] and was related to chemoresistance of breast cancer cells and metastasis [Tang et al., 2012; Wang et al., 2012]. The oncogenic function of miR-125b in breast cancer is further supported by our data resulting from the analyses of cancer stem-like SP cells in breast cancer cell lines. Interestingly, miR-125b overexpression in breast cancer cells induced EMT-like cellular marker alteration. EMT is considered to play an important role in chemoresistance [Zhang et al., 2012] and generates cells with stem-cell like properties [Mani et al., 2008]. miR-125b has been demonstrated to function as a key mediator for SNAIL-induced stem cell propagation and chemoresistance [Liu et al., 2013]. Thus, miR-125b may be a critical small non-coding RNA with potential to specifically target cancer stem-like SP cells and could be a new strategy for breast cancer treatment.

In summary, the present study provided evidence that SP cells were enriched CSCs and associated with chemoresistance in breast cancer. Elevated miR-125b expression was correlated with a high SP fraction. Further studies highlighted the importance of miR-125b for the maintenance of SP phenotype in breast cancer cells. Future work to identify the gene and protein networks directly targeted and affected and the entire roles of miR-125b in SP phenotype remain to be explored.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Drug resistance of FuR/MCF-7. 5-FU resistant FuR/MCF-7 and parental MCF7 cells were treated with different concentration of paclitaxel (A) or doxorubicin (B) for 48 h. Cell variability was determined by trypan blue dye exclusion assays and an inhibition rate was calculated as (1 − unstained treated cells/control cells) × 100. Data represented mean ± SD of triplicate independent experiments. $^{\#}P < 0.05$, $^{*}P \leq 0.01$ compared to parental MCF7.