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Original Research

The role of Iron on breast cancer stem-like cells

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Abstract: Iron is a fundamental nutrient that enables the functions of vital enzymes involved in cell replication, metabolism and growth. Cancer cells contain higher systemic iron levels relative to normal cells. In breast cancer cells, human epidermal growth factor receptor 2 (HER2) is overexpressed more than 30% of normal and its poorly prognosis results in elevated the proportion of cancer stem cells (CSCs) which are the main drivers in cancer recurrence. Finding a relation between increases of iron levels, HER2 expression and CSC population may provide new tools for breast cancer therapy. In this study, therefore, iron dependency in HER2 overexpression and CSC survival is examined in breast cancer cell line, MCF7. It has shown that cells overexpressing HER2 require iron more than their vector counterparts and HER2-increased CSCs are vulnerable to iron chelation. Additionally, this sensitivity of CSCs to iron reduction is obviously indicated in various breast cancer cell lines; HCC1954, MDA-MB-435 and Hs578T. Finally, the concept is also shown in neoplastically transformed breast cancer cell line, HMLER. Altogether, this study demonstrates that iron depletion causes toxicity for CSCs.

Key words: Iron, CSC, iron chelator, HER2.

Introduction

Breast cancer is the most common cancer worldwide in females accounting for more than 25% of total number of new cases from all cancer types (1). Highly heterogeneous breast tumor cells have greatly invasive capacity and self-renewal properties in which exist together with differentiated and non-invasive cells (2). Recent studies show that cancers arise from a small fraction of cancer initiating cells that are capable of giving rise to the heterogeneity (3). These capacities are in parallel with normal stem cells. Thus, these cells are named as stem cell-like cancer cells (CSCs) (4-7). They exist in a variety of cancers like ovarian cancer, prostate cancer, and breast cancer, and are not sensitive to standard chemotherapy and radiotherapy (5,8-10). CSCs are not only the initiator of cancer but also might be in charge of progression, metastasis, and the recurrence of cancer after treatment. Therefore, understanding needs for CSCs may be critical for the development of breast cancer prevention and treatments (11,12). Targeting essential trace nutrients like iron which enable vital functions in cells may provide new insights for cancer therapy.

Iron has crucial functions of iron- and haem-containing enzymes, including enzymes involved in DNA synthesis and the cell cycle, mitochondrial enzymes involved in cellular respiration, detoxifying enzymes such as catalase and peroxidase, enzymes involved in metabolism and many more (13-15). These beneficial effects of iron have a role in cancer such as tumor cell proliferation. Tumor cells store the majority of cellular iron and utilize them for metabolic functions like the synthesis of iron-sulfur clusters in mitochondria (16,17). They need more iron than their normal counterparts so as to fortify increased rates of cell proliferation (13). In cancer cells, cellular growth and the synthesis of iron-dependent enzymes rely upon intracellular iron levels (18,19). Depleting iron levels in tumor cells may abrogate cellular proliferation and metabolic processes.

Iron chelators are small molecules, exist naturally or in synthetic form, show high affinity for iron. Such molecules as desferrioxamine (DFO), deferiprone, deferasirox, and di-2-pyridylketone 4, 4-dimethyl-3-thiosemicarbazone (Dp44mT) are used in clinical applications for sequestering iron and for the treatment of disorders with increased systemic iron levels (20-22). Owing to their ability to inhibit cancer cell growth, these molecules have been used as a potential chemotherapeutic agents (23-25). It is not surprising that a drug modulating free iron levels in cells may particularly play a role in survival of transformed cells. Induction of iron depletion leads to G1-S cell cycle arrest followed by apoptosis (26,27). In patients with breast cancer, human epidermal growth factor receptor 2 (HER2) is overexpressed 30% more than that of in normal cells. Increase in tumorigenesis, metastasis and CSC population parallels to HER2 elevated levels (28). Several studies have recently reported that HER2 overexpression promotes augmentation of CSCs (28-30). Despite all these interesting findings, what remains less clear is understanding effects of iron chelators on cancer initiation, especially survival of CSCs.

In current study, mitochondrial and cytoplasmic iron levels were detected in breast cancer cells, including HER2 overexpressed cells and their non-tumorigenic counterparts. Alterations in CSC population of these cells were reported in treatment of iron chelators. HER2-positive and –negative breast cancer cell lines were used in order to substantiate results by different breast cancer cells. Additionally, nontumorigenic, immortalized

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human mammary epithelial cells, HMLEs (31) and its tumorigenic form, HMLER cells—transformed with a V12H-Ras oncogene (31) were used to better indicate impacts of iron chelators on CSCs. This work validates that iron is required in CSC survival and shows that iron chelators target not only transformed cells but also CSCs in breast cancer cells such as MCF7, HCC1954, MDA-MB-435, and Hs578T and ras-transformed tumorigenic cells, HMLER.

Materials and Methods

Cell lines and tissue culture

HMLE and HMLER cell lines were provided by Dr. RA Weinberg (MIT, Cambridge, MA). MCF7, MCF10A, MDA-MB-435, Hs578T and HCC1954 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific), 5 µg/mL insulin (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin solution (Invitrogen, Gaithersburg, MD, USA). HMLE and HMLER cells were cultured in DMEM/F12 with 10% fetal bovine serum (FBS), 5 µg/mL insulin, 10 ng/mL epidermal growth factor (EGF, Sigma), 0.5 µg/mL hydrocortisone (Sigma) and 1% penicillin/streptomycin solution. MCF10A cells were grown in in DMEM/F12 with 10% horse serum (HS, Gemini Bio-Products, West Sacramento, CA, USA) 5 µg/mL insulin, 10 ng/mL EGF, 0.5 µg/mL hydrocortisone and 1% penicillin/streptomycin solution. HCC1954 cells were cultured in RPMI with 10% FBS and 1% penicillin/streptomycin solution. All cell lines were cultured in 10 cm^2 tissue culture dishes (New York, USA) at 37°C in a humidified atmosphere of 5% CO_2. Sub-confluent cells (70–80% confluency) were sub-cultured following trypsinization (0.05% trypsin-ethylenediaminetetraacetic acid (EDTA), Invitrogen) every 3-4 days.

MTT cell proliferation assay

6,000 cells/well were seeded in a 96-well plate and incubated in interested growth media with varying concentrations of Dp44mT. After 4 days, medium was removed and 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well along with 90µL of fresh medium. The plate was incubated at 37°C for 4 hours and the medium was aspirated and washed with 1X PBS. 150 mL of dimethyl sulfoxide was added to each well and the plate were placed on an orbital shaker at 700 rpm for 5 min. The plate was read at wavelength of 590 nm using a microplate reader (BioTek Epoch, Winooski, VT, USA). Triplicate wells were used for each treatment, and experiments were repeated three times.

Flow cytometry for CSCs

Anti-human CD44-fluorescein (FITC, clone G44-26) and anti-human CD24-phycoerythrin (PE, clone ML5) (BD Biosciences, Franklin Lakes, NJ, USA) were used for analysis. Cells were digested with 0.25% trypsin to produce a single cell suspension and were washed twice with a staining buffer (PBS solution containing 0.1% FBS). The cell concentration was adjusted to 1×10^6 cells in a 100 µL buffer. Antibodies were added to the cell suspension at concentrations recommended by the manufacturer and staining was performed in dark at 4°C for 30 min followed by two washes with the same buffer. Samples were run with a BD Accuri C6 (Becton Dickinson, San Josè, CA, USA) and analysis was performed with the manufacturers' software (BD Accuri C6 software). CSC population represents $CD44^+/CD24^-$ cells on histograms.

Detection of iron levels by flow cytometry

Cells were collected with a 0.25% trypsin and were washed twice with PBS buffer. They were suspended with serum-free medium containing 0.5 µM calcein-AM (C-AM, Sigma, St. Louis, MO, USA) and 0.5 µM rhodamine B-[(1,10-phenanthroline-5-yl)-aminocarbonyl]benzyl ester (RPA, squarix biotechnology, Marl, Germany). Samples were incubated in dark at 37°C for 30 min followed by two washes with the buffer. They were suspended in a PBS buffer and were run with a BD Accuri C6 (Becton Dickinson, San Josè, CA, USA). Mean fluorescence for each treatment was obtained and analysis was performed with the manufacturers' software (BD Accuri C6 software). Calcein-AM and RPA fluorescence shown in the form of mean fluorescence were inversely correlated with intracellular iron.

Statistical Analysis

All experiments were separately repeated at least three times. Figures are representative of all the corresponding experiments performed and results are presented as the mean ± SEM. Statistical significance was calculated using a paired two-sided Student's t test. Differences with p values of 0.05 or less were considered statistically significant.

Results

HER2 overexpression increases iron dependency

Cancer cells require iron as a nutrient that nourishes cell proliferation and tumor initiation (27). HER2 overexpression in breast cancer cells enhances tumorigenesis, CSC population and metastasis (28). In order to determine the relation between HER2 overexpression and iron levels, normal breast epithelial cell line, including only vector (MCF10A-vec) and its HER2 overexpressed line (MCF10A-HER2) cells were treated with increasing concentrations (0.05 to 10 nM) of Dp44mT for 4 days and cell viability was measured. Control treatments were set as %100 and the percentage of viability was calculated in Dp44mT treatments. MCF10A-vec cells are much more sensitive (IC_{50}= 0.5 nM) to iron decrease compared to MCF10A-HER2 cells (IC_{50}= 4-5 nM), showing the effect of HER2 overexpression on iron chelation (Fig. 1A). In order to determine this relation in breast cancer cells, the same experiment was done with different concentrations (0.1 to 1000 nM) of Dp44mT in MCF7-vec and MCF7-HER2 cells as they are more resistant than normal breast cells. In these cells, the resistance of HER2 overexpressed cells to Dp44mT was weaker (Fig. 1B).

To test whether iron levels alter with HER2 overexpression, both mitochondrial and cytoplasmic iron

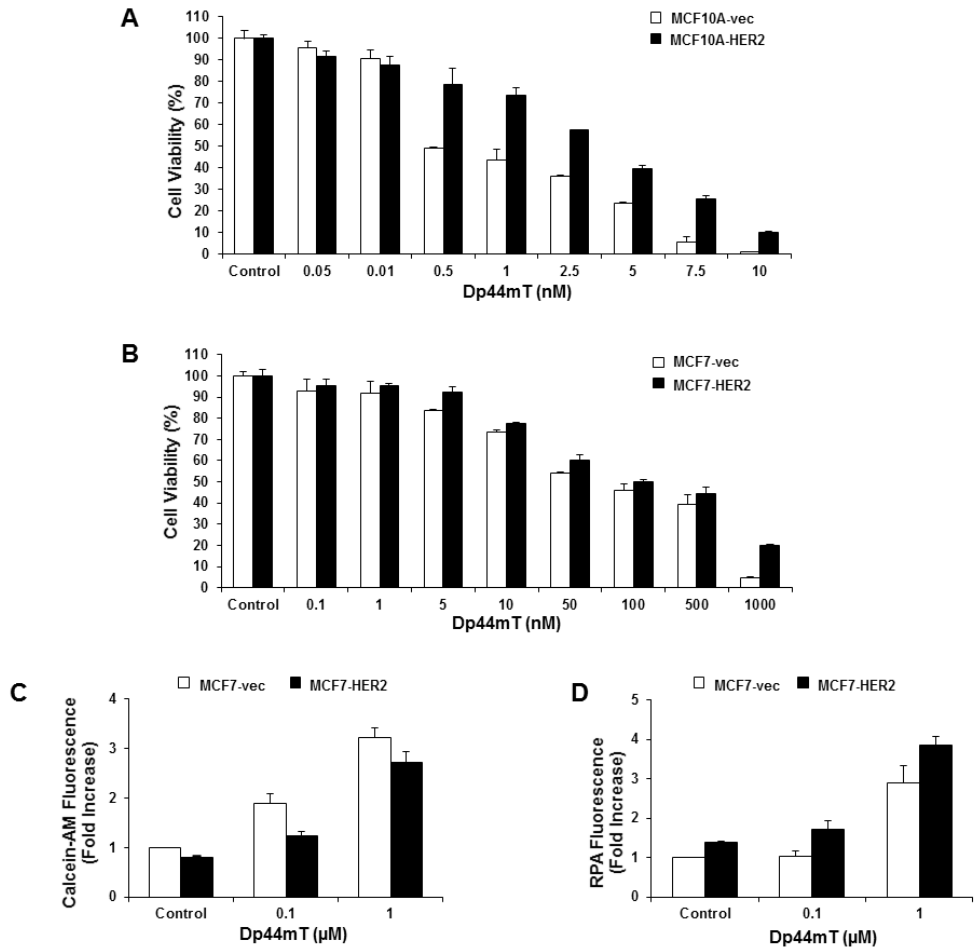

Figure 1. Cell viability in response to Dp44mT and iron measurement. **A.** Dp44mT treated to MCF10A (0.05 to 10 nM) and MCF7 cells (0.1 to 1000 nM) at 37°C for 4 days and then cells subjected to MTT cell viability assay. Bars represent the percentage of cell viability ± SEM from 3 experiments. **B.** MCF7 cells were treated with 0.1 and 1 μM Dp44mT for 24 hours and iron levels measured by C-AM and RPA staining utilizing flow cytometry assay. Bars represent fold increase of the mean fluorescence ± SEM from 3 experiments.

levels were measured in MCF7-vec and MCF7-HER2 cells. Cytoplasmic iron levels were comparatively high in MCF7-HER2 cells and treatments of 0.1 and 1 μM Dp44mT had a less effect on iron decrease in MCF7-HER2 cells (Fig. 1C). However, mitochondrial iron levels were less in MCF7-HER2 cells and treatments of 0.1 and 1 μM Dp44mT decreased iron levels more extensively in MCF7-HER2 cells (Fig. 1D).

Iron chelators decrease CSC population

Several recent studies have reported that regulation of CSC population is correlated with HER2 expression levels (8,28,29). To assess the ability of iron chelators to lessen the proportion of CSC with the $CD44^{+}/CD24^{-}$ antigenic phenotype (10) in breast cancer cells, MCF7-vec and MCF7-HER2 cells were treated with 2 nM Dp44mT for 3 days. Additionally, cells were treated with 20 μM $FeCl_3$ and its combination of Dp44mT to determine whether exogenous iron supplement rescue the effect of Dp44mT on CSC population. The effect was not easily readily seen in MCF7-vec cells since they have a small number of CSC population. In MCF7-HER2 cells, iron addition enhanced CSC population about 2-fold compared to control while it has been diminished by Dp44mT treatment. Iron and Dp44mT combination reversed the population decrease caused by Dp44mT (Fig. 2). To see the impact of specific iron chelator DFO, on CSC population, MCF7-HER2 cells were treated with 10 μM DFO or 20 μM $FeCl_3$ and their combinations for 5 days. DFO

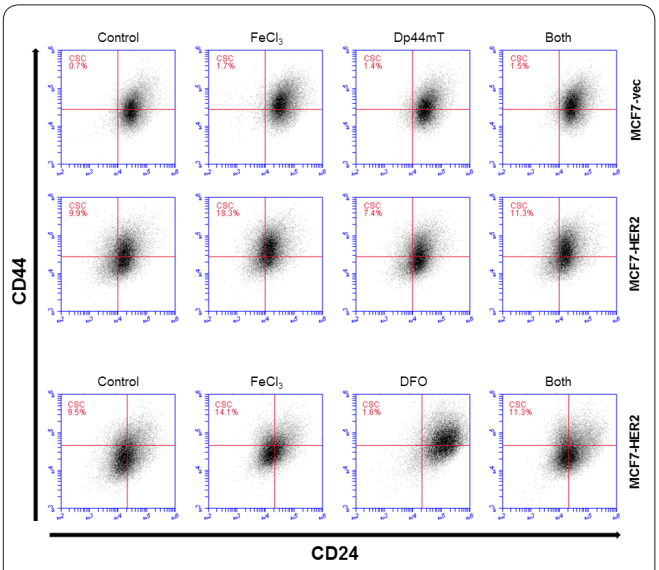

Figure 2. Effects of iron and iron chelators on the proportion of CSCs. Cells were grown ± 20 μM $FeCl_3$ and its combinations with 2 nM Dp44mT for 3 days, and 10 μM DFO for 5 days. They are stained with CD44-FITC and CD24-PE antibodies and then flow cytometry assay was done. Results represent 3 separately repeated experiments.

dramatically reduced CSC population about 6-fold and this reduction was rescued by iron combination (Fig. 2).

In order to show if this effect is seen in other breast cancer cells, HCC1954, Hs578T and MDA-MB-435

cell lines were exposed to 10 μM DFO or 20 μM $FeCl_3$ and their combinations for 5 days. In HCT1954 cell lines, iron only increased CSC population more than 2-fold while DFO decreased CSC population. This decrease was reversed by combination of exogenous iron. In Hs578T and MDA-MB-435 cell lines, basal CSC population was substantially high and therefore iron addition has no effect. Treatment of DFO diminished the population approximately 20% in MDA-MB-435 and 40% in Hs578T cells and these reductions were rescued by iron combination (Fig. 3).

DFO treatment decreases CSC numbers in HMLER breast cancer cells

Many breast cancer chemotherapeutic drugs like paclitaxel kill the mass of cancer cells, but not CSCs, so the therapy might eventually fail due to recurrence (32-34). HMLE is an immortalized nontumorigenic epithelial cell and has a low proportion of CSCs whereas its neoplastically transformed derivative (35), HMLER naturally contains a high proportion of CSCs (36). HMLE and HMLER cells were used to evaluate whether DFO treatment responses of these cells parallel the same responses of breast cancer cell lines used above. Here, paclitaxel is used to confirm that CSC proportion increased in total number of cancer cells owing to death of differentiated cells. HMLE cells were treated with increasing concentrations of paclitaxel (0.5, 1 and 2 nM) and of DFO (1, 5, 10 and 20 μM) for 4 days and then cells are recovered with only fresh medium for another 4 days. Paclitaxel treatment increased the proportion of CSCs by 8-fold compared to control treatment, but DFO showed no effect (Fig. 4A).

In order to determine whether DFO kills CSCs, highly enriched in HMLER cells, cells were treated with increasing concentrations of DFO (1, 5, 10 and 20 μM) for 4 days and recovered as described under M&M. DFO treatment reduced the proportion of CSCs compared to controls. In highest concentrations, CSC population was decreased by half of basal levels (Fig. 4A). To determine whether increase of CSCs in HMLER is due to iron levels, both cytoplasmic and mitochondrial iron levels were measured in HMLE and HMLER cells. Cytoplasmic and mitochondrial iron levels were 6-fold and 2-fold more in HMLER compared to HMLE cells, respectively (Fig. 4B).

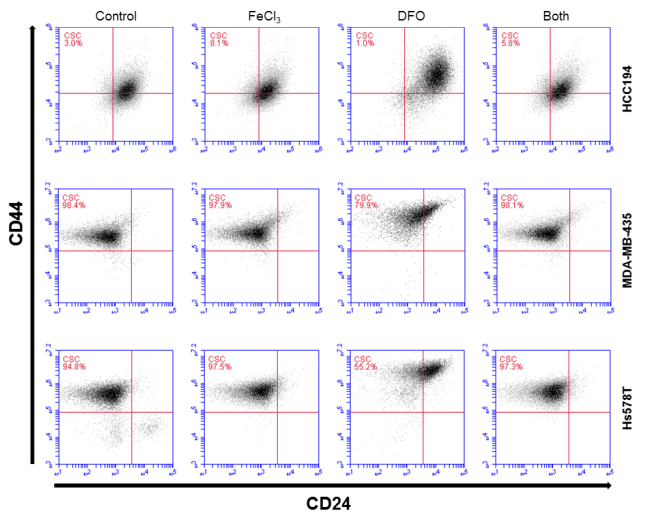

Figure 3. CSC population in response to DFO in different breast cancer cell lines. 10 μM DFO and ± 20 μM $FeCl_3$ were treated to cells for 5 days and they were exposed to flow cytometry assay. Results represent 3 separately repeated experiments.

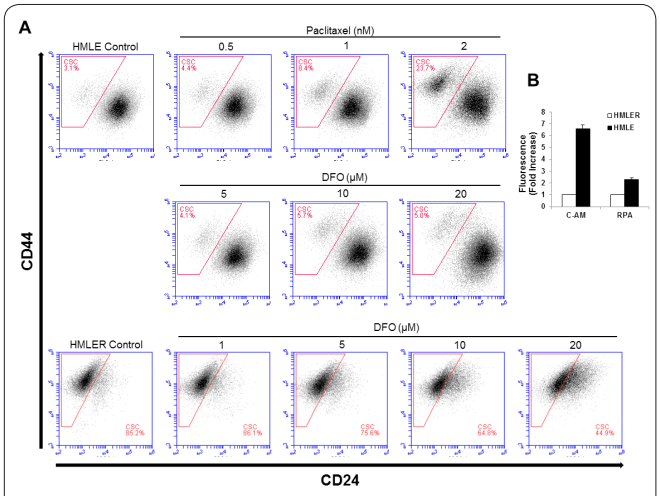

Figure 4. Effects of paclitaxel and DFO on CSC population in HMLE and HMLER cells. **A.** HMLE cells were treated with paclitaxel (0.5, 1 and 2 nM) and DFO (5, 10 and 20 μM) for 4 days and recovered with fresh media for another 4 days. HMLER cells were treated with DFO (1, 5, 10 and 20 μM) as the same way and then flow cytometry assay was done. Results represents 3 separately repeated experiments. **B.** Basal iron levels were measured with C-AM and RPA staining followed flow cytometry assay. Bars represent fold increase of the mean fluorescence ± SEM from 3 experiments.

Discussion

Iron is essential for cellular growth and metabolism since it is involved in many enzymes' reactions (13-17). It is usually maintained at higher concentrations in cancer cells relative to normal cells, supplying requirements for tumor initiation and progression. Especially cancer cells reliant upon iron must sustain levels of iron that are high enough to keep on robust cell proliferation. However, cellular growth and metabolic processes may be abrogated when iron levels are depleted to below the threshold. Thus, iron depletion might increase vulnerability of cancer cells to cell death. In parallel to elevated iron levels, cancer cells need increased levels of genes involved in cancerogenesis. HER2 gene is overexpressed in patients with breast cancer cells and promotes CSC increment compared to their normal counterparts (28-30). Targeting essential tools such as iron homeostasis, the proportion of CSC that enable vital functions in cancer cell survival and recurrence may provide new insights for breast cancer therapy.

In current study, the correlation between elevated levels of iron, HER2 expression and CSC population was depicted in breast cancer cell lines. It has shown that cells overexpressing HER2 were resistant to iron chelation by Dp44mT relative to vehicle controls. Nontumorigenic breast cancer cell line, MCF10A was much more sensitive to Dp44mT compared to breast cancer cell line, thereby indicating existence of higher iron load in cancer cells (Fig. 1A and B). Cytoplasmic iron levels were higher in HER2 overexpressed cancer

cells whereas their mitochondrial iron levels were lower compared to vehicle control (Fig. 1C and D). These observations strongly propose that iron levels are correlated with HER2 overexpression. Low levels of mitochondrial iron in MCF7-HER2 cells may be because of mitochondrial dysfunction via iron-sulfur cluster defect. It is evident that HER2 overexpression parallels to increased iron levels required for cell viability.

Exogenous iron supplement increases CSC population in MCF7 breast cancer cells. This was readily observable in MCF7-HER2 relative to MCF7-vec cells since HER2 induces the CSC population. In addition to partial decrease of the CSC population by Dp44mT, CSC population was greatly decreased by DFO treatment in MCF7-HER2 cells (Fig. 2). Combination of iron with Dp44mT and DFO reversed the decrease of cell population, indicating the significance of iron for CSC survival. DFO specifically chelates iron while Dp44mT chelates copper as well as iron; therefore, DFO was used in further experiments. To substantiate these results, other breast cancer cells; HCC1954, MDA-MB-435 and Hs578T were treated with iron, DFO and their combinations. DFO treatment reduced the proportion of CSC, which was rescued by exogenous iron supplement (Fig. 3). Altogether these findings show that iron is indispensable for CSC viability and its depletion eliminates CSC survival in breast cancer cell lines. This is consistent with requirement of cells overexpressing HER2 under iron. It looks there is a triangle relationship between levels of intracellular iron, HER2 and CSC population and this relation is seen as a positive feedforward loop.

It has already known that paclitaxel, like many drugs, shows no effect on CSC population because it kills the bulk of cancer cells (36). Therefore, treatment of paclitaxel increases the proportion of CSCs in total volume. This is confirmed in immortalized nontumorigenic HMLE cells that have well-separated populations in histogram (Fig. 4A). DFO treatment in HMLE cells has no effect on the proportion of CSCs as it is already low in basal levels. However, the treatment significantly eliminates CSC population in HMLER cells which have a high proportion of CSCs and these cells have more iron than HMLE cells (Fig. 4A and B). Comparison of paclitaxel and DFO indicates that iron is an important nutrient for CSCs. These findings emphasize that a massive proportion of cancer cells die upon treatment with drugs widely used in chemotherapy. However, CSCs persist, giving rise proliferation of cancer cells. To sensitize CSCs to cell death, drugs that selectively target their viability are required. Hence, further efforts may be aimed to better understand the impact of iron on CSC viability.

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