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Dynamic Phenotypic Transition of Breast Cancer Cells *In Vitro* Revealed by Self-floating Cell Culture

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Abstract. *Background: Breast tumor heterogeneity leads to phenotypic diversity, such as tumor-initiating and metastatic properties and drug sensitivity. Materials and Methods: We found that a self-floating cell (SFC) culture enriches a drug-resistant subpopulation in a HER2-positive breast cancer cell line. SFCs were analyzed for cancer stem cell markers, gene expression profiles, and sensitivity for anticancer drugs. Results: SFCs expressed cancer stem cell markers, such as aldehyde dehydrogenase (ALDH) activity and elevated HER2 autophosphorylation. Gene expression profiles of SFCs showed a dramatic difference compared to those of parental or forced floating cells. SFCs also expressed CD133, a marker of drug resistance, and resisted cytotoxic drugs by drug efflux transporters. In contrast, HER2 kinase inhibitors efficiently reduced SFC viability. Conclusion: SFCs enrich drug-resistant subpopulations even in vitro and might reflect the highly plastic nature of breast cancer cells even in vitro.*

Breast tumors are composed of subpopulations that show diverse characteristics in terms of tumor-initiation, metastasis, and drug resistance (1). Recent studies have demonstrated the plastic nature of cancer cells by non-genetic alterations (2). This plasticity seems to confer an evolutionary behavior on cancer cells and makes anticancer therapies complicated. Understanding the mechanisms of breast cancer heterogeneity may lead to sustained therapeutic responses. Simple *in vitro* models of tumor heterogeneity may reveal mechanistic insights underlying tumor plasticity and provide drug-screening systems for heterogeneity-targeting therapeutics.

A lack of contact inhibition and anchorage-independent proliferation are classical properties of cancer cells that may enrich cancer-initiating cell subsets from a long-term monolayer culture of *RB1*-deficient mouse fibroblasts (3). Focus in a cancer cell monolayer, that originates from a single cell with focus-initiating ability, is spontaneously shed from the cell sheet and sustains its survival in sphere suspension. This observation suggests that only subpopulations of cells have cancer-initiating properties and are enriched through changes in the microenvironment.

Here we report that a 3D culture system enriches a drug-resistant population with high expression of cancer stem-like cell markers. In our system, referred to as a self-floating cell (SFC) culture, cell aggregates were spontaneously shed from a confluent monolayer of HBC-5 cells, a human breast cancer cell line, and outgrew as spheres. They showed high aldehyde dehydrogenase (ALDH) activity and elevated HER2 signaling, known stem cell markers. SFCs showed altered gene expression profiles compared to that of parental cells. Although they resisted classical chemotherapeutics, suppression of elevated HER2 activity by HER2 tyrosine kinase inhibitors efficiently reduced the proliferation of SFCs. These results suggest that SFC enriches drug-resistant subpopulations and that the dynamic phenotypic transition of SFCs reflects the highly plastic nature of breast cancer cells.

Materials and Methods

Cell culture and chemicals. Adherent HBC-5 cells were grown as cell monolayers in RPMI1640 medium (Sigma, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, US) with penicillin/streptomycin (Thermo Fisher Scientific). Forced-floating cells (FFCs) were prepared on tissue culture plates were coated with anti-adhesive polymer poly-(2-hydroxyethyl methacrylate) as

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described elsewhere (4). HBC-5 monolayer cultured cells formed focus when cells reached confluence on the plates. To obtain SFCs, focus forming cells from HBC-5 monolayer culture were detached by gentle pipetting. Detached cells were transferred to a plate containing new medium. This operation was repeated every 2-3 days for approximately 10 times to enrich spheres (about 1 month). Sphere-like cells were usually maintained 3-4 months in the culture medium and used as SFCs. Medium was exchanged twice a week. Doxorubicin, cisplatin, cyclosporin A, and CI1033 were purchased from Sigma. 5-fluorouracil was purchased from Wako. Lapatinib and erlotinib were obtained from Selleck Chemicals (Houston, TX, USA).

Western blot. Western blot was performed as previously described (5). Proteins were separated by SDS-PAGE and blotted onto PVDF membrane (GE Healthcare, Buckinghamshire, UK). The following antibodies were used: 1/1,000 rabbit anti-CD133 antibody (abcam (Cambridge, UK) ab19898), 1/1,000 mouse anti-CD44 antibody (Ancell (Bayport, MN, USA) 352-0201), 1/5,000 mouse anti-tubulin antibody (Sigma B-5-1-2), 1/1,000 rabbit anti-phospho-HER2 Y1221/1222 antibody (Cell Signaling (Danvers, MA, USA) #2243S), rabbit anti-HER2 antibody (Cell Signaling #2165), rabbit anti-phospho-EGFR antibody Y1148 (Cell Signaling, #4404S), rabbit anti-EGFR antibody (Cell Signaling, #2232), 1/5000 anti-mouse IgG-HRP (GE Healthcare), and 1/5,000 anti-rabbit IgG-HRP (GE Healthcare). All antibodies were diluted with CanGetSignal reagent (TOYOBO, Osaka, Japan).

MTT assay. 3×10^3-10^4 cells suspended in RPMI-1640 containing 10% FBS were seeded on a well of 96-well plates. Cells were incubated in the presence of drugs for 3 days. 0.5 mg/ml MTT (Sigma) was added and incubated for 4 h. Formazan was solubilized by 8% SDS overnight. OD570 was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry. ALDH activity was visualized by ALDEFLUOR reagent (Stemcell Technologies, Vancouver, BC, USA) according to the manufacturer's instructions. Cells were dispersed with Accutase (BD, Franklin Lakes, NJ, USA) and re-suspended in staining buffer. Cells were stained with ALDEFLUOR reagent in the presence or absence of DEAB (ALDH inhibitor) at 37˚C for 30 min. Cells were stained with 7-AAD (BD) prior to analysis. Live cells were gated as 7-AAD-negative cells. Cells were analyzed by FACSAria (BD) using FACSDiva software (BD).

RNAseq. For fragment library preparation, a SOLiD total RNA-seq kit (Thermo Fisher Scientific) was used in accordance with the manufacturer's protocol. Sequencing was performed using a SOLiD5500 platform (Thermo Fisher Scientific). Differentially expressed genes in SFCs (more than two fold increase or decrease compared to both parental cells and forced floating cells) were extracted using Avadis NGS software (Strand Genomics, Inc., Hebbal, Bangalore, India).

qPCR. Total RNA were prepared with ISOGEN (Nippon Gene, Tokyo, Japan) and cDNA were synthesized with ReverTra Ace qPCR RT master mix with gDNA remover (TOYOBO). Real-time PCR was performed with QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) using the Eco Real Time PCR system (Illumina, San Diego, CA, USA). Primers for *BMP4, DKK1, FST* were purchased from Qiagen. The following primers were used for TGFB3 quantification. *TGFB3F*: AAGAAATCCATAAATTCG ACATGATC, *TGFB3R*: CACATTGAAGCGGAAAACCTT.

Results

Isolation and characterization of self-floating cells from the HBC-5 breast cancer cell line. The HBC-5 breast cancer cell line is a HER2-expressing cell line originally established from a papillotubular adenocarcinoma (6). During cell culture, we noted that cell aggregates were spontaneously shed from the confluent cell culture and grew as spheres on regular tissue culture dishes (Figure 1A). We termed such spheres "self-floating cells" (SFCs). SFCs could be maintained as spheres for at least 4-6 months. The characteristics of SFC cell growth were distinct from those of parental (adherent) cells or those grown on anti-adhesive polymer poly-(2-hydroxyethyl methacrylate) ("forced floating cells," FFCs) (Figure 1B).

We searched for the characteristic markers of SFCs and found that ALDH activity, a cancer stem-like cell marker in breast cancer, was increased in SFCs (Figure 1C). Overexpression of HER2 in mammary epithelial cells increases the stem-like cell population and the expression of ALDH (7); therefore, we examined the activation status of HER2. Elevated auto-phosphorylation levels in SFCs indicated activated HER2 signaling in them (Figure 1D). These results suggest that the SFCs enrich cell populations with high ALDH activity and activated HER2 signaling. These findings are consistent with higher ALDH activity in HER2-overexpressing cells (7). In addition, we found the increased expression of CD133 in SFCs (Figure 1E), which is a marker for drug resistance in breast cancer (8).

Furthermore, the gene expression profile of SFCs showed a drastic difference from those of adherent cells or FFCs (Figure 1F). Among them, the expression levels of *TGFB3*, *BMP4, FST*, and *DKK1* were significantly altered in SFCs (Figure 1G). These results showed global changes in gene expression in SFCs, reflecting highly dynamic phenotypic transition even *in vitro*.

SFCs are resistant to anticancer drugs in a drug efflux transporter-dependent manner. SFCs express CD133, a marker for drug resistance in breast cancer (Figure 1E). Consistent with this, they were tolerant to classical anticancer agents (Figure 2A). We then examined whether the inhibition of ABC transporter activity could reverse drug resistance. We used a pan-ABC transporter inhibitor, cyclosporin A (9). Cyclosporin A alone did not affect the viability of either adherent cells or SFCs; however, their doxorubicin resistance was partially diminished by co-treatment with cyclosporin A (Figure 2B). These results suggest that SFCs are resistant to anticancer drugs, at least in part, in an ABC transporter-dependent manner.

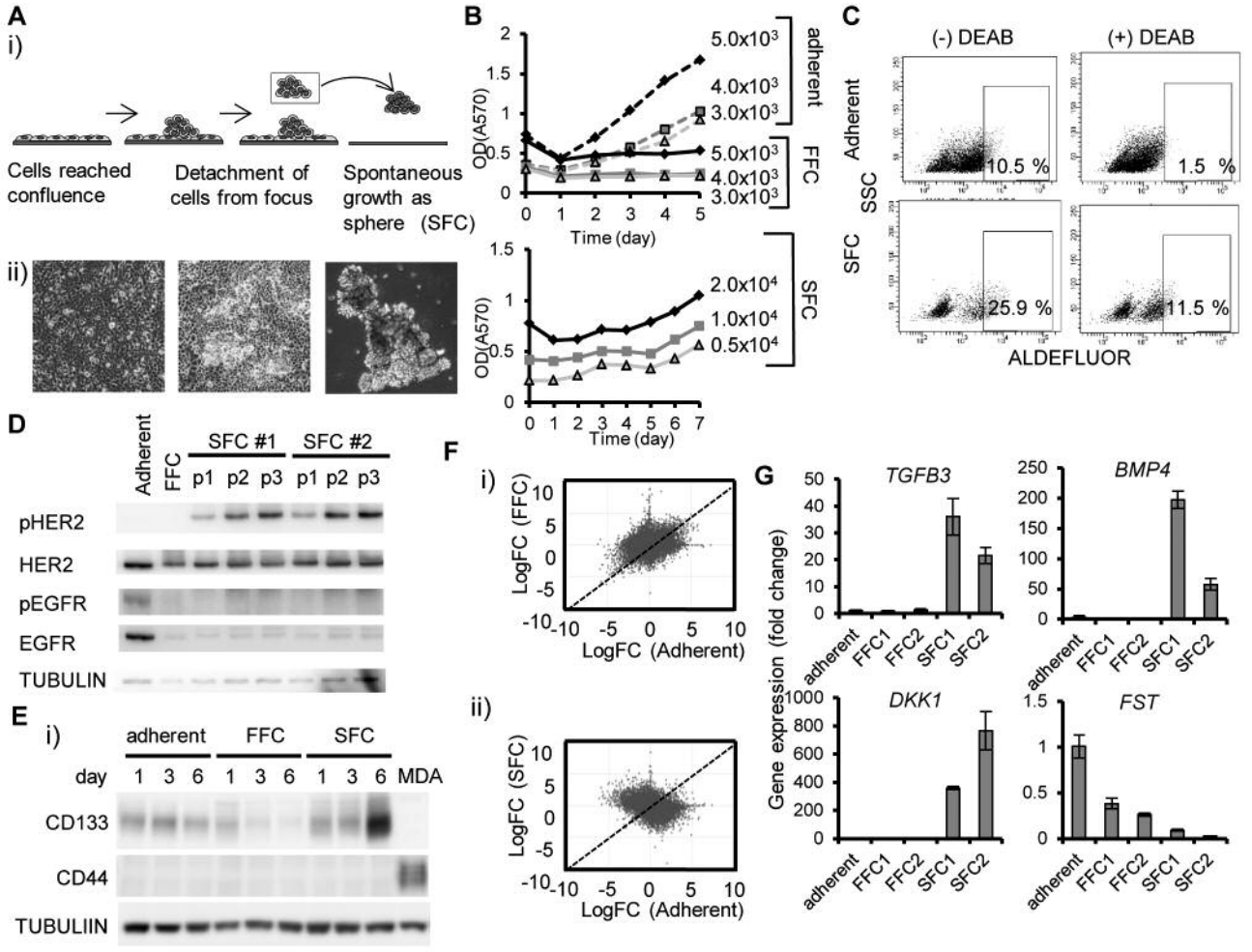

Figure 1. *Isolation of self-floating cells expressing stem cell markers from the HBC-5 breast cancer cell line. (A) Emergence of SFCs from HBC-5: i) Schematic diagram of the emergence of SFCs, (ii) representative picture of confluent culture (left), cells forming foci (middle), and spheroid growth of SFCs (right). (B) Growth of SFCs. Growth of adherent cells and FFCs (upper panel) and that of SFCs (lower panel). (C) Expression of ALDH in SFCs. DEAB (an ALDH inhibitor) was used as a negative control. (D) Increase in HER2 auto-phosphorylation in SFCs. FFCs were collected six days after seeding on poly-HEMA-coated plates. SFCs were collected at passages 1, 2, and 3 (p1, p2, p3). (E) Expression of CD133 and CD44 in SFCs. MDA: MDA-MB-231. (F) Global changes in gene expression profiles of SFCs: i) adherent vs. FFC, ii) adherent vs. SFC. (G) Differential expression of components of TGF-β and WNT signaling in SFCs. Data represent mean and standard deviation from triplicate. *p<0.05 by Student's T-test.*

Drug-resistant SFCs are highly sensitive to HER2 tyrosine kinase inhibitors. Our results indicate that HER2 signaling is activated in SFCs (Figure 1). On the other hand, inhibition of this signaling in HER2-expressing luminal cancer stem-like cells by trastuzumab impairs their self-renewal (10). Therefore we examined whether the inhibition of this signaling by small molecule kinase inhibitors impairs the growth of SFCs. CI1033 and lapatinib inhibit both EGFR and HER2, and strongly inhibited the cell survival of SFCs (Figure 3A and B). However, erlotinib, an EGFR-specific inhibitor, did not inhibit SFC cell growth (Figure 3C). These results suggest that activation of HER2 signaling plays an important role in the growth of SFCs, and that the addition of the inhibition of HER2 signaling offers a potential therapeutic strategy for heterogeneous tumors containing drug resistant subpopulations.

Discussion

Tumor heterogeneity is generated by genetic and non-genetic changes and makes cancer therapy complicated. In this study, we found an SFC population that enriched a drug-resistant subpopulation of breast cancer cells with stem-like cell markers. Consistent with high ALDH activity, HER2

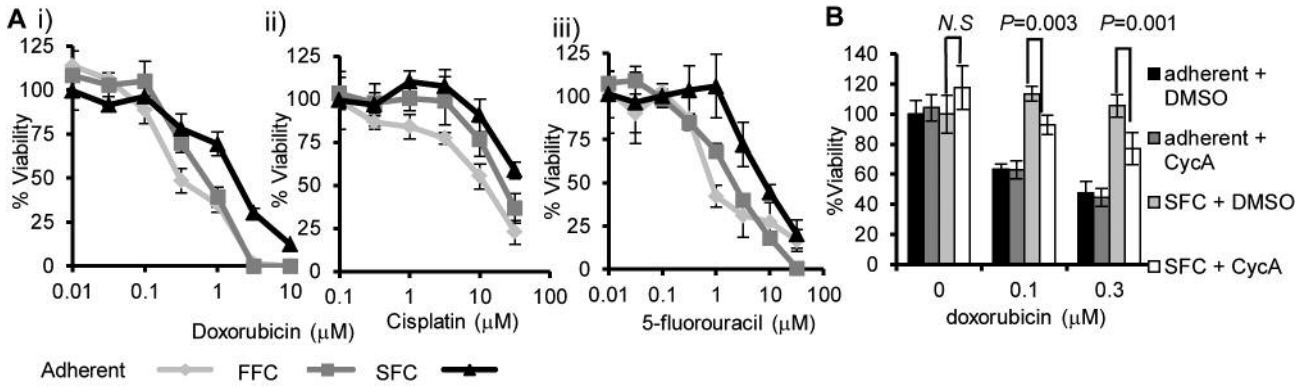

Figure 2. *Self-floating cells are resistant to anticancer drugs in a drug efflux transporter-dependent manner. (A) Resistance of SFCs to classical anticancer agents. Data represent mean and standard deviation of triplicates; i) doxorubicin, ii) cisplatin, and iii) 5-FU. (B) Doxorubicin resistance of SFCs was partially reversed by cyclosporin A. Cells were treated with doxorubicin with or without 1 μM cyclosporin A. Data represent mean and standard deviation of triplicates. *p<0.05 by Student's t-test. N.S.: Not statistically significant.*

phosphorylation was elevated in SFCs. SFCs expressed CD133, a predictive marker for drug resistance in breast cancers, and were resistant to classical anticancer drugs, while they were vulnerable to HER2 tyrosine kinase inhibition. These results revealed a dynamic phenotypic transition of breast cancer cells even *in vitro*. Thus, SFCs may be an *in vitro* model system for tumor heterogeneity and understanding the mechanisms of the phenotypic transition of breast cancer cells in tumors.

SFCs express stem-like cell markers, and show distinct gene expression profiles including WNT/β-catenin, TGF-β, and BMP4 signaling components (Figure 1). Their differential expressions are associated with malignant phenotypes in breast cancer (11-14). Differentiated tumor cells can also dedifferentiate into cancer stem cells without genetic changes (2). SFCs may represent a subpopulation with nongenetic alterations or one with rare mutations in the HBC-5 cell line. SFCs may be useful for discovering the molecular cues triggering phenotypic changes.

SFCs are resistant to classical anticancer agents (Figure 2A). Drug resistance was reversed by cyclosporin A, a pan-ABC transporter inhibitor (Figure 2B). Consistent with drug resistance, they also expressed CD133, a putative drug resistance marker in breast cancers (Figure 1E). Side-population cells are drug resistant and express ABC transporters, leading to poor responses or relapse after intensive chemotherapy (15). Drug resistance mediated by ABC transporters can be canceled by cyclosporin A or other drugs (16). Together with the changes in marker expression and transcriptional profile, a subpopulation of cells with altered drug sensitivity may be selected by changes in the microenvironment. Although SFCs are resistant to classical anticancer drugs, our findings indicate they become sensitive to anti-HER2 therapy (Figure 3). Auto-phosphorylation of

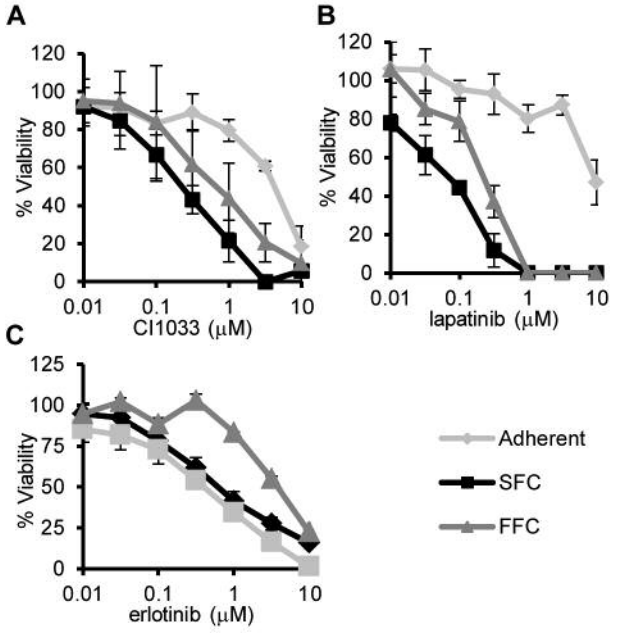

Figure 3. *Self-floating cells are highly dependent on HER2 activation. SFCs were highly sensitive to the inhibition of HER2 by small-molecule inhibitors. Data represent mean and standard deviation of three independent experiments. (A) CI1033 (pan-ErbB inhibitor), (B) lapatinib (EGFR, HER2 inhibitor), and (C) erlotinib (EGFR-specific inhibitor).*

HER2 is elevated in SFCs, consistent with high ALDH activity (Figure 1D). Other targets for drug resistant subpopulations of breast cancer cell lines may be discovered using chemical or genetic screening with the SFC system or their gene-expressing profiles. From another point of view, our results demonstrated that an anti-HER2 therapy-sensitive

subpopulation was selected by the SFC culture. Breast tumors contain HER2-expressing subpopulations and this subpopulation exhibits CSC-like phenotypes. Trastuzumab can inhibit their CSC-like properties (10). Heterogeneous expression of HER2 in *HER2* amplification-negative breast cancer is associated with poor prognosis (17). Indeed a population of patients with low HER2 still benefits from trastuzumab (18). Given that SFCs are drug-resistant and anti-HER2 therapy-sensitive, our results further support the usefulness of combination or sequential therapy using anti-HER2 drugs and classical anticancer drugs even for HER2-low breast cancers, which is under clinical trial (ClinicalTrial.gov Identifier: NCT01275677).

In summary, we showed the dynamic phenotypic transition of SFCs *in vitro*. SFCs may become a useful tool for understanding the mechanisms of phenotypic change in tumors and for discovering therapeutic options for targeting drug-resistant subpopulations.

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