Apoptosis Reversal Promotes Cancer Stem Cell-Like Cell Formation

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Abstract

It has long been a puzzle in cancer treatment that despite the initial appearance of apoptosis, the process could be reversed in some cancer cells and often results in more aggressive tumors and metastasis. The mechanism for this recurrence is yet unknown. Here we report that human mammary carcinoma cells induced to undergo apoptosis could recover with increased tumorigenicity in vitro and in vivo, and induced lymph node metastasis. Specifically, the reversed cells underwent epithelial-to-mesenchymal transitions in the primary tumors in situ, and mesenchymal-to-epithelial transitions in the metastatic cells. Flow cytometry confirmed an elevated percentage of cells carrying cancer stem cells (CSCs) markers (CD44+/CD24-) in the reversed breast cancer cell population, with hypomethylated CD44 promoters and hypermethylated CD24 promoters. More importantly, CSCs were generated anew from non-stem cancer cells after apoptosis reversal possibly through epigenetic modifications. The results from this study can open doors to discovering more effective cancer treatments by suppressing apoptosis reversal.

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Introduction

Cancer is among the leading causes of mortality worldwide [1]. The greatest therapeutic challenge it poses is its cellular heterogeneity, probably the product of clonal evolution [2] and/or cancer stem cells (CSC) [3]. CSCs are more resistant to conventional cancer therapies than non-CSC populations [4–6] and more likely to cause cancer recurrences [7]. Current cancer treatments including chemotherapy, γ-irradiation and immunotherapy eliminate cancer cells mostly through activating apoptosis pathways [8–10]. However, recent studies demonstrated that cancer cell lines could survive apoptosis induction and the reversed cancer cells possessed some features of CSCs and became more aggressive [11–13].

Here, we showed that breast cancer cells undergoing reversal of apoptosis acquired increased tumorigenicity both in vitro and in vivo and there was a correlated elevation of the percentage of cells with CSC markers in the reversed cells comparing with the untreated ones. Importantly, this reversal process triggered a transition from non-stem cancer cells (NSCCs) to CSCs, which could be suppressed by pre-treatment with epigenetic modulators. These results suggest that targeting the epigenetic regulation could be a promising strategy for decreasing CSCs and hence tumorigenicity and/or metastasis.

Materials and Methods

Cell Culture and Apoptosis Induction

The culture of MCF-7, T47D and MDA-MB-231 (American Type Culture Collection, Manassas, VA, USA) were done as described in the Supplementary data. 2.5 μM staurosporine (STS) (Sigma-Aldrich, St. Louis, MO, USA) and 5 μM paclitaxel were added to MCF-7 cultures to induce apoptosis. After 24 hours, cultures were harvested and used for subsequent experiments.

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(Sigma-Aldrich) were applied to MCF-7 cells for 6 hours and to T47D cells for 10 hours, respectively, to induce apoptosis when cell density reached 70% confluency. Solvent controls of STS and paclitaxel were 0.25% v/v and 0.05% v/v dimethyl sulfoxide (DMSO) (Sigma-Aldrich) respectively.

**CellEvent™ Staining and Fluorescence-Activated Cell Sorting (FACS)**

Apoptotic MCF-7 and T47D cells were stained with 3 μM CellEvent™ (Invitrogen, Carlsbad, CA, USA) at a cell concentration of 10^5 cells/mL in the dark for 30 minutes at 37°C. Cells were then filtered through a 40-μm nylon mesh (BD Biosciences, Bedford, MA, USA) before running on the Bio-Rad S3 sorter (Bio-Rad, Hercules, CA, USA) (refer to Supplementary data for details on cell sorting and the subsequent procedure for culturing of cells undergoing apoptosis reversal).

**Propidium Iodide (PI) Staining for Live/Dead Cell Detection**

Cells were collected, washed twice with phosphate buffered saline (PBS) and stained with 2 μg/mL PI (Thermo Fisher Scientific, Waltham, MA, USA) just before running on the BD FACSVerse flow cytometer (BD Biosciences).

**In Vivo Tumorigenicity Assay**

The following experimental procedures were approved by the Animal Ethics Committee, the Chinese University of Hong Kong and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011). Single MCF-7 cells were resuspended in 50 μL serum-free RPMI 1640 and mixed with Growth Factor Reduced Matrigel Matrix (BD Biosciences) at 1:1 ratio (v:v) and injected subcutaneously into the mammary fat pad of anesthetized 6-week old female nude mice (refer to Supplementary data for details on animal surgery). The control group received only solvent-free RPMI 1640 medium and Matrigel mixture (1:1). Tumor volumes were determined every 3 days. Tumor volume (mm^3) = 0.52 × width (mm)^2 × length (mm).

**Mammosphere Culture**

Single cell suspension was plated in an ultra-low attachment 6-well plate (Corning, NY, USA) at a density of 4 × 10^4 cells/mL in the primary or 10^4 cells/mL in the secondary and tertiary mammosphere formation experiments. The culture condition for mammosphere formation was described in the Supplementary data. Mammosphere formation efficiency (MFE) (%) = (number of mammospheres per well / number of cells seeded per well) × 100. Only mammospheres with a diameter ≥60 μm were counted at the end of each passage (day 7).

**Immunohistochemical Analysis**

Tissues and xenograft tumors were fixed and sectioned for analysis (refer to Supplementary data for details on slice preparation). For detection of CSC markers, fluorochrome-conjugated antibodies against human CD44 (PERCP-CY5.5, BD Biosciences) and CD24 (PE, BD Biosciences) were added at 1:40 and 1:10 dilution, respectively. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 1:5000 dilution. For EMT markers, sections were incubated with mouse anti-E-Cadherin (BD Biosciences) and rabbit anti-N-Cadherin antibodies (Abcam, Cambridge, UK) at 4°C overnight, followed by goat anti-mouse Alexa Fluor® 488- and goat anti-rabbit Alexa Fluor® 594-labeled secondary antibodies (Thermo Fisher Scientific).

**Cell Staining and Fluorescence-Activated Cell Sorting**

For preparing cells for flow cytometry, cells were blocked with 1:50 diluted Fc block (Miltenyi Biotec, Auburn, CA, USA) for 20 minutes. For preparing cells for confocal microscopy, cells were fixed, permeabilized and blocked with 1% BSA and 5% normal goat serum (Invitrogen) for 1 hour. In both cases, antibodies against human CD44 (PERCP-CY5.5, BD Biosciences) and CD24 (PE, BD Biosciences) were added at 1:40 and 1:10 dilution, respectively, and incubated at 4°C in dark. For flow cytometry, cells were analyzed on FACSVerse equipped with FACSuite software (BD Biosciences). For confocal analysis, images were acquired using the Olympus FluoView FV1000 confocal laser scanning microscope with a 60X objective and analyzed using the FV1000 software (Olympus, Tokyo, Japan) (refer to Supplementary data for details).

**FACS of NSCCs**

CSCs in breast cancer were defined as CD44^–/CD24^–, and NSCCs were defined as CD44^+ /CD24^+ cells. Cells were immunostained under identical conditions as described above and CSCs and NSCCs were collected using Bio-Rad S3 sorter. Sorted NSCCs were cultured in the collection medium for 7 days before STS (or solvent control) treatment as described in the ‘basic procedure for apoptosis induction and reversal of apoptosis’ in Supplementary data.

**Isolation of Limited Numbers of CSCs by Magnetic-Activated Cell Sorting (MACS) Using Dynabeads**

NSCCs in MCF-7 were obtained through FACS and reversal of apoptosis was done as described above. Reversed NSCC (NSR) cells were harvested at day 7. Then, MACS using Dynabeads^® (Invitrogen) was first applied for CD24 negative selection to collect CD24^– cells, followed by a CD44 positive selection to eventually obtain the CD44^+ /CD24^– (CSC) population. Cells remaining after this sorting procedure were named NCSCs.

**Reverse Transcription Followed by Real-time PCR (RT-qPCR)**

mRNA was extracted and reverse-transcribed to cDNA using SuperScript^® III 1st Strand Synthesis System (Invitrogen). Real-time PCR was performed as described in the Supplementary data.

**Bisulfite Conversion and Methylation-Specific PCR (MSP)**

Genomic DNA was extracted and bisulfite conversion of DNA was performed using the EpiTect Bisulfite Kit (Qiagen, Duesseldorf, Germany). For methylation analyses of the promoter regions of CD44 and CD24, two rounds of end-point PCR were performed (refer to Supplementary data for details).

**Epigenetic Modulator Experiments**

FACS-sorted NSCCs were treated with 5 μM 5-Azacytidine (5-Aza) (Sigma-Aldrich) or 0.5 mM S-(5′-Adenosyl)-L-methionine chloride dihydrochloride (SAM) (Sigma-Aldrich). Solvent was used as control. The media containing the drugs were replaced every 24 hours and the cells were treated for a total of 72 hours before procedure for apoptosis reversal (refer to Supplementary data for details).

**Live Cell Imaging**

Cells were stained sequentially with CellEvent™, Mitotracker Red CMXRos (Invitrogen) and Hoechst 33342 (Invitrogen) (refer to Supplementary data for details). Cell morphology was observed using...
the Olympus FluoView FV1000 confocal laser scanning microscope with a 60X objective and further analyzed using the FV1000 software.

Western Blotting

Total proteins were extracted from cells and analyzed by Western blotting. Immunodetection were performed using anti-PARP at 1:1000 dilution(Cell Signaling, Danvers, MA, USA) and anti-β-tubulin (1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (refer to Supplementary data for details).

Results

**Apoptosis Reversal Increased Tumorigenicity of Breast Cancer Cells In Vitro and In Vivo**

First, to show how apoptosis reversal affects tumorigenesis, we used the MCF-7 cell line and apoptotic inducer, STS [14], to build an apoptosis reversal model (Supplementary Figure 1A). Typical apoptotic morphological changes, including cell shrinkage, membrane blebbing, mitochondrial fragmentation and nuclear condensation, were observed 6 hours after STS treatment. Twenty-four hours after replacing STS with fresh medium, cells appeared to recover (Supplementary Figure 1B). Western blotting revealed that poly(ADP-ribose) polymerase (PARP) was cleaved after STS treatment, confirming the activation of caspase, a biochemical marker of apoptosis (Supplementary Figure 1C). To show that apoptosis reversal induced transformation of NSCCs to CSCs is not restricted to a particular cell line, another breast cancer cell line T47D was treated with the chemotherapeutic drug paclitaxel. Chemotherapy so far has been the mainstay in breast cancer treatment and paclitaxel is emerging at 2 weeks after injection while visible tumors did not appear until after 4 weeks for the solvent-control and untreated groups (Figure 1D). The mean final tumor size (Figure 1C and Supplementary Figure 5) and mean final tumor weight (Figure 1D) were significantly greater in the reversed group than in the control groups. Among the 10,000-injected-cell groups, tumors were first detected at about 4 weeks after injection of the reversed cells while no tumor was found in the control groups throughout the experiment (Figure 1C and Supplementary Figure 5). Tumors derived from all 3 cell types possessed similar morphologies (Figure 1E).

Autopsy revealed that in the 50,000-reversed-cell group, lymph node metastasis was detected in 4 out of 8 mice (Figure 2A). Immunostaining of epithelial-mesenchymal transition (EMT) markers revealed that primary tumors formed from both solvent control and untreated cells expressed the epithelial marker E-cadherin [27] while those from reversed cells expressed the mesenchymal marker N-cadherin (Figure 2B). Interestingly, the metastasis detected in the lymph node re-expressed E-cadherin (Figure 2C). The same epithelial/mesenchymal expression patterns were also found in cells undergoing apoptosis reversal in vitro (Figure 2D). Transcript levels of N-cadherin and other mesenchymal markers including fibronectin and vimentin [28] in cultured reversed cells were all elevated when compared to those in solvent control cells (Figure 2E).

**CD44⁺/CD24⁻ CSC-Like Cells Were Transferred from NSCCs During Apoptosis Reversal**

Next, we used flow cytometry to evaluate the percentage of tumor-initiating or CSC-like cells before and after apoptosis reversal, based on the expression of two cell-surface markers, CD44 and CD24 [8]. Cells with the CD44⁺/CD24⁻ configuration were designated as NSCCs, while those with CD44⁻/CD24⁺ were designated as CSC-like cells [29]. We found that, as in previous studies [30], <2% (~0.1% in our case) of untreated MCF-7 cells were CD44⁻/CD24⁺ (Figure 3A), whereas the percentage of reversed cells that were CD44⁺/CD24⁻ was significantly elevated (Figure 3A and B). Higher CD44 and lower CD24 expressions were separately observed in the
reversed MCF-7 population than in the control groups (Figure 3C and D). This was further confirmed by immunostaining of these two markers in cultured MCF-7 cells and the corresponding tumor sections (Supplementary Figure 6B). Although the increased percentage of CSC-like cells in the reversed population could be attributed to the enrichment of pre-existing CSCs present in the cell population before treatment [31], an alternative explanation could be that some of the original NSCCs were transformed into new CSCs by apoptosis reversal. To test the latter hypothesis, we induced apoptosis in NSCCs from untreated cells. CellEvent™ staining and FACS were used to isolate caspase-activated cells. Solvent-treated NSCC controls were sorted in parallel (Supplementary Figure 7). We were able to show that new CSCs could originate from NSCCs after apoptosis reversal (NSR), since no CSC was observed among NSCCs treated with solvent only (NSSC) or untreated NSCCs (NSUT) (Figure 3E and F). Moreover, these NSRs formed stable spheroids in the in-vitro mammosphere assay while NSSCs and NSUTs failed to yield any mammosphere (Figure 3G and H).

The discovery on NSCC to CSC transition during apoptosis reversal prompted us to investigate whether this phenomenon was repeatable in different apoptotic induction condition and in other cancer cell line. Unlike MCF-7 cells, there was no CSCs detectable in untreated T47D population (Figure 3I). Results from T47D-paclitaxel model showed that after reversal of apoptosis, CD44+/CD24- cells appeared in the population, which could not be detected in the solvent control group (Figure 3J). Consistently, the increase of CD44 and decrease of CD24 expressions were significant in the reversed T47D cells when compared to untreated and solvent control groups (Figure 3K and L). These results suggested that reversal of apoptosis assists the transformation of NSCCs to CSCs and this is a general phenomenon in breast cancer cells.

The Expression of CSC Markers CD44 and CD24 Was Correlated with DNA Methylation Status at Their Promoters During Apoptosis Reversal

During cancer development, cells acquire capabilities for malignant growth through both genetic and epigenetic mechanisms [32]. However,
genetic advantages take longer to accumulate under selective pressure, whereas epigenetic changes are faster-acting. To test if the transformation from NSCCs to CSCs by apoptosis reversal involves methylation/demethylation of specific genes, we first investigated the transcript levels of $\text{CD44}$ and $\text{CD24}$ in the reversed versus control MCF-7 cells. Consistent with flow cytometry results, the mRNA level of $\text{CD44}$ was elevated while that of $\text{CD24}$ was decreased in the reversed cells when compared to control (Figure 4A). The expression data were confirmed by the methylation statuses of $\text{CD44}$ and $\text{CD24}$ in these two groups using methylation-specific PCR (MSP). In control cells, $\text{CD44}$ were hypermethylated while $\text{CD24}$ were hypomethylated (Figure 4B). In contrast, cells having undergone apoptosis reversal showed hypomethylation of $\text{CD44}$ and hypermethylation of $\text{CD24}$ (Figure 4B). We then induced apoptosis reversal on FACS-sorted NSCCs. Reversed cells from NSCCs (CellEvent™-positive) (NSRs) were further sorted into CSC-like cells (CSC) and non-CSC cells (NCSCs) (Supplementary Figure 8), and the mRNA levels of $\text{CD44}$ and $\text{CD24}$ in each group were consistent with their corresponding protein expressions (Figure 4C). Accordingly, hypomethylation of $\text{CD44}$ and hypermethylation of $\text{CD24}$ were observed in CSCs when compared to NCSCs (Figure 4D). These results clearly indicated that methylation statuses of $\text{CD44}$ and $\text{CD24}$ determined the expression levels of these genes, and that apoptosis reversal drove the epigenetic modifications of $\text{CD44}$ and $\text{CD24}$ and gave rise to cells with CSC properties. The contribution by epigenetic regulations during the reversal of apoptosis and CSC generation was further confirmed by pretreating NSCCs with the methylation inhibitor, 5-azacytidine (5-Aza) [33] (ANSRs), or the methyl group donor, S-(5'-adenosyl)-L-methionine chloride dihydrochloride (SAM) [34] (SNSRs), before the induction of apoptosis (Supplementary Figure 9). Both drugs suppressed the formation of CSC-like cells after apoptosis reversal, as indicated by the lower percentage of $\text{CD44}^+$/$\text{CD24}^-$ cells in ANSRs and SNSRs (Figure 4E and F). Meanwhile, $\text{CD44}$ expression was suppressed in SNSRs and $\text{CD24}$ expression was up-regulated in ANSRs (Figure 4G and H). These results suggested that the NSCC-to-CSC transition during apoptosis reversal is at least partially attributable to epigenetic regulations.
Discussion

While previous studies showed that apoptosis reversal increased drug resistance, cell migration and invasion in vitro [13], our study is the first to demonstrate that cancer cells recovering from apoptosis reversal have higher tumorigenicity and metastatic potential in vivo, and that apoptosis reversal actually induced the formation of new CSCs from NSCCs in...
Figure 4. Dynamic regulation of CD44 and CD24 methylation statuses during apoptosis reversal. (A and B) Relative expressions (A) and methylation statuses by methylation-specific PCR (B) of CD44 and CD24 in reversed versus solvent control cells. (C and D) Relative expressions (C) and methylation statuses (D) of CD44 and CD24 in CSCs and NCSCs sorted from NSRs. (B and D) MCtrl and UCtrl: methylated and unmethylated bisulfite-converted control human DNA; CD44 M and CD44 U: CD44 methylated- and unmethylated-specific primers; CD24 M and CD24 U: CD24 methylated- and unmethylated-specific primers. (E) FACS analyses of the expressions of CD44 and CD24 in NSR, ANSR (5-Aza-pre-treated NSR) and SNSR (SAM-pre-treated NSR). (F-H) Effects of 5-Aza or SAM pre-treatment on the percentage of CD44+/CD24- cells (F), CD44+ cells (G) and CD24+ cells (H) in NSR. For A, C, F, G and H, Data represent mean±SEM (n = 3). *P < .05; **P < .01; ***P < .001.

Figure 3. Reversed breast cancer cells have higher percentage of cells with CSC markers (CD44+/CD24-). (A) Distributions of MCF-7 cells expressing CD44 and CD24 in reversed, solvent control or untreated group. More cells had CSC markers (CD44+/CD24-) (Q3) after apoptosis reversal. (B-D) Percentage of MCF-7 cells expressing CD44+/CD24- (B), CD44+ (C) and CD24+ (D). Data represent mean±SEM. (n = 6). **P < .01; ***P < .001. (E) Expressions of CD44 and CD24 in reversed NSCC (NSR), solvent control NSCC (NSSC) and untreated NSCC (NSUT) cells by FACS analyses. (F) Percentages of CD44+/CD24- cells in all three groups. Data represent mean±SEM (n = 3). ***P < .001. n.d.: not detected. (G) Inverted microscopic images of mammospheres formed by NSRs, NSSCs and NSUTs. Scale bar: 500 μm. (H) Mammosphere formation efficiencies of the three passages of NSRs, NSSCs and NSUTs. Data represent mean±SEM (n = 3). ***P < .001. n.d.: not detected. (I) Distributions of T47D cells expressing CD44 and CD24 in reversed, solvent control or untreated group. Some cells possessed CSC markers (CD44+/CD24-) (Q3) after apoptosis reversal. (J-L) Percentage of T47D cells expressing CD44+/CD24- (J), CD44+ (K) and CD24+ (L). Data represent mean±SEM. (n = 3). *P < .05; **P < .01.
breast cancer cells. It has long been thought that cancer cell development is uni-directional: with CSCs at the apex, giving rise to progenitors and other differentiated cells [3]. Nevertheless, more recent studies have proposed the concept of CSC plasticity in which cells can transit between the non-CSC and the CSC states in both directions [35–37]. Our current study has clearly shown that the percentage of CSC-like cells (CD44+/CD24−) increased in the cell population which had undergone apoptosis reversal, and that these CSCs originated from NSCCs.

Besides, here we show that by inhibiting DNA methylation or demethylation before apoptosis induction, the CSC formation rate after apoptosis reversal is also reduced. Other epigenetic mechanisms [38] may also be involved. Therefore, it is critical to consider the involvement of chromatin remodeling during apoptosis reversal.

This study has clearly shown that through apoptosis reversal, NSCCs are transformed into CSCs, at least partly by the epigenetic modifications of CSC marker genes, and the resulting apoptosis-reversed cells undergo EMT [39] and possess enhanced tumorigenicity and metastatic properties in vivo. Thus when applying the commonly used tactic of eliminating cancer cells by triggering apoptosis [8–10], one needs to be mindful of at least ensuring cancer cells undergoing treatment have passed the point-of-no-return before terminating the treatment, in order to minimize the chances of relapse and/or metastasis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.01.005.

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Conflict of Interest Statement
We declared no conflict of interest.

References
[1] McGuire S (2016). World Cancer Report 2014. Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO Press, 2015, Adv Nutr 7, 418–419.
[2] Nowell PC (1976). The clonal evolution of tumor cell populations. Science 194, 23–28.
[3] Bonner D and Dick JE (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3, 730–737.
[4] O’Brien CA, Kreos A, and Dick JE (2009). Cancer Stem Cells in Solid Tumors: An Overview. Semin Radiat Oncol 19, 71–77.
[5] Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, Laino I, De Francesco F, and Papaccio G (2013). Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J 27, 13–24.
[6] Yu Y, Ramena G, and Ebble RC (2012). The role of cancer stem cells in relapse of solid tumors. Front Biosci 4, 1528–1541.
[7] Visvader JE and Lindeman GJ (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8, 755–768.
[8] Fulda S and Debatin KM (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25, 4798–4811.
[9] Wong RS (2011). Apoptosis in cancer: from pathogenesis to targeting therapy. J Exp Clin Cancer Res 30, 87.
[10] Hassan M, Watari H, AbuAlmaaty A, Ohba Y, and Sakuragi N (2014). Apoptosis and molecular targeting therapy in cancer. Biomed Res Int 2014, 150845.
[11] Tang HL, Tang HM, Mak KH, Hu S, Wang SS, Wong KM, Wong CS, Wu HY, Law HT, and Liu K, et al (2012). Cell survival, DNA damage, and oncogenic transformation after a transient and reversible apoptotic response. Mol Biol Cell 23, 2240–2252.
[12] Tang HL, Yuen KL, Tang HM, and Fung MC (2009). Reversibility of apoptosis in cancer cells. Br J Cancer 100, 118–122.
[13] Wang SS, Xie X, Wong CST, Choi PY, and Fung MC (2014). HepG2 cells recovered from apoptosis show altered drug responses and invasiveness. HBPD Int 13, 293–306.
[14] Grant S (2009). Therapeutic Protein Kinase Inhibitors. Cell Mol Life Sci 66, 1163–1177.
[15] Holmes FA, Walters RS, Theriault RL, Forman AD, Newton LK, Raber MN, Buzdar AU, Frye DK, and Hortobagyi GN (1991). Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. J Natl Cancer Inst 83, 1797–1805.
[16] Reichman BS, Seidman AD, Crown JPA, Heelan R, Hakes TB, Lebowohl DE, Gilewski TA, Surbone A, Currie V, and Hudis CA, et al (1993). Paclitaxel and docetaxel in advanced breast cancer: a pivotal study. J Clin Oncol 11, 1943–1951.
[17] Schiff PB, Fanti J, and Horwitz SB (1979). Promotion of microtubule assembly in vitro by taxol. Nature 277, 665–667.
[18] Schiff PB and Horwitz SB (1980). Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci U S A 77, 1561–1565.
[19] Haldar S, Jena N, and Croce CM (1995). Inactivation of Bel-2 by Phosphorylation. Proc Natl Acad Sci U S A 92, 4507–4511.
[20] Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, and Daidone MG (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 65, 5506–5511.
[21] Shaw FL, Harrison H, Spence K, Ablert MP, Simoes BM, Farnie G, and Clarke RB (2012). A Detailed Mammosphere Assay Protocol for the Quantification of Breast Stem Cell Activity. J Mammary Gland Biol Neoplasia 17, 111–117.
[22] Donutz G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, and Wicha MS (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Gene Dev 17, 1253–1270.
[23] Ponti D, Zaffaroni N, Capelli C, and Daidone MG (2006). Breast cancer stem cells: An overview. Eur J Cancer 42, 1219–1224.
[24] Veenmeulen L, Mele FDSE, van der Heijden M, Cameron K, de Jong HJ, Borovski T, Tuyenman JB, Todaro M, Merz C, and Rodermond H, et al (2010). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 12, 468–476.
[25] Plaks V, Hong NW, and Werb Z (2015). The Cancer Stem Cell Niche: How Essential Is the Niche in Regulating Stemness of Tumor Cells? Cell Stem Cell 16, 225–238.
[26] O’Brien CA, Pollett A, Gallinger S, and Dick JE (2007). A human colon cancer cell capable of initiating tumour growth in immune-deficient mice. Nature 445, 106–110.
[27] Lu L, Zhou D, Jiang X, Song K, Li K, and Ding W (2012). Loss of E-cadherin in multidrug resistant breast cancer cell line MCF-7/Adr: possible implication in the enhanced invasive ability. Eur Rev Med Pharmacol Sci 16, 1271–1279.
[28] Nieman MT, Prudoff RS, Johnson KR, and Wheelock MJ (1999). N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol 147, 631–643.
[29] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, and Clarke MF (2003). Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100, 3983–3988.
[30] Yin H and Glass J (2011). The Phenotypic Radiation Resistance of CD44(+)/CD24(−) low Breast Cancer Cells Is Mediated through the Enhanced Activation of ATM Signaling. PLoS One 6, e24080.
Borst P (2012). Cancer drug pan-resistance: pumps, cancer stem cells, quiescence, epithelial to mesenchymal transition, blocked cell death pathways, persisters or what? *Open Biol* 2, 120066.

Sadikovic B, Al-Romaih K, Squire JA, and Zielenska M (2008). Cause and Consequences of Genetic and Epigenetic Alterations in Human Cancer. *Curr Genomics* 9, 394–408.

Christman JK (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21, 5483–5495.

Laduron P (1972). N-methylation of dopamine to epinine in brain tissue using N-methyltetrahydrofolic acid as the methyl donor. *Nat New Biol* 238, 212–213.

Chaffer CL, Brueckmann I, Scheel C, Kaestli AJ, Wiggins PA, Rodrigues LO, Brooks M, Reinhardt F, Su Y, and Polyak K, et al (2011). Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proc Natl Acad Sci U S A* 108, 7950–7955.

Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F, D’Alessio AC, Young RA, and Weinberg RA (2013). Poised Chromatin at the ZEB1 Promoter Enables Breast Cancer Cell Plasticity and Enhances Tumorigenicity. *Cell* 154, 61–74.

Gupta PB, Fillmore CM, Jiang GZ, Shapira SD, Tao K, Kuperwasser C, and Lander ES (2011). Stochastic State Transitions Give Rise to Phenotypic Equilibrium in Populations of Cancer Cells. *Cell* 146, 633–644.

Cantone I and Fisher AG (2013). Epigenetic programming and reprogramming during development. *Nat Struct Mol Biol* 20, 282–289.

Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou Y, Brooks M, Reinhard F, Zhang CC, and Shippitsin M, et al (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133, 704–715.