

Enrichment and Selective Targeting of Cancer Stem Cells in Colorectal Cancer Cell Lines

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Abstract

Cancer stem cells (CSCs) are the subpopulation of cells within a tumor proposed to be responsible for tumor initiation, relapses, and resistance to chemotherapeutic drugs. Here we optimized sphere culture conditions to isolate and enrich CSCs from colorectal cancer cell line IMCE-Ras. Spheroid cells that developed in culture expressed high levels of putative stem cell markers, and showed stronger anchorage-independent growth abilities and resistance to conventional chemotherapeutic drugs compared with the initial monolayer adherent cells. Xenograft transplantation assays further demonstrated that IMCE-Ras spheroid cells are highly enriched in CSCs. To develop CSC-targeted therapy, we found that the relative percentage of CSC in IMCE-Ras cells was significantly decreased after a short duration exposure to DNA Methylation inhibitor 5-aza-2'-deoxycytidine (5-Aza-dC), indicating that DNA Methylation may be critical for self-renewal and maintenance of CSCs. Indeed, double knockout of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3b (DNMT3b) in colon cancer cell line HCT116 resulted in loss of >95% DNA Methylation and complete loss of tumorigenicity both *in vitro* and *in vivo*. These data suggest that DNA Methylation is critical for maintenance of the colon CSC population, and a combination of classical chemotherapeutic drugs and DNA Methylation inhibitors may be an effective treatment of colon cancer.

Keywords: Colon cancer stem cell; Tumorsphere; Chemotherapy; Methylation; 5-aza-2'-deoxycytidine

Introduction

Cancer stem cells (CSCs) are defined as “a small subset of cancer cells” within a cancer, which can self-renew and replenish the heterogeneous lineage of cancer cells that comprise the tumor [1]. Identification and isolation of CSCs from primary tumor tissues or cell lines have so far depended on fluorescence-activated cell sorting (FACS) using a variety of different cell surface markers followed by tests of propagation in immunodeficient mice [2-8]. However, CSC markers are expressed in a complex pattern; neither single marker expression nor simple combinations can be universally used for isolation and enrichment of CSC from different sources of tumor cells [9].

Colon CSCs were first identified and enriched by FACS sorting of human tumors using putative stem cell marker CD133 in 2007 [3,10]. The percentages of CD133⁺ cells in the tumorigenic populations ranged from 3.2-24.5%. Using established cell lines, Ieta et al. [11] found that CD133⁺ cells in the HT29 colon cancer cell line were more tumorigenic than CD133⁻ cells both *in vitro* and *in vivo*, suggesting that CD133 may also mark CSCs in colon cell lines. However, Shmelkov et al. [12] argued that expression patterns of CD133 were ubiquitous in differentiated epithelial cells and were not restricted to the CSC fraction in metastatic colon cancers. Further, CD133⁺ HCT116 colon cancer cells were found not to be radio resistant [13]. These data challenged the view that CD133 was an effective marker of colon CSCs. In another study, Dalerba et al. [14] employed CD44 and epithelial surface antigen (ESA) as stem-cell-specific markers to isolate colorectal CSCs. Recently, Yeung et al. [15] demonstrated that colorectal cell lines contain CSC populations that can be enriched by the use of an *in vitro* Matrigel-based differentiation assay together with selection for expression of the CD44 and CD24 cell surface markers. Despite these lines of evidence demonstrating that CSCs exist in different sources of colon tumor cells, most of these CSC assays are cumbersome and expensive, not ideal for

screening and testing of drugs for development of CSC-based therapy.

We adopted a sphere culture to isolate and enrich colon CSCs from colon cell lines. Sphere cultures have been used to isolate CSCs from different types of cancer cell lines including breast [16,17], renal [18], liver [19], prostate [20], pancreatic [21], and brain [22] cancers along with melanoma [23]. Tumorsphere culture in serum-free medium was also used to isolate and propagate colon CSCs from primary tumors, but has not been optimized to enrich CSCs from colon cancer cell lines [3,24]. For this study, we optimized the suspension culture conditions for culture of colon cancer cell lines. We demonstrate that this modified sphere culture system can be used to isolate and enrich CSCs from colon cancer cell lines. Using the isolated cells, we provide evidence to show that the colon CSCs are resistant to chemotherapeutic drugs such as 5-FU but sensitive to DNA Methylation inhibitor 5-aza deoxycytidine. Our results imply that a combination of conventional chemotherapeutic drugs and DNA Methylation inhibitors may be an effective treatment for colon cancer.

Material and Methods

Cell lines and cell cultures

Human colon cancer HCT-116, DLD1 and HT-29 cells were

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obtained from the American Type Culture Collection (ATCC, Rockville, MD). Double knockout (DKO) cells (HCT116 cells with genetic disruption of DNMT1 and DNMT3b) were kindly provided by Dr. Bert Vogelstein and cultured as previously described [25]. In brief, cells were maintained in Dulbecco's modified Eagle medium (DMEM; 4.5 g/L D-glucose) supplemented with 10% FBS and 1% antibiotic/antimycotic in tissue culture flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Mouse colon cancer cells IMCE-Ras cells were kindly provided by Dr. Robert H. Whitehead at the Vanderbilt University [26]. These cells are only conditionally immortalized and cultured at the permissive temperature of 33°C in Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L D-glucose) containing 5% fetal calf serum, 1 µg/ml insulin, 10 µM α-thioglycerol, 1 µM hydrocortisone and 5 units per ml of mouse gamma interferon, which is used to up regulate the immortalizing gene within the cells. The medium was changed two times a week, and cells were passaged using 0.05% trypsin/EDTA.

In vitro propagation of tumorsphere

Cells were counted and plated in petri dishes at a constant density of 40,000 viable cells per ml. Cells were grown in serum-free DMEM medium (Gibco), which was supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma), 0.4% bovine serum albumin (BSA, Sigma), and 5 µg/ml insulin (Sigma). 1 × F12 (Gibco) and/or N2 (Stemcell Technologies Inc.) supplements were added to the treatment group to investigate their effect on tumorsphere formation. Human colon cancer HCT-116, DLD1 and HT-29 cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 6 days, and collected by gentle centrifugation. Mouse colon cancer cells IMCE-Ras cells were incubated at the permissive temperature of 33°C. The pelleted cells were enzymatically dissociated with Accutase (Innovative Cell Technologies) for 10 minutes at room temperature, and mechanically dispersed by gently pipetting through a 23-gauge sterile needle. Single-cell suspensions were plated at the same density and culture conditions as described above, to generate the second generation of tumorspheres, and so forth. The number of spheres formed in each well was determined after 6 days. Tumorspheres from every passage were dissociated and assayed for tumorsphere-forming efficiency.

MTT assay

Inhibition of cell growth in response to chemotherapeutic drugs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously [27,28]. Briefly, cells at 2.5 × 10⁴ cells per milliliter were seeded into 96-well culture plates with six replicates. After 24 hours of plating, cells were treated with different agents as described in figure legends. At 48-h post treatment, the culture medium was removed, 50 µl of MTT solution (5 mg/ml) added to each well, and cultures incubated for 3-4 hours. The formazan crystals were then dissolved by adding 0.1 ml of dimethyl sulfoxide. The intensity of the color that developed, which reflects the number of live cells, was measured at a wavelength of 570 nm. All values were compared with the corresponding controls.

Quantitative real-time PCR analysis

Total RNA from parental cells and tumorspheres was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions [29]. 1 µg of total RNA was used for the reverse transcription assay to generate cDNA using M-MLV reverse transcriptase (Invitrogen). 1 µl of cDNA was used for a single PCR

reaction to determine the expression of self-renewal markers and stem cell markers. The quantitative RT-PCR was performed using the fluorescent dye SYBR Green Master Mix following standard protocols on an ABI PRISM 7300 sequence detection system (Applied Biosystems, CA) [29]. The data were first analyzed using Sequence Detector Software SDS 2.0 (Applied Biosystems). Results were calculated and normalized relative to the GAPDH control using the Microsoft Excel program. The relative expression values were calculated relative to GAPDH by using the 2^{-ΔCT} method [29]. The data shown here represent the average of three independent experiments.

Soft-agar assay

Cells (5 × 10³ cells per 35-mm well) were resuspended in complete medium containing 0.35% agarose. Cells were grown on tissue culture dishes containing a 2-mL layer of solidified 0.7% agar in a complete medium. After 14 days, number of colonies was quantified from two randomly taken micrographs per well (original magnification, ×20). For visualization, foci were methanol-fixed and stained with 0.005% crystal violet [28].

In vivo tumorigenesis assays

Cells were resuspended in 50 µl DMEM/F12 medium and mixed with 50 µl Matrigel (Becton Dickinson) at a 1:1 ratio and held on ice. The entire 100 µl sample was injected into each flank of 6-8 weeks old NOD/SCID mice anesthetized with isoflurane according to the animal protocol approved by the USC committee for research in vertebrate animals. Tumor sizes in two dimensions were measured twice weekly, and volumes were estimated using the formula (L × W²) × 0.5, wherein L is length and W is width, as previously described [28,30].

Statistical analysis

The results were expressed as mean ± SD. The data were treated by Student's t test to determine statistical significance. We used nonparametric tests (Mann-Whitney test), if appropriate, to compare differences. P < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 12.0 software [29].

Results

Spheroid culture for enrichment of CSCs from colon cancer cell lines

In order to develop a method to isolate and propagate CSCs from colon cancer cell lines *in vitro*, we optimized the tumorsphere culture conditions. We cultured colon cancer cell line HCT116 in serum-free DMEM medium containing bFGF and EGF, and further supplemented with N2 or/and F12 nutrients. Although HCT116 cells could form tumorspheres in culture media supplemented with either N2 or F12, tumorsphere forming ability was gradually lost during the serial passages of tumorspheres, indicating that sphere-forming cells can survive for a few passages under these conditions but fail to undergo self-renewal proliferation (Figure 1A). In the presence of both N2 and F12, the tumorsphere forming efficiency continued to increase with each passage, suggesting self-renewal proliferation (Figure 1B). To investigate whether the same medium could be used to grow tumorspheres from other colon cancer cell lines, we cultured two more human colon cancer cell lines, DLD1 and HT29, and one mouse colon cancer cell line, IMCE-Ras [26]. All of these cell lines could form tumorspheres and maintain their tumorsphere-forming ability over long-term culture in the presence of both N2 and F12 (Figure 1C).

To test whether these tumorspheres are enriched for CSCs,

we examined the expression profiles of putative stem cell markers including Lgr5 [31,32], CD133 [3], Aldh2 [33] and Sca1[34,35], and self-renewal genes including Ascl2 [36,37], Bmi [38], Nanog [39] and Oct3/4 [39] in tumorspheres derived from colon cancer cell line IMCE-Ras (Figure 2A). Quantitative real-time PCR results revealed that the spheroid cells expressed 47-fold higher levels of putative stem cell maker Sca1. The expression levels of other putative stem cell markers and self-renewal genes are also significantly higher in tumorspheres compared to monolayers (Figure 2B). These gene expression profiles indicated that the tumorspheres may be enriched for CSCs.

Spheroid IMCE-Ras cells showed increased tumorigenic potential both *in vitro* and *in vivo*

To further address whether tumorspheres are enriched for CSCs, we first performed soft-agar assays. IMCE-Ras derived spheroid cells formed about 2.5-fold more colonies than the monolayer cells (Figure 3A). We then tested whether these spheroid cells also showed enhanced tumorigenicity *in vivo*, by injection of spheroid or monolayer cells into the flank region of adult NOD/SCID mice. Figure 3B illustrates the ability of serially diluted spheroid and monolayer cells to form tumors. Monolayer IMCE-Ras cells were able to form tumors when at least 50K

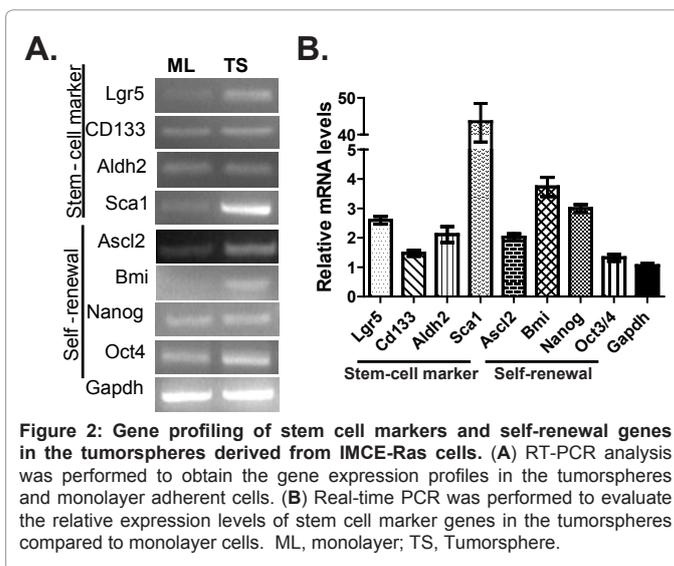


Figure 2: Gene profiling of stem cell markers and self-renewal genes in the tumorspheres derived from IMCE-Ras cells. (A) RT-PCR analysis was performed to obtain the gene expression profiles in the tumorspheres and monolayer adherent cells. (B) Real-time PCR was performed to evaluate the relative expression levels of stem cell marker genes in the tumorspheres compared to monolayer cells. ML, monolayer; TS, Tumorsphere.

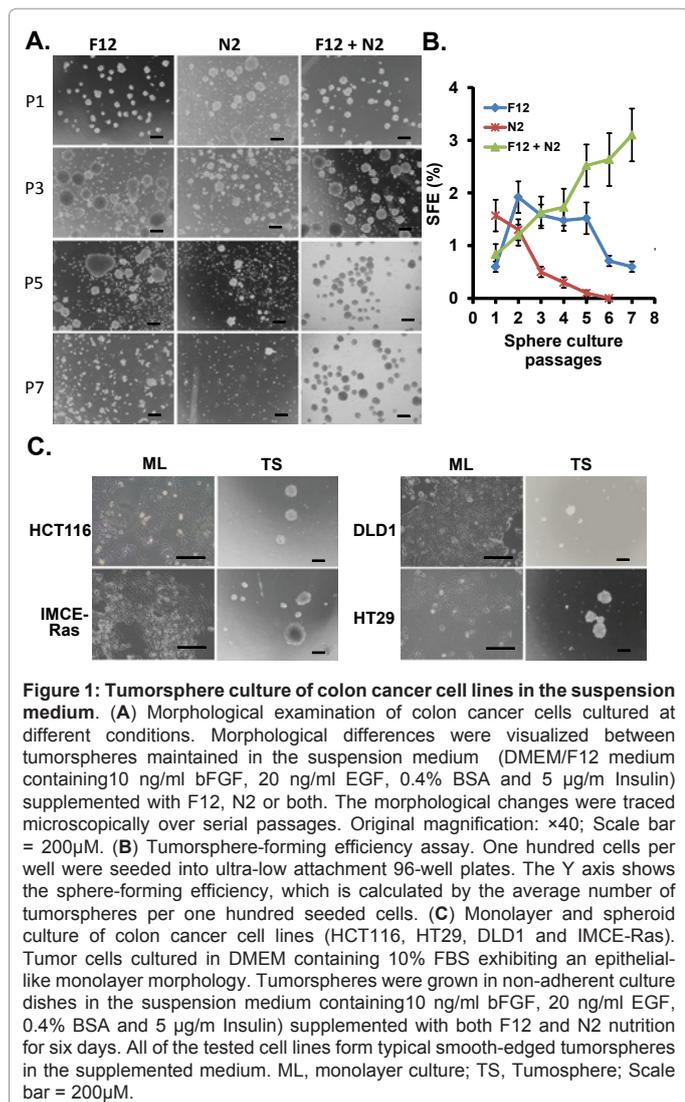


Figure 1: Tumorsphere culture of colon cancer cell lines in the suspension medium. (A) Morphological examination of colon cancer cells cultured at different conditions. Morphological differences were visualized between tumorspheres maintained in the suspension medium (DMEM/F12 medium containing 10 ng/ml bFGF, 20 ng/ml EGF, 0.4% BSA and 5 µg/ml Insulin) supplemented with F12, N2 or both. The morphological changes were traced microscopically over serial passages. Original magnification: ×40; Scale bar = 200µM. (B) Tumorsphere-forming efficiency assay. One hundred cells per well were seeded into ultra-low attachment 96-well plates. The Y axis shows the sphere-forming efficiency, which is calculated by the average number of tumorspheres per one hundred seeded cells. (C) Monolayer and spheroid culture of colon cancer cell lines (HCT116, HT29, DLD1 and IMCE-Ras). Tumor cells cultured in DMEM containing 10% FBS exhibiting an epithelial-like monolayer morphology. Tumorspheres were grown in non-adherent culture dishes in the suspension medium containing 10 ng/ml bFGF, 20 ng/ml EGF, 0.4% BSA and 5 µg/ml Insulin supplemented with both F12 and N2 nutrition for six days. All of the tested cell lines form typical smooth-edged tumorspheres in the supplemented medium. ML, monolayer culture; TS, Tumorsphere; Scale bar = 200µM.

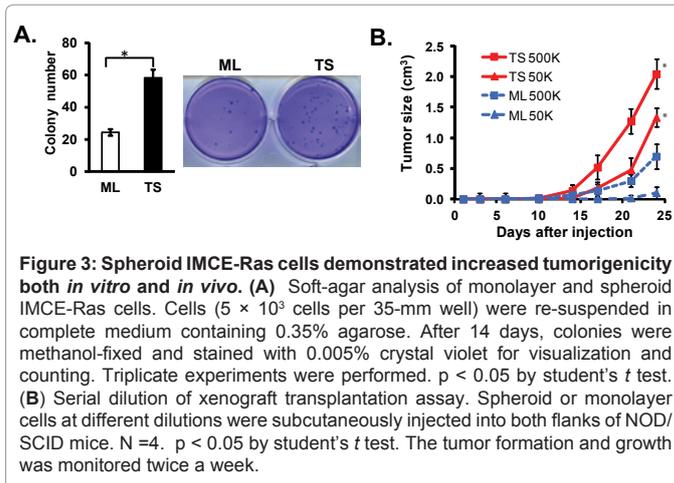


Figure 3: Spheroid IMCE-Ras cells demonstrated increased tumorigenicity both *in vitro* and *in vivo*. (A) Soft-agar analysis of monolayer and spheroid IMCE-Ras cells. Cells (5×10^3 cells per 35-mm well) were re-suspended in complete medium containing 0.35% agarose. After 14 days, colonies were methanol-fixed and stained with 0.005% crystal violet for visualization and counting. Triplicate experiments were performed. $p < 0.05$ by student's t test. (B) Serial dilution of xenograft transplantation assay. Spheroid or monolayer cells at different dilutions were subcutaneously injected into both flanks of NOD/SCID mice. $N = 4$. $p < 0.05$ by student's t test. The tumor formation and growth was monitored twice a week.

cells were injected, but failed to do so at lower cell doses (10K, 5K, 500). In contrast, spheroid cells were able to generate tumors in all animals when 500K, 50K, 10K, or 2K cells were injected (Table 1). As few as 500 spheroid cells derived from the IMCE-Ras cell line were capable of forming tumors: one of two mice injected with 500 spheroid cells developed a tumor. Statistical calculation reveals that tumorspheres from IMCE cells are up to about 450-fold enriched in tumor-initiating capacity in comparison with cells in monolayer culture (Table 1).

Spheroid IMCE-Ras cells show resistance to chemotherapeutic agents and remain sensitive to methylation inhibitor 5-Aza-2'-deoxycytidine

CSCs are characterized by their high resistance to chemotherapeutic agents [1]. To test whether the spheroid cells derived from IMCE-Ras share this property, we treated spheroid IMCE-Ras cells and the cells in monolayer culture with 5-FU and Doxorubicin. The cell viability was measured by MTT assay. As shown in Figure 4A and B, spheroid cells manifested significantly higher resistance to chemotherapeutic drugs 5-FU and Doxorubicin than the control monolayer cells (Figure 4A and B). However, MTT assay did not show any differences in the sensitivities to the DNA Methylation inhibitor 5-aza-2'-deoxycytidine

Injected cells	Tumors/Injections					Frequency of CSC ^a	p-value
	5x10 ⁵	5x10 ⁴	1x10 ⁴	2x10 ³	500		
Tumorsphere	2/2	2/2	2/2	2/2	1/2	1/890	0.0013
Monolayer	2/2	1/2	0/2	0/2	0/2	1/390,850	

^aThe limit-dilution calculation was based on a Poisson probability distribution: $\ln(y) = rx + \ln(a)$, where y is the ratio of non-tumor-generating injections, x is the number of cells injected, and r is the frequency of CSC; a is the y intercept and it was assumed that with no cells injected, no tumors are formed (therefore at 100% non-tumor generation, $a = 1$). The frequency of CSCs (r) was calculated from the slope of line best fit to the data points [56,57].

Table 1: Tumorspheres displayed increased tumorigenicity in xenograft transplantation assay.

(5-Aza-dC). Treatment of cells with 0.5 - 2 μ M of 5-Aza-dC resulted in a weak inhibition of cell growth (Figure 4C).

To further test whether the drug treatments affect the relative content of CSCs, we treated IMCE-Ras cells with each drug, 5-FU or 5-Aza-dC, for two days and then washed out the drug to allow recovery of the surviving cells for an additional 5 days (Figure 5A). The relative CSC population was assessed by sphere formation and soft-agar assays. At 2 days post-treatment, we observed that more cells were killed by exposure to 5-FU than 5-Aza-dC. After the drug washout, surviving cells can resume proliferation quickly in both drug-treated plates. Interestingly, exposure of cells to 5-Aza-dC treatment resulted in > 90% reduction in tumorsphere-forming ability compared to mock-treated cells (Figure 5B), while cells surviving 5-FU treatment displayed slightly increased tumorsphere formation efficiency (Figure 5B). The colony formation assay shows a similar pattern (Figure 5C). Taken together, our data suggest that Methylation inhibitor 5-Aza-dC can effectively eliminate CSCs, although they are not as effective at reducing the total population of cancer cells. Conventional chemotherapeutic drugs like 5-FU preferentially kill non-stem cancer cells, leading to enrichment of CSCs after treatment.

DNA methylation is critical for maintenance of cancer stem cell properties

5-Aza-dC is a DNA methyltransferase (DNMT) inhibitor that blocks DNA Methylation [40]. To further demonstrate that DNA Methylation is critical for maintenance of colon CSCs, we studied the CSCs in a DNA methyltransferase 1 and 3b double knockout cell line (DKO) and the wild-type HCT116 colon cancer cell line. Again, the relative CSC population was assessed by ability to form tumorspheres in serum-free medium and colonies in soft-agar plates. In parallel with loss of more than 95% of genome-wide DNA Methylation [25], DKO cells almost completely lost their tumorsphere-forming and colony-forming abilities (Figure 6B and C). Furthermore, animal injection experiments confirmed that wild type HCT116 cells were able to generate tumors in all animals when 10K, 1K and 500 cells were injected, whereas one million of the DKO cells failed to generate tumors in the injected animals (Figure 6D). These data suggest that a CSC population may not exist in DKO cells. The loss of CSCs cannot be simply explained by the slightly impaired proliferation ability of these cells (Figure 6A). Overall, these data suggest that DNA Methylation is critical for self-renewal of cancer stem cells. Inhibition of DNA Methylation may lead to exhaustion of the CSC population.

Discussion

Previous studies have demonstrated that intestinal cancer may originate from transformed crypt stem cells [5,31] and be maintained by a rare population of CD133+ tumor initiating cells within the tumor mass [3,10,14], suggesting the existence of a cellular hierarchy in colon cancers. However, the validity of surface protein CD133 as a CSC marker was questioned [12,13,41]. Besides CD133, CD44, CD24, CD166 and

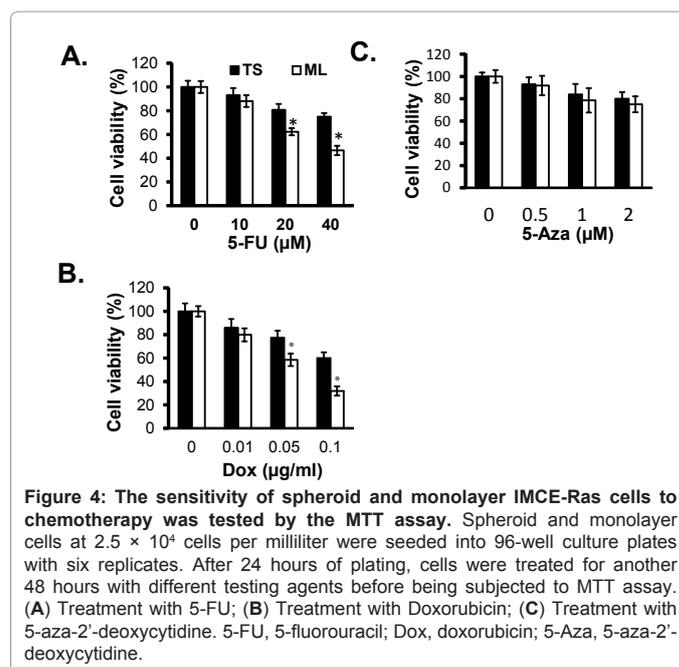


Figure 4: The sensitivity of spheroid and monolayer IMCE-Ras cells to chemotherapy was tested by the MTT assay. Spheroid and monolayer cells at 2.5×10^4 cells per milliliter were seeded into 96-well culture plates with six replicates. After 24 hours of plating, cells were treated for another 48 hours with different testing agents before being subjected to MTT assay. (A) Treatment with 5-FU; (B) Treatment with Doxorubicin; (C) Treatment with 5-aza-2'-deoxycytidine. 5-FU, 5-fluorouracil; Dox, doxorubicin; 5-Aza, 5-aza-2'-deoxycytidine.

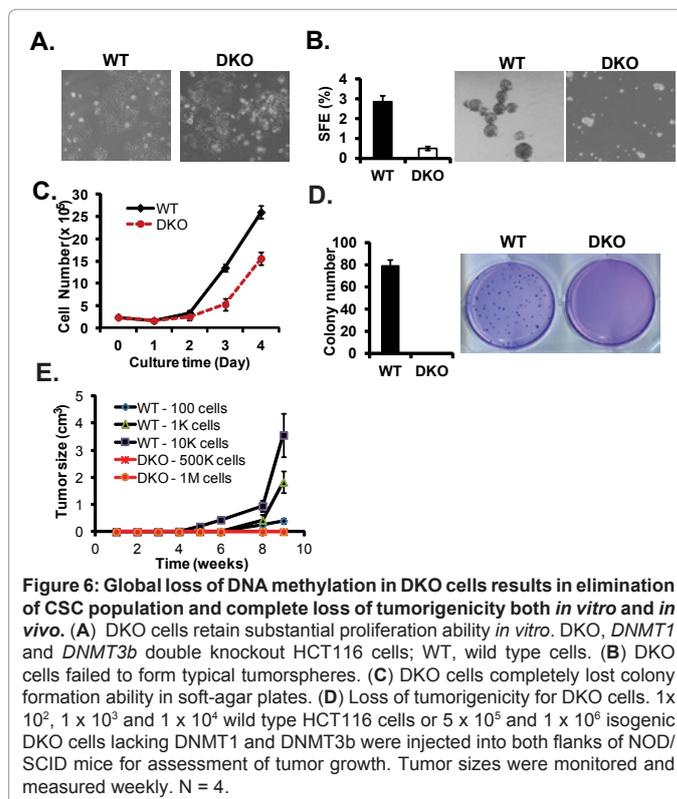
ESA have been used individually or in combination for isolation and enrichment of colon CSCs [6-8,15,42]. Again, no simple combination can be used for identification of CSCs from different sources of tumor cells [9]. The tumorsphere assay has been used for more than a decade as a suitable surrogate assay for *in vivo* serial transplantation to verify self-renewal potential of normal and cancer stem cells. Several previous studies have utilized the spheroid culture to isolate and culture CSC from primary colon carcinoma [3,42]. However, this method has not been optimized for enrichment of CSC from colon cancer cell lines.

Our previous study showed that supplemental B27 can sustain the growth of tumorspheres derived from primary tumors of MMTV-Her2/neu transgenic mice [43]. In this study, we further tested another growth supplement, N2, for the continued growth of tumorspheres from colon cancer cell lines. We found that basic medium supplemented with either N2 or F12 is sufficient for formation of tumorspheres for a few passages. However, in the presence of both N2 and F12, the sphere forming efficiency continues to increase over the passages, indicating that the CSCs are expanding with each passage. Our data indicates that optimized spheroid culture conditions can be potentially used to propagate and expand CSC from colon cancer cell lines and provide a practical method for development of relatively high-throughput drug screening assays for CSC-targeted therapy [44].

While the presence of self-renewing cells in tumorspheres is supported by their ability to form new generations of tumorspheres, we provide the following evidence to support our speculation that

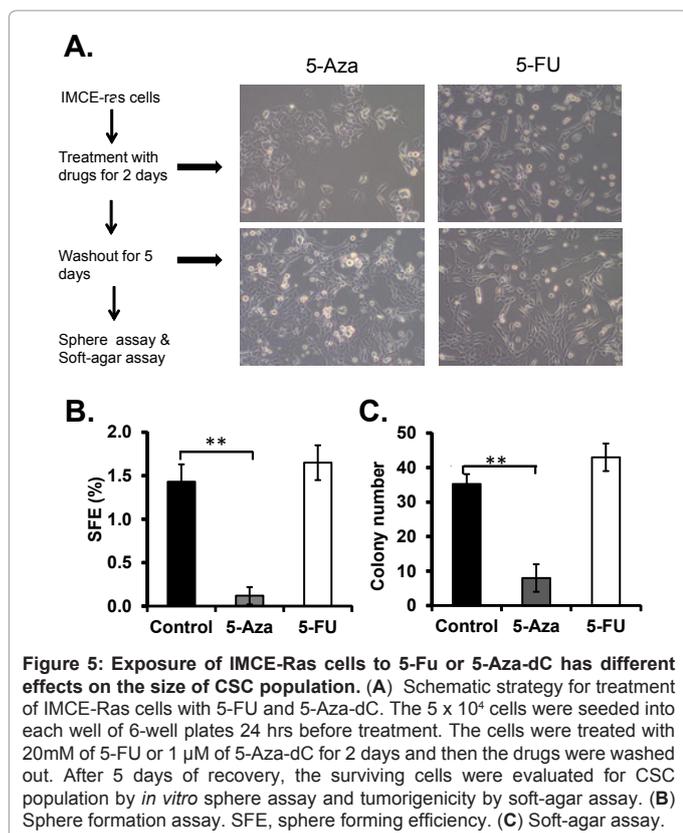
CSCs are not only present but actually enriched in tumorspheres. First, a higher level of expression of putative stem cell markers in tumorspheres further corroborates the presence of CSC-like cells in the tumorspheres. However, Sca1 was the only stem cell marker whose mRNA was present at dramatically higher levels in tumorspheres compared to monolayer cultures. Sca1 has been recognized as an adult and cancer stem cell marker from different tissues [34,35]. Further studies will clarify whether Sca1 can be used a maker to isolate CSCs from colon cancer cell lines and primary colon carcinomas. Next, we performed both *in vitro* and *in vivo* experiments to demonstrate that tumorspheres are highly tumorigenic. We found that tumorspheroid cells displayed increased colony formation abilities in soft-agar assay compared with adherent cells cultured as monolayer. *In vivo* transplantation assay data shows that tumorspheres from IMCE-Ras cells are up to about 450-fold enriched in tumor-initiating capacity in comparison with cells in monolayer culture. Taken together, our data suggest that tumorspheres derived from IMCE-Ras cells are enriched for CSCs.

CSCs are suggested to be slow cycling and to express high levels of drug transporters and anti-apoptotic proteins. Consequently, they are resistant to chemotherapeutic drug-induced cell death [45-48]. Presently, there are only a few studies reporting on the chemo sensitivity of colorectal CSCs. Most of these studies suggest that CSCs are more resistant to chemo- and radio-therapy, being responsible for the recurrence of the disease after treatment [42,49-52]. In line with these findings, our data showed that spheroid cells are more resistance to conventional chemotherapeutic drugs 5-FU and Doxorubicin. However, the sensitivity of spheroid cells to the DNA methylation inhibitor 5-Aza-dC is the same as cells in monolayer culture by MTT assay. The MTT assay can only measure the survival rate of the total cell



population and thus may not accurately reflect the sensitivity of CSCs to chemotherapies. To functionally demonstrate that CSCs are resistant or sensitive to chemotherapies compared to the bulk of tumor cells, we briefly exposed the colon cancer cells to chemotherapeutic drugs and then measured the relative percentages of CSCs in the surviving cells. We reasoned that if CSCs were more resistant to treatment, they should be enriched among the cells that survive. Indeed, short duration exposure to 5-FU led to relative enrichment of colon CSCs as previously reported in the literature [49]. In contrast, exposure to 5-Aza-dC resulted in dramatic decreases in colony and sphere formation abilities, strongly suggesting that colon CSCs are selectively eliminated by the DNA methylation inhibitor 5-Aza-dC.

The question remains why 5-Aza-dC can selectively target colon CSCs. 5-Aza-dC is a DNA methyl-transferase inhibitor and is also known to strongly induce cell differentiation, but it is not a strong apoptotic inducer [40]. Several recent studies indicate that epigenetic therapy could potentially also be used to induce CSC differentiation, thereby rendering these aggressive cells more susceptible to conventional cytotoxic treatment [53-55]. We hypothesized that DNA Methylation is critical for maintenance of the colon CSC population. In line with this hypothesis, DKO cells with loss of >95% of genome-wide Methylation almost completely lost their sphere-forming abilities and failed to form colonies on soft-agar plates. In addition, *in vivo* transplantation assays show that DKO cells completely lost their tumorigenicity, which is the main feature of CSCs. Since DKO cells retain their long-term proliferation ability *in vitro*, severely reduced DNA Methylation levels in DKO cells may account for the loss of tumorigenicity. This data is in line with recent findings that the transition of stem cells from self-renewal to differentiation is accompanied by extensive changes in their Methylation pattern [54]. In hematopoietic stem cells (HSCs), the self-renewal program requires



constitutive maintenance of a critical threshold of Methylation. More importantly, hypomethylation impairs malignant self-renewal of AML cells and completely blocks initiation of acute B-lymphoid leukemia (B-ALL) from transformed stem cells [54]. Overall, these data suggest that retention or re-establishment of stem cell-specific Methylation patterns is an important step in the development and maintenance of CSCs. Thus, demethylating chemicals may be important for the consideration of using “epigenetic” therapy to target CSCs.

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