CANCER GENOMICS & PROTEOMICS 6: 19-30 (2009)

Phenotypic Subpopulations of Metastatic Colon Cancer Stem Cells: Genomic Analysis

INNA L. BOTCHKINA[1], REBECCA A. ROWEHL[2], DAVID E. RIVADENEIRA[3], MARTIN S. KARPEH JR.[4], HOWARD CRAWFORD[5], ANTOINE DUFOUR[6], JINGFANG JU[7], YUAN WANG[7], YAN LEYFMAN[3] and GALINA I. BOTCHKINA[3]

[1]Department of Physiology, University of California, San Francisco, CA; Departments of [2]Microbiology, [3]Surgery/Surgical Oncology, [5]Pharmacology, [6]Chemistry, and [7]Pathology, Stony Brook University, NY; [4]Beth Israel Medical Center, NY, USA

Abstract. *Background: Human cancer is characterized by high heterogeneity in gene expression, varieties of differentiation phenotypes and tumor–host interrelations. Growing evidence suggests that tumor-initiating, or cancer stem cells (CSCs), may also represent a heterogeneous population. The present study was undertaken to isolate and characterize the different phenotypic subpopulations of metastatic colon cancer and to develop a working colon CSC model for obtaining highly tumorigenic and clonogenic cells in sufficient numbers. Materials and Methods: Different phenotypic cell subpopulations were isolated based on differential levels and patterns of expression of several stemness markers, including CD133, CD44, CD166 and CD49b. Stemness properties of isolated cells were tested by analysis of their ability to form floating colonospheres in vitro, to induce tumors in NOD/SCID mice after transplantation at relatively low cell numbers, and to produce progenitors of different phenotypes. Results: The metastatic colon cancer HCT116 cell line, which expressed a majority of known CSC markers, closely resembling the patterns of expression in exfoliated peritoneal cells from several metastatic colon cancer patients, was selected as a reference material. Genome-wide microarray analysis (Affymetrix; DAVID) of CD133^{high} CSC-enriched versus CSC-depleted cell populations revealed 4,351 differentially expressed genes with an overrepresentation of those responsible for apoptosis resistance, regulation of cell cycle, proliferation, stemness and developmental pathways. Simultaneous analysis of 84 stem cell- and metastasis-related genes with corresponding PCR arrays identified genes differentially expressed in several colon CSC phenotypic populations versus bulk tumor cells, and in relation to each other. It was found that colonospheres induced by tumorigenic cells with the highest expression of CD133 and those which were induced by CD133/CD44-negative cells possessed profoundly different stem cell-related gene expression profiles. Conclusion: The proposed approaches allow for reliable isolation and propagation of highly tumorigenic and clonogenic cells of different phenotypes. Genomic analysis of several candidate CSC phenotypic populations may contribute to the identification of novel targets for colon cancer stem cell-targeted drug development and treatment.*

Correspondence to: Galina I. Botchkina, Department of Surgery/Surgical Oncology, HSC-18, 050, SBUMC, Stony Brook, NY 11794, U.S.A. Tel: +1 6314442076, Fax: +1 6314446348, e-mail: gbotchkina@notes.cc.sunysb.edu

Key Words: Cancer stem cells, phenotypes, metastatic colon cancer, HCT116, microarray, DAVID.

The limited effectiveness of standard anticancer therapies has been attributed to the existence of rare, highly drug resistant cells within tumors, operationally called cancer stem cells (CSCs) (1). This concept was firmly supported by the recent isolation and characterization of tumorigenic cells from all major human hematopoietic and solid tumors, which led to the important conclusion that not every cancer cell, but only CSCs are responsible for tumor initiation, development and response to treatment (2, 3). CSCs share some basic features and signal transduction pathways, such as Wnt, Shh, Notch, Bmi-1 and others with normal stem cells (4, 5). Thus, it is widely accepted that CD133 [known as prominin-1 or AC133, a pentaspan glycoprotein (6) originally classified as a marker of primitive haematopoietic and neural stem cells] is also a marker of organ-specific adult stem cells and CSCs, and several types of tumorigenic cells, including brain tumors (7-9), kidney (10), hepatocellular (11, 12), colon (13, 14), pancreatic (15, 16) and prostate (17) carcinomas were isolated using this marker. These studies have demonstrated that the minor CD133$^+$ cell population

possesses much higher tumorigenic and clonogenic potential compared to their $CD133^-$ counterparts or unsorted cells, and that the $CD133^+$ cells are highly resistant to chemo- (18-19) and radiotherapy (20).

Human cancer is characterized by high heterogeneity in gene expression, varieties of differentiation phenotypes and interactions between cells and the extracellular matrix, which tightly regulate tumor features, growth rates, drug sensitivity, *etc*. There is accumulating data that CSCs may also represent heterogeneous phenotypic populations. Thus, several distinct CSC populations were found in breast tumors: $CD133^+$ and $CD44^+/CD24^-$ (21); and $CD24^+/CD29^+$ or $CD24^+/CD49f^+$ (22). A subpopulation of migrating $CD133^+/CXCR4^+$ CSCs was identified in pancreatic tumors (15). Colon cancer specimens possess high variability in the patterns and levels of expression of some stem cell markers, such as CD133 (23, 24), which is in line with data showing that the percentage of $CD133^+$ cells correlates with tumor aggressiveness and clinical outcome (9, 25-27). Although the isolation of $CD133^+$ CSC is based on the assumption that differentiated cells lose their CD133-positivity, several reports suggested that CD133 is rather widely expressed in some epithelial tissues, including kidney cancer and lactiferous ducts of mammary glands (28), mature epithelium of pancreatic ducts (29) and primary colon cancer cells (24). Since not all solid colon tumors express CD133, the combined expression of $EpCAM^{high}/CD44^+/$ $CD166^+$ was suggested as being more robust for the isolation of colon tumor-initiating cells (23). There is growing evidence that both $CD133^+$ and $CD133^-$ populations are tumorigenic in some cases of advanced metastatic glioblastoma (30-32) and colon cancer (24), although the lack of CD133 by itself does not indicate an exact phenotype of this candidate CSC population. It is yet to be established what cancer cell subpopulations are responsible for initiation and progression of each tumor type, and what the nature of each type of tumor-initiating cell is. The present study was undertaken to isolate and characterize the different phenotypic subpopulations of metastatic colon cancer and to develop a working colon CSC model for obtaining highly tumorigenic and clonogenic cells in sufficient numbers. The metastatic colon cancer HCT116 cell line, which expresses a majority of known CSC markers, and possesses a constitutively larger proportion of several stemness markers was selected as a reference.

Materials and Methods

Isolation of CSCs from peritoneal wash of colon cancer patients. Patients with clinically advanced metastatic colon cancer subjected to surgical treatment were asked to participate in this study by providing peritoneal washing fluid and signing an informed consent in accordance with the local IRB requirements. At the beginning of surgery, 700 ml of sterile saline were instilled into the peritoneal cavity, gently agitated by rotation of the operative table and aspirated. Peritoneal washes were immediately delivered from the operating room and centrifuged at 300 x*g* for 6 min at 4˚C. The cell pellet was reconstituted in PBS and gently loaded onto a layer of Histopaque-1077 gradient (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 400 × *g* for 30 minutes at room temperature. RBC, dead cells and debris were removed from the bottom of the tube and live nucleated cells collected at the interface.

Cells. Standard HCT116 cells derived from a poorly-differentiated human colonic adenocarcinoma were grown under standard conditions (DMEM with 10% FCS, on uncoated flasks or dishes). Isolated $CD133^+$ cell populations were cultured in serum-free medium supplemented with 20 ng/ml EGF, 10 ng/ml FGF-2 and 10-20 ng/ml LIF (optional), on collagen type I-coated dishes.

Magnetic Cell Sorting (MACS) and antibodies. Dissociated cells were centrifuged at 300 xg for 6 min at 4˚C, rinsed with sterile MACS buffer and labeled with CD133 antibodies (Abs) directly or indirectly conjugated with ferromagnetic beads (Miltenyi Biotec, CA, USA) as recommended by the manufacturer. For up to 10 million cells, 350 μl MACS buffer, 100 μl of the blocking reagent and 50 μl of the biotinylated CD133 were added, mixed well and incubated for 15 min at 4˚C. After washing twice with 7 ml MACS buffer cells were incubated for 20 min at 4˚C with 100 μl Anti-Biotin in 400 μl MACS buffer. Washed labeled cells were resuspended in 500 μl of buffer and sorted with MACS devices.

Flow cytometry (FACS) and Abs. Cells were prepared as described above and labeled with one or several markers conjugated with different fluorescent dyes, including anti-human CD133/2-APC (clone 293C3; Miltenyi Biotec, CA, USA); CD166-PE (clone 105902; R&D Systems, MN, USA); CD44-FITC (clone F10-44-2), CD44-PE (clone F10-44-2; Invitrogen/Biosources, USA); CD44v6-FITC (clone 2F10; R&D Systems, USA), EpCAM-FITC (Biosources). Antibodies were diluted in buffer containing 5% BSA, 1mM EDTA and 15-20% blocking reagent (Miltenyi Biotec) to inhibit unspecific binding to non-target cells. After 15 min incubation at 4˚C, stained cells were washed, resuspended in 500 μl of MACS buffer and sorted with multiparametric flow cytometry with BD FACS*Aria* cell sorter (Becton Dickinson, CA, USA) under sterile conditions.

In vivo tumorigenicity. All experiments involving the use of animals were performed in accordance with SBU institutional animal welfare guidelines. NOD/SCID mice (Charles River Laboratories, Wilmington, MA, USA) were maintained under defined conditions at the SBU animal facility. Immediately after sorting, aliquots of the particular cell populations were counted and cell viability was determined by conventional trypan blue test. Cells were centrifuged at 300 xg for 6 min at 4˚C, suspended in ice-cold 1:1 mixture of growth medium and Matrigel Matrix (BD Biosciences) and 40 μl were injected subcutaneously into the flanks of 8-week-old mice. The primary tumor sizes were measured with a caliper on a weekly basis. All mice were sacrificed by cervical dislocation after 6 weeks post-transplantation.

Analysis of mouse tumor xenografts. Tumors were minced with scissors under sterile conditions, rinsed with Hank's balanced salt solution (HBSS) and incubated for 2 hours at 37˚C in serum-free RPMI-1640 medium supplemented with 200 units/ml Collagenases type II and type IV (Sigma-Aldrich, USA), 120 μg/l penicillin and

100 μg/mL streptomycin. Cells were further disaggregated by pipetting and serial filtration through cell dissociation sieves (size 40 and 80 meshes; Sigma-Aldrich). Contaminating erythrocytes were lysed by incubation in ammonium chloride hypotonic buffer for 5 min on ice. Single cell suspensions were sorted with BD FACS*Aria* and subjected to further analysis.

Sphere formation assay. The clonogenic potential of different phenotypic populations was analyzed in 3D non-adherent culture conditions using 10% Matrigel matrix (BD Biosciences) in MSCBM (Lonza, MD, USA). Cells were counted, resuspended and plated on 6-well plates at a final count of 300 and 1×10^3 cells per well. One and two weeks after initiation, plates were inspected for colony (sphere) growth: the number of colonies within each well was counted under a microscope and representative fields were photographed.

Affymetrix GeneChip human genome U133A array. Five μg of good quality total RNA was added to a cDNA reaction using a T7 dT_{24} primer and SuperScript II RT (Invitrogen, USA). The cDNA was purified utilizing a phenol/chloroform extraction followed by an ethanol precipitation. An *in vitro* transcription (IVT) reaction was performed using the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Life Sciences, USA). The IVT reaction products were purified using RNAeasy purification kit (Qiagen, USA) with a DNAase treatment step and analyzed as recommended by the manufacturer (Affymetrix, USA). For each sample an Affymetrix U133 Plus 2.0 array was hybridized overnight at 45˚C in an Affymetrix Hybridization Oven 640. The arrays were then washed, stained in an Affymetrix GeneChip® Fluidics Station 450, scanned in Affymetrix GeneChip® Scanner 3000 7G and the images were analyzed using the Affymetrix GCOS software version 1.4. The gene list with 2-fold cut-off was then further subdivided into functional categories with the bioinformatic analysis resource DAVID (Database for Annotation, Visualization and Integrated Discovery) of the Advanced Biomedical Computing Center (NCI, Frederick, http://david.abcc.ncifcrf.gov). Gene Pathway analysis was also analyzed by DAVID.

Results

Phenotypic analysis. It was found that a majority of colon cancer cells, either isolated from peritoneal washes of metastatic colon cancer patients (5 of 5 tested specimens; representative experiment is shown in Figure 1 A), or from HCT116 cell line (Figure 1B), were positive for CD133 and CD44, expressing low-to-moderate levels of these two markers and high levels of CD166 (Figure 1 C) and EpCAM (not shown). Both types of cells also possessed several distinctive minority cell populations with highest levels of expression of some stemness markers, including $CD133^{high}/CD44^{high}$, $CD166^{high}/CD44^{high}$, as well as minor populations of cells lacking CD133 ($CD133^{-}/CD44^{high}$ and $CD133^{-}/CD44^{-}$; Figure 1); however, the expression levels in clinical specimens were variable. Since the HCT116 cell line possessed all these minority phenotypic populations at higher ratios than, for example SW480 and HT29 cells, it was selected as a major source for further studies.

Since CSCs usually represent a very minor population of cancer cells, several experimental approaches which promoted CSC self-renewal and allowed significant enrichment of CSC-associated phenotypic populations were tested. Thus, culturing cells at low density (30-50% confluent) before sorting led to a significant increase in the yield of MACS-$CD133^{+}$ cells, ranging from 20.5% to 50.7% (38.5±12.7) compared to 4.9±1.1% if cells were cultured at 75-90% confluent culture. Since not all isolated $CD133^{+}$ cells are putative stem cells (although enriched, it is a mixture of stem cells with their early progeny, which still retain CD133-positivity), it was important to determine conditions which could increase the proportion of putative CSCs. It was found that repeated sets of cell sorting followed by culturing at low cell density in serum-free MSCB medium led to a significant increase in the proportion of $CD133^{high}$ cells (Figure 1 D, E), which was retained to some extent after transplantation to NOD/SCID mice (Figure 1F).

Analysis of stem cell properties. The isolated phenotypic cell population was tested for stem cell properties, *i.e*. thir ability to form floating colonospheres in 3D culture systems (clonogenic potential), their ability to induce tumors in NOD/SCID mice after transplantation of a relatively low cell number (*in vivo* tumorigenicity) and their ability to produce different cells phenotypes (plasticity). To examine their *in vitro* clonogenic potential, an equal number of the freshly FACS purified cells (300 and 1×10^3 cells per well) were seeded on Ultra-Low Attachment 6-well plates (Corning, NY, USA) in 10% Matrigel matrix in serum-free stem cell medium. In 10 days, all produced spheres were counted and representative fields were micro-photographed. It was found that four phenotypic populations, including $CD133^{high}$, $CD133^{-}$, $CD133^{high}/CD44^{high}$ and $CD166^{high}/CD44^{high}$, possessed much higher efficiency in forming floating colonospheres (Figure 2 A, B; only spheres induced by $CD133^{high}/CD44^{high}$ and $CD133^{-}$ populations are shown), compared to unsorted bulk tumor cells, which produced predominantly flat, 2-dimensional colonies at non-adherent culturing conditions. The $CD133^{-}$ cells induced lower numbers, but larger sizes of colonospheres, with a higher number of produced cells (Figure 2 C, D). It was noted that long passaging of HCT cells as floating spheres, as well as repeated cell sorting, led to the shift of the major cell population toward higher levels of expression of CD133 and gradually increasing clonogenic capacity.

To compare phenotypic composition of colonospheres induced by different populations of colon cancer cells, all spheres of each phenotype were collected, dissociated in enzyme-free dissociation reagent (Chemicon Int.) and analyzed, sorted and counted with the BD FACS*Aria* for further tests. Surprisingly, FACS analysis of dissociated spheres induced by $CD133^{high}$, $CD133^{-}$, $CD133^{high}/CD44^{high}$ and $CD166^{high}/CD44^{high}$ subpopulations revealed a strikingly

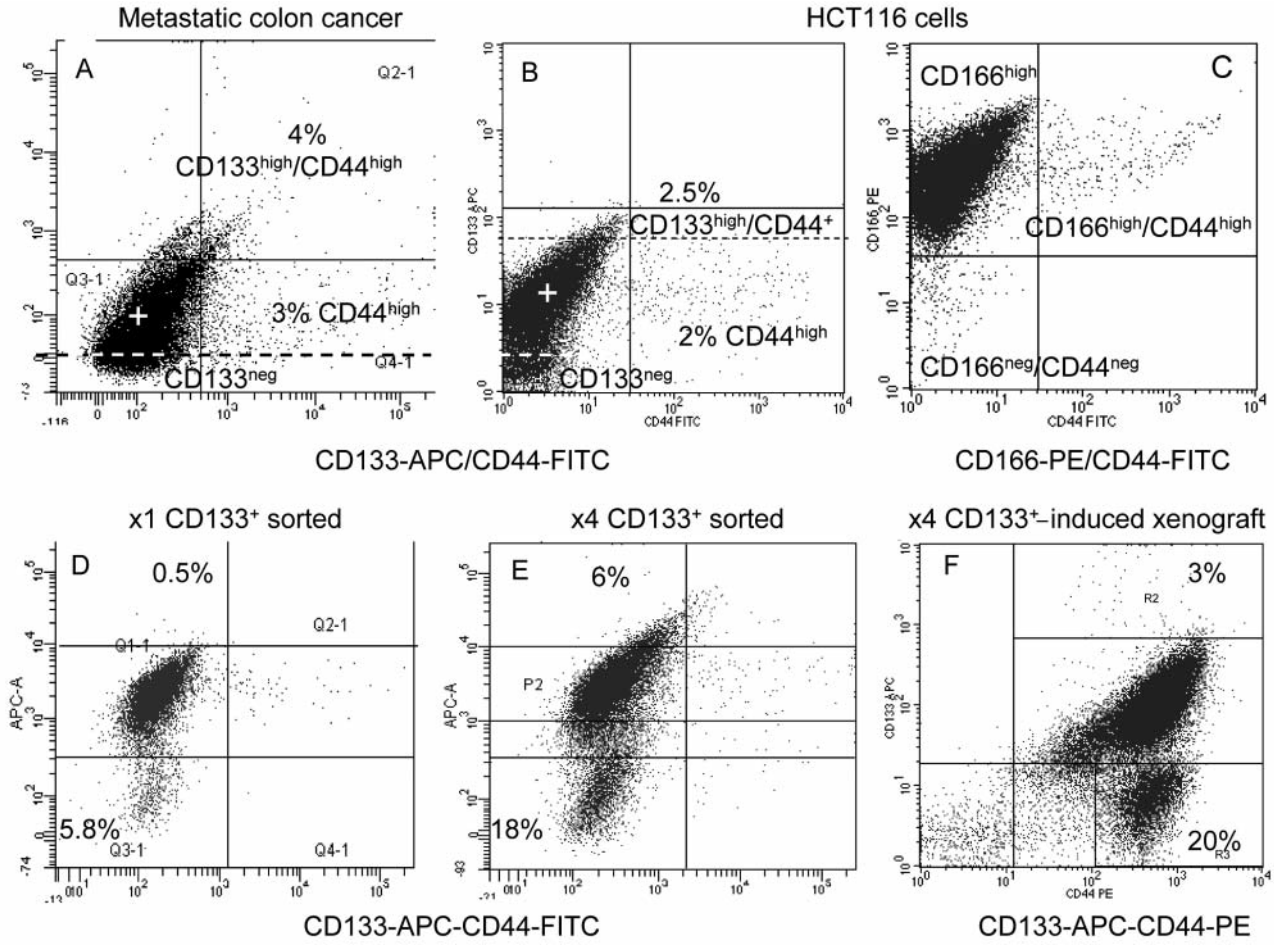

Figure 1. *Phenotypic subpopulations of metastatic colon cancer cells. The majority of epithelial MACS-EpCAM isolated cells from peritoneal wash of metastatic colon cancer patients (A) and established metastatic colon cancer HCT116 cells (B, C) expressed low-to-moderate levels of CD133 and CD44 (marked with +). They also possessed distinctive minority phenotypic subsets with highest expression of CD133, with or without co-expression of high levels of CD44 (A, B), highest combined expression of CD166 and CD44 (C), and subpopulation of cells negative for these markers. Repeated MACS-CD133 cell sorting led to a significant increase in the number of cells with high levels of CD133 expression and this shift was retained after 2 days culturing under standard conditions (C, D) and transplantation to NOD/SCID mice (F). Note that an increased proportion of CD133^{high} cells led to increased CD133^{-} cell population (D-F; lower quadrants).*

similar pattern of expression of all tested cell surface CSC-related markers, with quite similar ratios of the minority phenotypic populations (Figure 2 A, B). All dissociated sphere cells possessed an additional distinctive minority population with high levels of expression CD49b (right column). These data indicate that the above-listed subpopulations of metastatic colon cancer cells have similar abilities to form colonospheres in 3D culture system and similar plasticity (ability to give rise to other phenotypes) under the described conditions.

In vivo tumorigenic potential. Cells of a particular phenotype were transplanted into NOD/SCID mice in quantities which were known to reliably and reproducibly induce mice tumor xenografts and, on the other hand, could also reveal possible differences in their tumorigenic potential. Thus, it was shown previously that 1,000 cells of several CSC phenotypes induced tumors in the majority, but not in all injected mice (23). Therefore, 1,500 cells of several candidate phenotypes were transplanted. It was found that MACS-CD133^{+} and FACS purified CD133^{high}/CD44^{high}, as well as cells which were repeatedly depleted of CD133^{+} population, uniformly induced tumors in NOD/SCID mice, whereas no tumors were developed after injection of 5×10^{5} unsorted bulk HCT116 cells by 6 weeks after transplantation. Importantly, cells repeatedly sorted and cultured at low density possessed a much higher ability to form tumors compared to single-time sorted MACS-

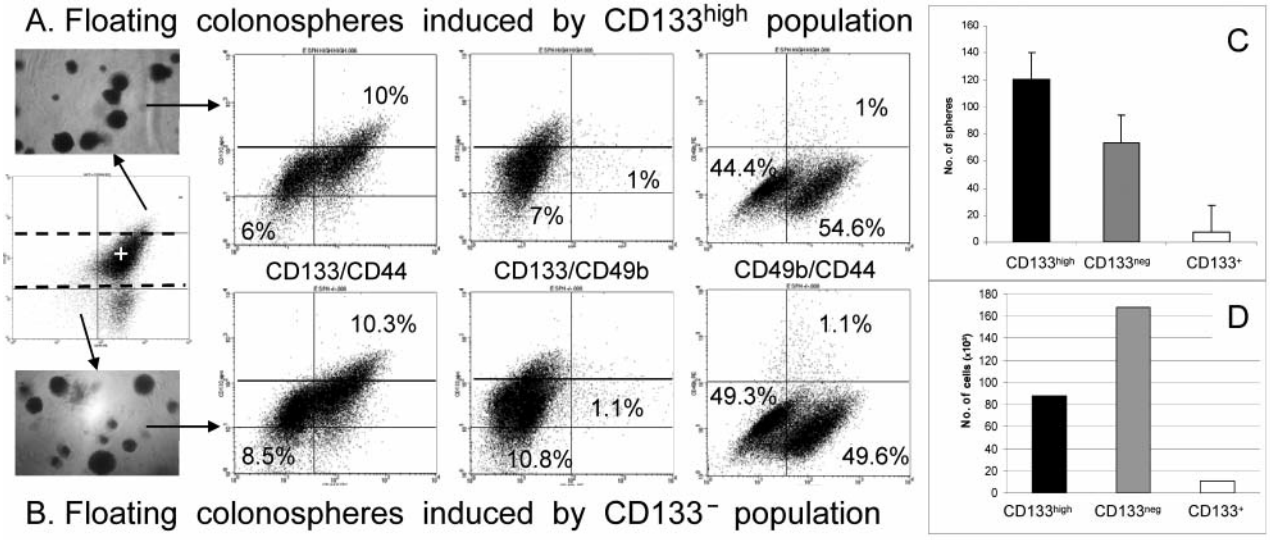

Figure 2. *Sphere-forming capacity of different phenotypic subpopulations. Equal numbers of CD133^{high} and CD133^{-} cells (10^3 cells per well; A and B, respectively) produced large numbers of floating colonospheres, whereas cells expressing moderate levels of CD133 produced predominantly flat, two-dimensional colonies in non-adherent conditions. Although CD133^{neg} cells produced slightly lower numbers of floating spheres, they were of larger size and contained more cells (summary at C and D). FACS analysis revealed similar patterns and similar ratios of different markers expression in both types of spheres.*

CD133^{+} cells, which means that most likely, they had an enriched proportion of putative tumorigenic cells, or CSCs. Thus, at 6 weeks after transplantation, 6 of 6 mice developed relatively small tumors after injection of x1 MACS-CD133^{+} cells, whereas repeatedly sorted cells induced significantly larger and more vascularized tumors in all mice (Figure 3A, B). Dissociated and MACS-CD133-sorted tumor xenograft cells also possessed significantly different sphere-forming capacities (Figure 3C), which means that an increased ratio of CD133^{high} in transplanted cells was retained to some extent in mouse tumors. FACS analysis revealed that, similarly to floating colonospheres, dissociated mouse tumors induced by different subpopulations also possessed all the original studied phenotypes, including a majority population which was low-to-moderately positive for CD133 and CD44, and highly positive for CD166, as well as minority subpopulations expressing highest levels of CD133^{high}, lack of it (CD133^{-}), and these which were CD166^{high}/CD44^{high} (Figure 1D and Figure 4). Repeatedly sorted CD133^{high} cells produced a significantly larger proportion of CD133^{-} cells as early as 2 days after culturing of isolated cells in adherent conditions (5.8% *versus* 18%), and such an increased CD133^{-} ratio was retained in mouse tumor xenografts induced by repeatedly sorted cells (Figure 4; right panel; lower right quadrant), as well as in floating colonospheres. As mentioned above, the CD133^{-} subpopulation possessed profound clonogenic and tumorigenic properties.

Genome-wide gene expression profiling. Using metastatic colon cancer HCT116 cells as a reference material, genome-wide and several pathway-specific gene expression profiles of CSC-enriched CD133^{high} cell population *versus* bulk tumor cells were analyzed. Expression of several stemness-related genes was also tested with real-time RT-PCR. Using the Affymetrix GeneChip Human Genome U133A assay it was found that freshly isolated CSC-enriched CD133^{high} cells possessed 988 differentially expressed genes compared to their bulk counterparts (CD133^{high}-depleted cells). Of these genes, CSCs showed significantly increased activity of 826 genes and reduced activity in 162 genes. The CD133^{high}-induced 3D floating spheroids revealed 4,351 differentially expressed genes with more than 500 significantly up-regulated (3- to 120-fold) and 436 down-regulated (by 3- to 1,500-fold) genes. The majority of the most up-regulated genes were those related to: anti-apoptosis (*APP*, *Bcl3/NFkappa B2 complex*, *BDNF, BIRC3, BIRC4, BTRC3, CBX4, CCAR1, CCPG1, CD74, DHCR24, FOXO3, HSPA1B, IGFBP3, IF16, NFKB1A, TBX3, TNFAIP3, TRIB3* and others), stemness (*NOTCH; APP, MIB1; Wnt* receptors *TGFB1I1, CSNK1D*), cell cycle/cell proliferation (*FOSB, IL-8, CCNG2, IGFBP3, TGFBP1, MXD1, INSIG1, EHF, CD74, CDC25A, HSMPP8*), transcription factors (*ID2, ID2B, DENR, MXD1*), DNA repair and many others. Overexpression of some stemness-related genes (*NOTCH, Shh, Wnt, Oct4*) was determined with RT-PCR (data are not shown). The most significantly down-regulated genes in HCT floating spheres were *HLI4* (by 1,500-fold), which is responsible for

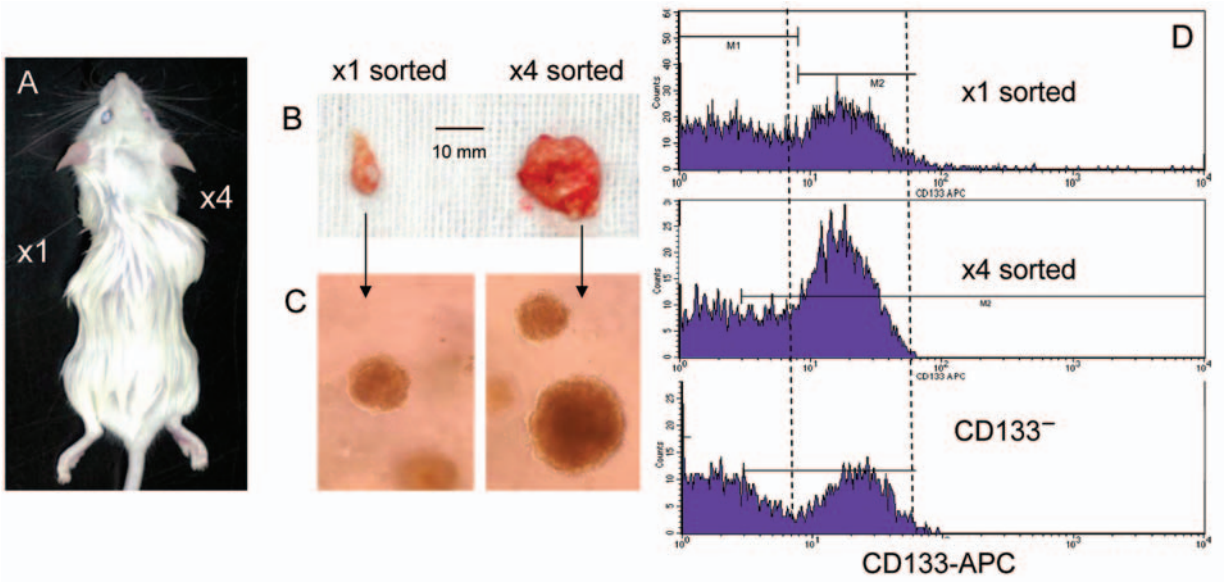

Figure 3. *Increased in vivo tumorigenicity of repeatedly MACS-CD133⁺-sorted HCT116 cells. To minimize possible host differences, the same number (1.5×10^3) of one- and four-times sorted cells was injected into opposite flanks of NOD/SCID mice. Six out of six mice uniformly developed relatively small tumors after injection of one-time immunomagnetically sorted cells (A; left injection site, x1 CD133⁺), whereas repeatedly sorted cells induced significantly larger tumors in six of six out mice (A; right injection site; ×4 CD133⁺). Removed tumors initiated by transplantation of repeatedly sorted CD133⁺ cells also displayed significantly higher levels of vascularization (B). Dissociated mouse tumor xenografts induced by repeatedly sorted CD133^{high} cells also possessed a higher ability to form floating spheres (C) and contained many more cells with high levels of CD133 (D).*

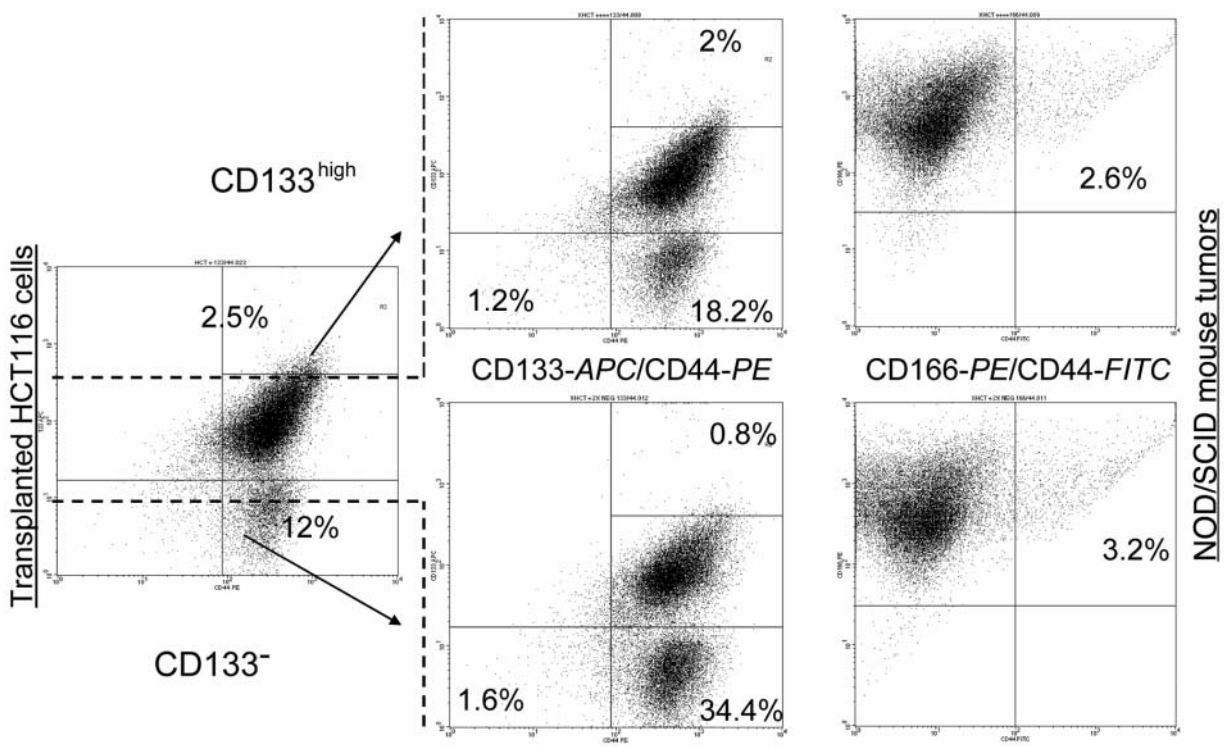

Figure 4. *FACS analysis of mouse tumor xenografts. Dissociated cells from mouse tumors induced by both CD133^{high} and CD133⁻ cells revealed similar patterns and levels of expression of CD133, CD44 and CD166. Middle panels show that both phenotypes produced both CD133^{high} and CD133⁻ cells, as well as all the minority subpopulations (ratio of each population is shown at each panel).*

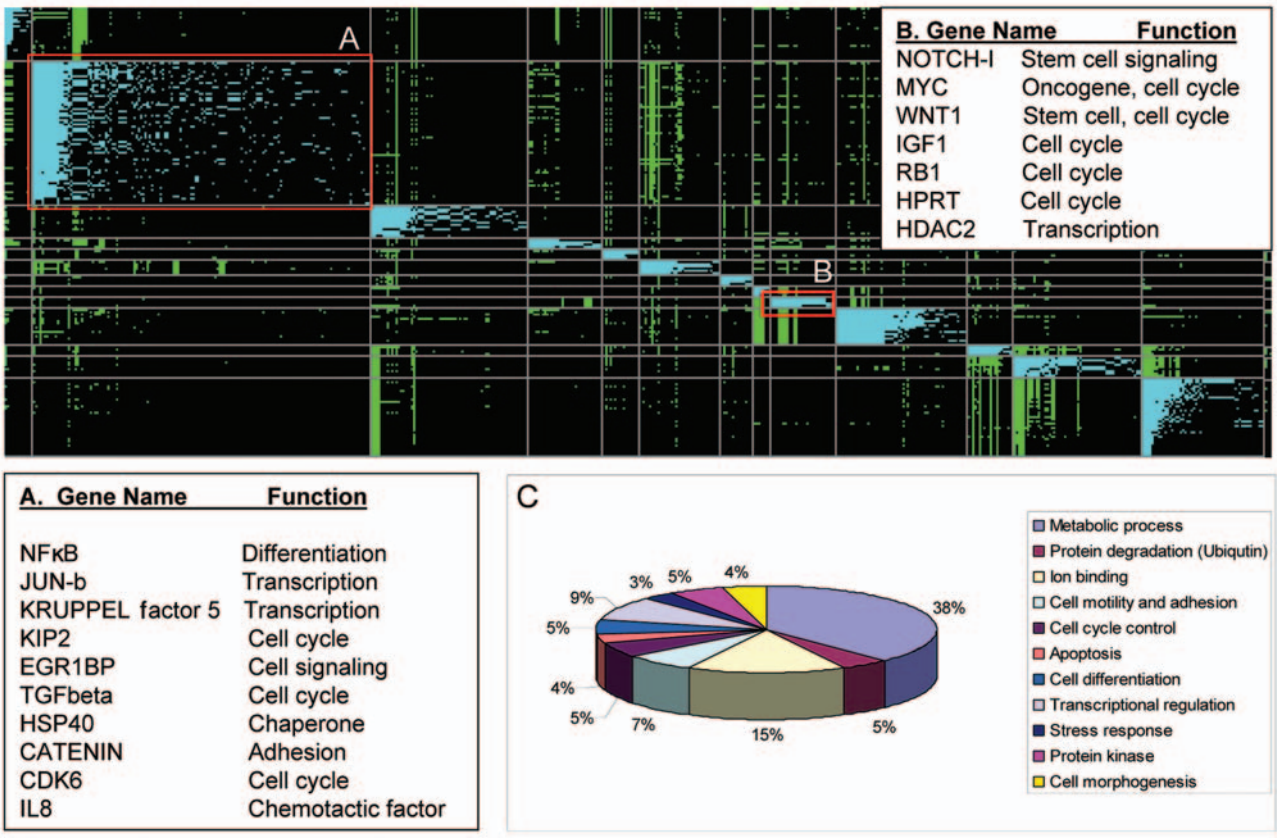

Figure 5. *DAVID analysis of genome-wide gene expression profiling of CSC-enriched CD133^{high} cells versus bulk tumor HCT116 cells with Affymetrix microarray assay. A fragment of the global view of genes is shown on the fuzzy heat map (A, B: two fields with red outline are described). A summary of several functional gene clusters is shown on C.*

heterophilic cell adhesion (motility, migration, metastasis); apoptosis-related cytochrome *c*, *COX6A1* (by 300-fold) and *BCL2L1*, which regulates the release of cytochrome *c* from mitochondria; *CXCL14* involved in cell-cell signaling (by 100-fold) also responsible for cell motility, migration and metastasis; apoptosis-related *AP15, BAX, CASP2, CFL1, ENO1, FXR1, HSPD1, HSP90B1, FAS,* Fas-binding (*FBF1, NPM1*), *MVEGFA, RAD21, RHOB, SOCS2, VDAC1* and many others; cell cycle/cell proliferation (*ras RHOB, CDV3, CDK8, NFYC*); cell motility (TPM1, VIM, MACF1); genes involved in negative regulation of cell growth (*DCBLD2, POSTN, CDH11*); signal transduction (ATP binding: *SPARC, MAP3K2, HSP90AB1*) and heat-shock protein genes (*HSP90B1, HSPD1*), which are required for antigen presentation (which is usually lost in advanced cancer).

Affymetrix microarray data were analyzed with DAVID (NIH, Frederik). The gene list with 2-fold cut-off was then further subdivided into functional categories with this bioinformatic analysis resource. All genes were grouped based on functional similarity using novel clustering algorithms. A portion of the global view of genes is shown on the fuzzy heat map in Figure 5 A, B (two fields are described; red outline). A summary of several functional gene clusters is shown in Figure 5 C.

Pathway-specific gene expression profiling. To characterize more precisely the alterations in the pathway-specific gene expression, the SYBR Green-based RT Profiler PCR Array System (SA Biosciences) was used. Stem cell-related and metastasis-related gene expression profiles were analyzed in two candidate colon CSC phenotypes, CD133^{high} and CD133^{-} (which are also negative for CD44 and CD166) compared to their bulk counterparts, and in relation to each other. Both CD133^{high} and CD133^{-} colonospheres revealed dramatic up-regulation of the majority of stemness-related genes (51 and 63 out of 84 analyzed stem cell-associated genes, respectively) compared to the bulk HCT116 cells (Figure 6 A, B). It was determined that CD133^{-}/CD44^{-}/CD166^{-} colon cancer cells have distinct growth characteristics and gene expression profiles. Thus, PCR array analysis revealed that the expressions of several stem cell-associated genes, including *ABCG2, NOTCH1, NOTCH2,*

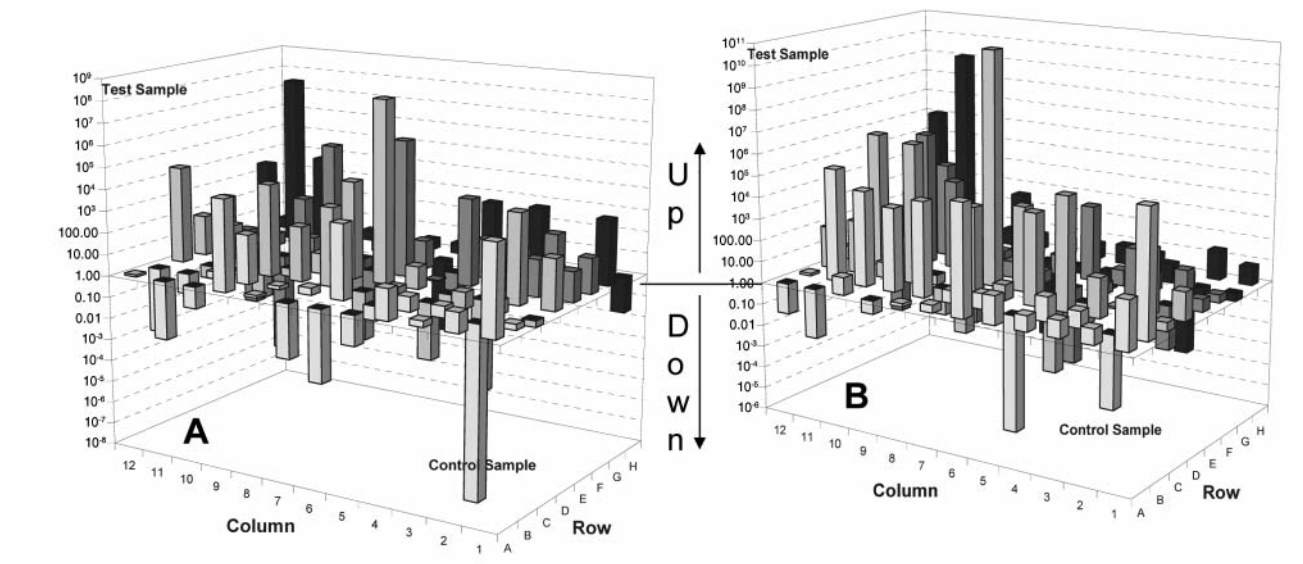

Figure 6. *Stem cell-related gene expression profiling with PCR array assay. Floating colonospheres induced by two phenotypic populations of colon tumorigenic cells, CD133^{high} (A) and CD133^{-}/CD44^{-} (B) were analyzed compared to the bulk HCT116 cells. Both types of spheres show dramatic up-regulation of stemness genes.*

WNT1, SOX2, DLL1, DLL3, DTX1, DTX2, FGF3, FRAT, IGF1, CD44, CXCL12, BMP2, COL2A1, MYOD1, DVL1, CD3D, CD4, CCND2, CD8A, CDH2 and *TERT,* were more significantly up-regulated in colonospheres induced by CD133^{-} cells compared to those induced by CD133^{high}. On the other hand, CD133^{high}-induced colonospheres also possessed a different set of genes, which were more significantly up-regulated compared to CD133^{-} spheres, including *ALPI, ASCL2, AXIN1, BMP1, BMP3, BTRC, CD30, CDC2, CTNNA1, EP300, FGF1, FGF4, FGFR1, FAG1, NEUROG2, PPARD, RB1, S100B, SOX1* and others. In contrast to the stem cell-related genes, the majority of the metastasis-related ones were slightly down-regulated in CSC-enriched populations *versus* bulk tumor cells; however, several genes were moderately up-regulated in CD133^{high} -induced colonospheres (Figure 7), including *CXCR4, VEGF* and its receptor *FLT4, CTSL, CXCL12, MMP3, MMP7, SYK, TIMP3, TRPM1* and *TSHR*.

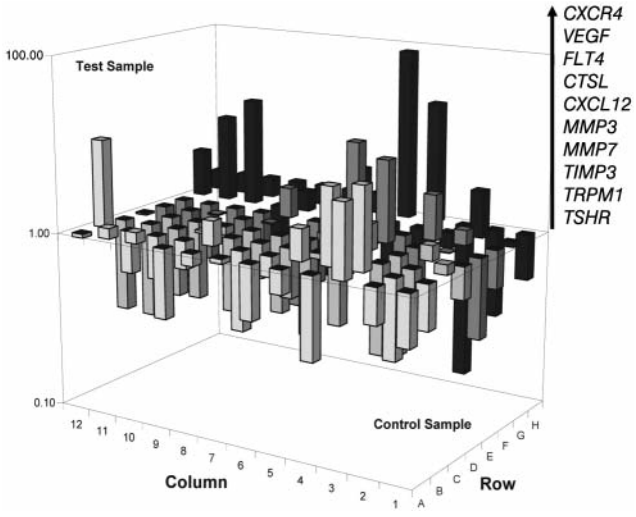

Figure 7. *Metastasis-related gene expression profiling with PCR array assay. Although the majority of metastasis-related genes in floating colonospheres induced by CSC-enriched CD133^{high} cell population were slightly down-regulated compared to the bulk HCT116 cells, several genes (listed on the right) were overexpressed.*

Discussion

Currently, there are controversial data concerning the difference in the expression of the most commonly used CSC marker, CD133, as well as other markers, between colon tumorigenic cells, bulk tumor cells and normal colonic epithelium. It remains to be established which is more important: the presence/absence of a particular marker or levels of its expression. The objective of this study was to identify, isolate and characterize the phenotypic, genomic and some biological features of tumorigenic subpopulation(s) from the HCT116 metastatic colon cancer cell line, which can be used as an alternative, relatively standardized approach compared to the isolation of colon CSCs from clinical specimens. Presently, the isolation of CSCs from solid tumors has certain limitations, such as a lack of unique CSC markers, high heterogeneity of human cancer and high variability in

gene expression, including the expression of stemness markers, resulting in difficulties in data interpretation and comparative analysis of the basic CSC features. Several different approaches are currently used for the isolation of colon CSCs, including $CD133^+$ as a single marker (13, 14), combined expression of $EpCAM^{high}/CD44^+CD166^+$ (23), lack of the CD133 expression (24), and side population (SP), based on the efflux of Hoechst 33342 (33, 34). The results clearly demonstrate that, in contrast to other cancer cell types, the majority of both exfoliated cells from peritoneal washes of metastatic colon cancer patients and cells from established metastatic colon cancer HCT116 cell line express low/moderate levels of CD133/CD44 and high levels of EpCAM/CD166. Therefore, positivity alone for these markers is not adequate as a criterion of stemness, at least for colon cancer, which is in line with the suggestion that quantitative differences in gene expression may be of high functional significance (35).

Several distinctive minority phenotypic populations have been identified, including $CD133^{high}$, $CD133^-/CD166^-/CD44^-$, $CD133^{high}/CD44^{high}$ and $CD166^{high}/CD44^{high}$, which possessed significantly higher clonogenic and tumorigenic potentials compared to their bulk (although CD133-positive) counterparts. A high proportion of $CD133^+$ cells in colon cancer HCT116 cells is not unique, and similar findings were recently reported for a liver cancer cell line (Huh-7), which exhibits as much as 50% $CD133^+$ cells (11). There is accumulating data that the percentage of $CD133^+$ CSCs correlates with tumor aggressiveness (8, 18, 25) and poor prognosis (19, 36), so it is not surprising that clinical specimens of colon tumors displayed high variability in $CD133^+$ content, which was reported from 1.8 to 24.5% (13), 2.5% (14), and from entirely negative to predominantly $CD133^+$ (23). Similar variability was reported for established colon cancer cell lines (37), showing that only 6 out of 12 lines expressed CD133 mRNA, and *CD133* protein expression was detected in 5 out of those 6 cell lines. In contrast, exfoliated cancer cells isolated from the peritoneal cavity of patients with metastatic colon cancer represent a unique and naturally relatively enriched population of cells committed to induce metastasis after acquiring all the necessary mutations and migratory potential. Therefore, to determine possible differences in clonogenic and tumorigenic properties of several candidate phenotypic populations of colon cancer cells, the established metastatic colon cancer HCT116 cells were chosen as a reference line expressing a majority of commonly used stem cell markers, which closely mimics the basic phenotypic patterns observed in clinical specimens of the peritoneal washes.

MACS-$CD133^+$ cells repeatedly sorted/cultured at low density in serum-free MSCBM revealed much higher *in vivo* tumorigenic potential and ability to form floating spheres, and the major population of repeatedly sorted cells generally expressed higher levels of CD133 as compared to the original unsorted cells, and a higher proportion of $CD133^{high}$ cells. It is suggested that this is the cell population with the highest levels of CD133 which represent putative colon tumorigenic cells, *i.e*. CSCs. This approach, however, did not affect the proportion of cells with combined highest expression of CD44/CD166. Since $CD44^{high}/CD166^{high}$ cells did not co-express the highest levels of CD133, it can be suggested that they most likely represent two separate tumorigenic subpopulations. It has been determined that after 6-10 passages of colonospheres induced by $CD133^{high}$ cell population, a majority of cells acquired high clonogenic potential comparable the potential of $CD133^{high}$ cells. However, these data are in line with observations that malignant potential usually increases in consequent passages *in vivo* and *in vitro* due to the high rates of genetic and epigenetic changes. Thus, increased tumorigenicity and clonogenicity of cancer cells was reported after transplantation to mice (38). Moreover, secondary, tertiary and quaternary xenografts were shown to be increasingly tumorigenic, and doubling time within ten passages of colon cancer spheroid aggregates was longer than after 30 passages (39). It is known also that recurrent cancer is associated with more malignant phenotype with increased intrinsic or acquired drug resistance and high mortality rates (3).

It has been determined here that several distinctive phenotypic populations, including $CD133^{high}$, $CD133^-/CD166^-/CD44^-$, $CD133^{high}/CD44^{high}$, $CD166^{high}/CD44^{high}$ and $CD49b^{high}$ have similar abilities to form colonospheres in 3D culture systems and similar plasticity (ability to give rise to other phenotypes) under the described conditions. These data are in line with another study on glioma (31), which demonstrated that both $CD133^+$ and $CD133^-$ cells can produce both phenotypes. However, it contradicts another report on colon cancer (24) which suggested that the $CD133^-$ population can produce only $CD133^-$ cells. It remains to be established whether or not these candidate colon tumorigenic and clonogenic subpopulations have a common stem cell predecessor and what their exact functional role in primary and metastatic colon cancer is. Although these subpopulations have similar plasticities, dramatic genomic differences between several candidate phenotypic populations, as well as between CSC-enriched and bulk colon cancer cells, have been found using genome-wide and stem cell pathway-related gene expression analyses. Thus, up-regulation of the *CXCR4* gene in $CD133^{high}$ cells compared to their bulk counterparts, which was identified in a subpopulation of migrating CSCs in pancreatic tumors (15), highlights the importance of acquiring a migrating phenotype by colon CSCs as a prerequisite for metastasis.

In conclusion, the present study suggests a working *in vitro* CSC model for obtaining a sufficient number of highly tumorigenic and clonogenic cells from a readily available metastatic colon cancer HCT116 cell line with further

propagation of CSCs as colonospheres of different phenotypes in 3D culture. Significant up-regulation of many genes involved in distinct stem cell-related and developmental signaling pathways, in addition to the activation of anti-apoptotic pathways in metastatic colon CSCs, suggests that these features may contribute to sustained tumor growth and drug resistance. The combination of the genome-wide and pathway-specific analyses with microarray and PCR-array assays can provide extremely important and accurate information for the identification of potential targets for the development of effective anticancer therapies and obtaining gene signature patterns for each clinicopathological case. Further study is necessary to determine the biological significance of each phenotypic cell population.

Acknowledgements

We thank Todd Rueb for extraordinary technical support with flow cytometry and cell sorting experiments.

This work was supported by the NYSTAR-designated Center for Advanced Sensor Technology, NY LILAC foundation, and SBU SOM.

References

1 Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer and cancer stem cells. Nature *414*: 105-111, 2001.

2 Dalerba P, Cho RW and Clarke MF: Cancer stem cells: models and concepts. Annual Rev Med *58*: 267-284, 2007.

3 Mimeault M, Hauke R, Mehra PP and Batra SK: Recent advances in cancer stem/progenitor cell research: therapeutic implications for overcoming resistance to the most aggressive cancers. J Cell Mol Med *11*: 981-1011, 2007.

4 Pardal R, Clarke MF and Morrison SJ: Applying the principles of stem cell biology to cancer. Nat Rev Cancer *3*: 895-902, 2003.

5 Reya T and Clevers H: Wnt signaling in stem cells and cancer. Nature *434*: 843-850, 2005.

6 Shmelkov SV, Clair R, Lyden D and Rafii S: AC133/CD133/Prominin-1. Int J Biochem Cell Biol *37*: 715-719, 2005.

7 Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J and Dirks PB: Identification of a cancer stem cell in human brain tumours. Cancer Res *63*: 5821-5828, 2003.

8 Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD and Dirks PB: Identification of human brain tumour initiating cells. Nature *432*: 396-401, 2004.

9 Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL and Yu JS: Analysis of gene expression and chemoresistance of CD133$^+$ cancer stem cells in glioblastoma. Mol. Cancer *5*: 67, 2006.

10 Bussolati B, Bruno S, Grange C, Buttiglieri S, Deregibus MC, Cantino D and Camussi G: Isolation of renal progenitor cells from adult human kidney. Am J Pathol *166*: 545-555, 2005.

11 Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T and Moriwaki H: Characterization of CD133$^+$ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem and Biophys Res Comm *351*: 820-824, 2006.

12 Yin S, Li J, Hu C, Chwen X, Yao M and Yan M: CD133 positive hepatocellular carcinoma cells posess high capacity for tumorigenicity. Int J Cancer *120*: 1444-1450, 2007.

13 O'Brien CA, Pollett A, Gallinger S and Dick J: A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. Nature *445*: 106-110, 2007.

14 Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R: Identification and expansion of human colon cancer-initiating cells. Nature *445*: 111-115, 2007.

15 Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ and Heeschen C: Distinct populations of cancer stem cells determine tumor growth and metastatic aActivity in human pancreatic cancer. Cell Stem Cell *1*: 313-323, 2007.

16 Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF and Simeone DM: Identification of pancreatic cancer stem cells. Cancer Res *67*: 1030-1037, 2007.

17 Collins AT, Barry PA, Hyde C, Stower MJ and Maitland NJ: Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res *65(23)*: 10946-10951, 2005.

18 Frank NY, Pendse SS, Lapchak PH, Margaryan A, Shlain D, Doeing C, Sayegh MH and Frank MH: Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. J Biol Chem *278*: 47156-47165, 2003.

19 Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmelad AB, Dewhirst MW, Bigner DD and Rich JN: Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*: 756-760, 2006.

20 Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, Sayegh MH, Sadee W and Frank MH: ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. Cancer Res *65*: 4320-4333, 2005.

21 Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV and Varticovski L: BRCA1 breast tumors contain distinct CD44$^+$/CD24$^-$ and CD133$^+$ cells with cancer stem cell characteristics. Breast Cancer Res *10(1)*: R10, 2008.

22 Vassilopoulos A, Wang RH, Petrovas C, Ambrozak D, Koup R, Deng CX: Identification and characterization of cancer initiating cells from BRCA1-related mammary tumors using markers for normal mammary stem cells. Int J Biol Sci *4*: 133-142, 2008.

23 Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C and Clarke MF: Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA *104*: 10158-10163, 2007.

24 Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, St Clair R, Baljevic M, White I, Jin DK, Chadburn A, Murphy AJ, Valenzuela DM, Gale NW, Thurston G, Yancopoulos GD, D'Angelica M, Kemeny N, Lyden D and Rafii S: CD133 expression is not restricted to stem cells, and both CD133$^+$ and CD133$^-$ metastatic colon cancer cells initiate tumors. J Clinical Investigation *118*: 2111-2120, 2008.

25 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA *100*: 3983-3988, 2003.

26 Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, Lichter P, Unterberg A, Radlwimmer B and Herold-Mende CC: Stem cell marker CD133 affects clinical outcome in glioma patients. Clinical Cancer Res *14*: 123-129, 2008.

27 Maeda S, H Shinchi H, Kurahara H, Y Mataki Y, Maemura K, Sato M, Natsugoe S, Aikou T and S Takao S: CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. British J Cancer *98*: 1389-1397, 2008.

28 Florek M, Haase M, Marzesco AM, Freund D, Ehninger G and Huttner WB: Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer. Cell Tiss Res *319*: 15-26, 2005.

29 Lardon J, Corbeil D, Huttner WB, Ling Z and Bouwens L: Stem cell marker prominin-1/AC133 is expressed in duct cells of the adult human pancreas. Pancreas *36*: e1-6, 2008.

30 Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, Aigner L, Brawanski A, Bogdahn U and Beier CP: $CD133^+$ and $CD133^-$ glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res *67*: 4010-4015, 2007.

31 Wang J, Saakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, Prestegarden L, Rosland G, Thorsen F, Stuhr L, Molven A, Bjerkvig R and Enger PO: $CD133^-$ glioma cells form tumors in nude rats and give rise to $CD133^+$ cells. Int J Cancer *122*: 761-768, 2008.

32 Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, Jeon JW, Kim MH, Kang BG, Jing Y, Jin J, Hong SC, Park WY, Lee DS, Kim H and Nam DH: Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Invest *88*: 808-815, 2008.

33 Haraguchi N, Utsunomiya T, Inoue H, Fumiaki Tanaka F, Koshi Mimori K, Barnard GF and Mori M: Characterization of a side population of cancer cells from human gastrointestinal system. Stem cells *24*: 506-513, 2005.

34 Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tag S, Reilly JG, Ghandra D, Zhou J, Claypool K, Coghlan L and Tang DG: Highly purified $CD44^+$ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. Oncogene *25*: 1696-1708, 2006.

35 Velcich A, Corner G, Paul D, Zhuang M, Mariadason JM, Laboisse C and Augenlicht L: Quantitative rather than qualitative differences in gene expression predominate in intestinal cell maturation along distinct cell lineages. Exp Cell Res *304*: 28-39, 2005.

36 Mehra N, Penning M, Maas J, Beerepoot LV, Van Daal N, Van Gils CH, Giles RH, and Voest EE: Progenitor marker *CD133* mRNA is elevated in peripheral blood of cancer patients with bone metastases. Clin Cancer Res *12*: 4859-4866, 2006.

37 Ieta K, Tanaka F, Haraguchi N, Kita Y, Sakashita H, Minori K, Matsumoto T, Inoue H, Kuwano H and Mori M: Biological and genetic characteristics of tumor-initiating cells in colon cancer. Ann Surg Oncol *15*: 638-648, 2008.

38 Kang Y, Omura M, Suzuki A, Oka T, Nakagami Y, Cheng C, Nagashima Y and Inoue T: Development of an orthotopic transplantation model in nude mice that simulates the clinical features of human lung cancer. Cancer Sci *97*: 996-1001, 2006.

39 Todaro M, Alea MP, Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP and Stassi G: Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell *1*: 389-402, 2007.

Received November 27, 2008
Accepted December 24, 2008