CANCER GENOMICS & PROTEOMICS 5: 301-310 (2008)

Genomic Analysis of Prostate Cancer Stem Cells Isolated from a Highly Metastatic Cell Line

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Abstract. *Background: Tumor-initiating or cancer stem cells (CSCs) were recently isolated from all major human cancers, including prostate cancer. However, the extreme heterogeneity of tumor cells in terms of biological behavior and gene expression patterns and difficulties isolating a pure population of CSCs from tumor tissues significantly impede a comparative analysis of CSCs. Materials and Methods: Different phenotypic populations were isolated from a metastatic derivative of PC-3 cell line, PC3-MM2, and tested for their ability to form tumors in NOD/SCID mice and floating spheroids in 3D culture systems. Results: All tested cell lines possessed minor populations of cells with highest expression of CD133, CD44 and CD166, whereas the vast majority of cells were CD133-negative. Several experimental approaches promoted a higher proportion of CD133-positive cells with increased in vivo tumorigenicity and the ability to produce floating spheres. Genome-wide microarray analysis (Affymetrix; DAVID) of CSC-enriched versus CSC-depleted cell populations revealed 213 genes with 10-100 fold increased activity out of 8994 differentially expressed ones and 87 genes with 5-50 fold decreased activity. Conclusions: The proposed in vitro prostate CSC model allows for reliable isolation and propagation of highly tumorigenic cells. This study may contribute to the identification of novel targets for CSC-targeted prostate cancer treatment.*

The recent isolation and characterization of rare stem-like cells from the majority of human cancers led to the important conclusion that not every cancer cell, but only CSCs, are responsible for tumor development, response to treatment and relapse (1-3). CSCs share many basic features and signal transduction pathways with normal stem cells (4-6), and most of the CSC types have been identified and isolated using common cell surface markers. Thus, it has been widely accepted that CD133 [known as Prominin-1 or AC133; a glycoprotein comprising five trans-membrane domains and two large glycosylated extracellular loops (7)], originally classified as a marker of primitive haematopoietic and neural stem cells, is also a marker of organ-specific adult stem cells and CSCs. Its function is still unknown, however CD133-positivity is largely used for the isolation of self-renewing and highly tumorigenic cells from different human cancer types, including brain tumors (8-10), kidney (11), prostate (12), liver (13-14), colon (15-16), melanoma (17), lung (18) and pancreatic (19) carcinomas. However, there are accumulating data that CSCs may represent heterogeneous phenotypic populations. 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 (20), mature epithelium of pancreatic ducts (21) and primary colon cancer cells (22). There is also growing evidence that both $CD133^+$ and CD133-negative populations are tumorigenic in some advanced metastatic cases of glioblastoma (23-25) and colon cancer (22). Although the existence of normal prostate stem cells was predicted in classic androgen cycling experiments by John Isaaks and colleagues (26) and later was demonstrated in human (27-29) and mouse prostate (30), there is only limited data on the isolation and characterization of prostate tumorigenic cells (31, 32). Collins *et al*. have shown that prostate cancer tumorigenic cells have a $CD44^+/\alpha2\beta1^{high}/CD133^+$ phenotype (31). Patrawala *et al*. have demonstrated that a small subset of $CD44^+$ cells in prostate cell cultures and xenograft tumors is more proliferative, clonogenic, tumorigenic and metastatic compared to $CD44^{(-)}$ population; and this $CD44^+$ subset expressed higher mRNA levels of several genes characteristic to embryonic stem cells (32).

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Key Words: Cancer stem cells, prostate cancer, PC3MM2, genom-wide microarray, DAVID analysis.

It becomes increasingly evident that effective anti-cancer therapies should be targeted to the cancer-specific tumor-initiating cells (2-3, 33, 34). $CD133^+$ cells were shown not only to be highly resistant to chemo- and radiotherapy (9, 33, 35-37), but their numbers increased after treatment (37). Since each particular cancer case is unique pathologically and highly heterogeneous biologically in terms of gene expression levels and interactions between cells and the host environment, comparative analysis of the basic features and common mechanisms of CSC drug resistance is difficult. In addition, at the present time, there is a lack of unique cell surface markers for CSCs, which makes it difficult to discriminate between normal and cancerous stem cells in solid tumors. Although both *in vitro* culturing and *in vivo* passaging of human cancer xenografts as solid tumors in non-syngenic host (immunodeficient mice) can change an original malignant phenotype, the same is true for original human primary tumors, because it is a general feature of cancer cells to have high rates of genetic and epigenetic changes (38-39). Therefore, it would be useful to develop working CSC models based on established aggressive cancer cell lines, providing highly tumorigenic CSCs in sufficient quantities necessary for CSC-targeted drug development and research. The objective of this study was to identify, isolate and characterize the phenotypic, genomic and some biological features of tumorigenic subpopulation(s) from highly metastatic prostate cancer cell lines, which can be used as an alternative, relatively standardized approach compared to and in addition to isolation of prostate CSCs from highly heterogeneous clinical specimens.

Materials and Methods

Cells. Standard parental metastatic prostate cancer PC-3 cells (ATCC) were maintained at SBU Cell Culture Facility. Two progressive PC-3 derivatives, metastatic PC3P and PC3MM2 cells, were obtained from MD Anderson Cancer Center (Houston, TX, USA). Before sorting, cells were grown at standard conditions (DMEM with 10% FCS, on uncoated flasks or dishes).

Magnetic cell sorting (MACS) and Abs. Dissociated cells were centrifuged at 300 g for 6 min at 4˚C, rinsed with sterile MACS buffer and labeled with CD133 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. Alternatively, cells were sorted with multiparametric flow cytometry with BD FACS*Aria* cell sorter (Becton Dickinson, CA) at sterile conditions. 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 (Biosource, CA, USA). 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.

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 SBU animal facility. Immediately after sorting, aliquots of positive, negative and unsorted cell populations were counted and cell viability was determined by conventional trypan blue test. Cells were centrifuged at 300 *g* 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 terminated by cervical dislocation after 6 weeks post-transplantation.

Analysis of mouse tumor xenografts. Tumors were minced with scissors at 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.

Sphere formation assay. Clonogenic potential of different phenotypic populations was analyzed in 3D non-adherent culture conditions using 1:1 Matrigel matrix (BD Biosciences) or 0.5% soft agar in MSCBM. Cells were counted, resuspended and plated on 48-well plates at a final count of 300 $CD133^+$ cells per well or 1×10^3 cells per well in a 6-well plate. Two weeks after initiation plates were inspected for colony (sphere) growth. The number of colonies within each well was counted under the microscope and representative fields were photographed. First passage floating spheres were removed, gently disaggregated with a new Matrigel/MSCS medium and reseeded.

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 reaction was performed using the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Life Sciences, USA). The IVT reaction products were purified using RNeasy purification kit (Qiagen, USA) with a DNase 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,

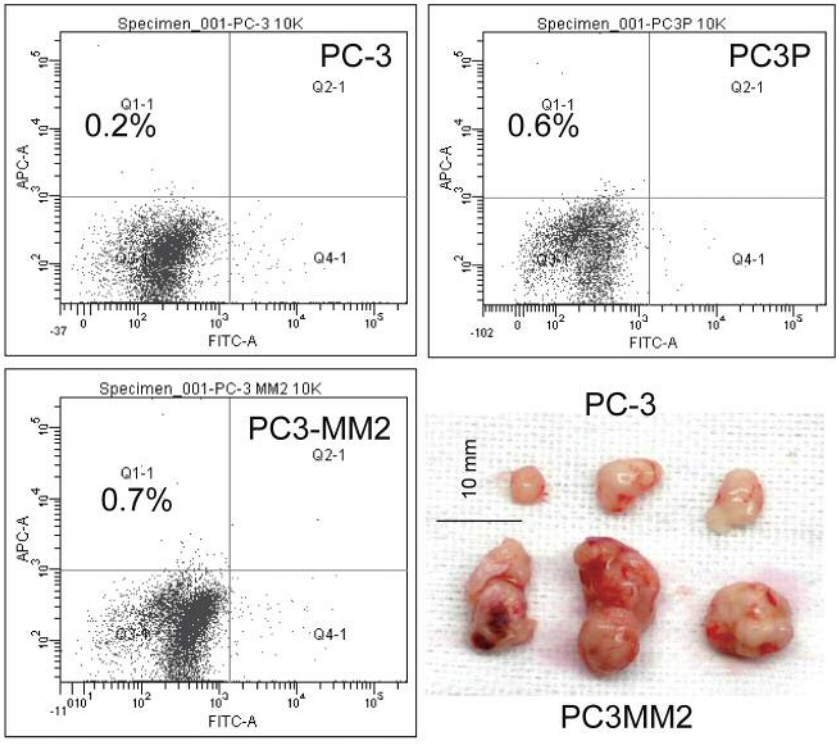


Figure 1. *Higher constitutive proportion of CD133^{high} cells correlates with increased in vivo tumorigenic potential. Parental prostate cancer PC-3 cells with constitutively lower percent of CD133^{+} cells compared to its progressive derivatives, PC3P and PC3MM2, induced smaller and less advanced tumors after transplantation of the equal number of cells (1 million of each cell type) to NOD/SCID mice (lower right panel). All cells were grown till fully confluent to avoid any influence of cell density on proportion of CD133^{+} cells.*

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 characterization. In order to select prostate cancer cells with the highest tumorigenic potential, the expression profiles of several common cell surface markers, including CD133, CD44, EpCAM and CD166 were examined in the established prostate PC-3 cell line and its progressively more metastatic derivatives, PC3MM2 and PC3P, as well as their ability to form tumors in NOD/SCID mice. All tested cell lines possessed a very minor population of cells with high expression of CD133 (CD133^{high}), whereas the majority of cells did not express this marker, but were CD44 and CD166-positive (Figure 1; only CD133-APC and CD44-FITC are shown). A higher constitutive proportion of CD133^{high} cells in PC3MM2 line correlated with its higher *in vivo* tumorigenic potential. Thus, tumor xenografts induced by transplantation of the equal number of either unsorted (1 million of each type) or 1,500 MACS-CD133^{+} cells isolated from PC-3 (which contain ~0.2% of CD133^{high} cells at fully confluent cultures) and PC3MM2 (which contain ~0.7% of CD133^{high} cells) possessed dramatically different tumor volumes, level of vascularization and latency of their appearance (only CD133^{+} mice xenografts are shown). Since PC3MM2 cells possessed highest tumorigenic potential and intrinsically higher proportion of CD133^{high} cells, all further experiments were carried out on this cell line alone.

Since even CD133^{high} cells represent a heterogeneous population of putative CSCs and their more numerous progenitors which still retain CD133-positivity, several experimental conditions which can promote CSC self-renewal were tested. Thus, culturing cells at low density in a serum-free medium (MSCB; Lonza, MD, USA), and selection of the fast adherent to collagen type I cells before cell sorting allowed for a significant increase in the yield of CD133^{+} cells. However, it was found that the most efficient increase can be achieved by repeated sets of MACS sorting followed by culturing in serum-free medium, which not only led to a

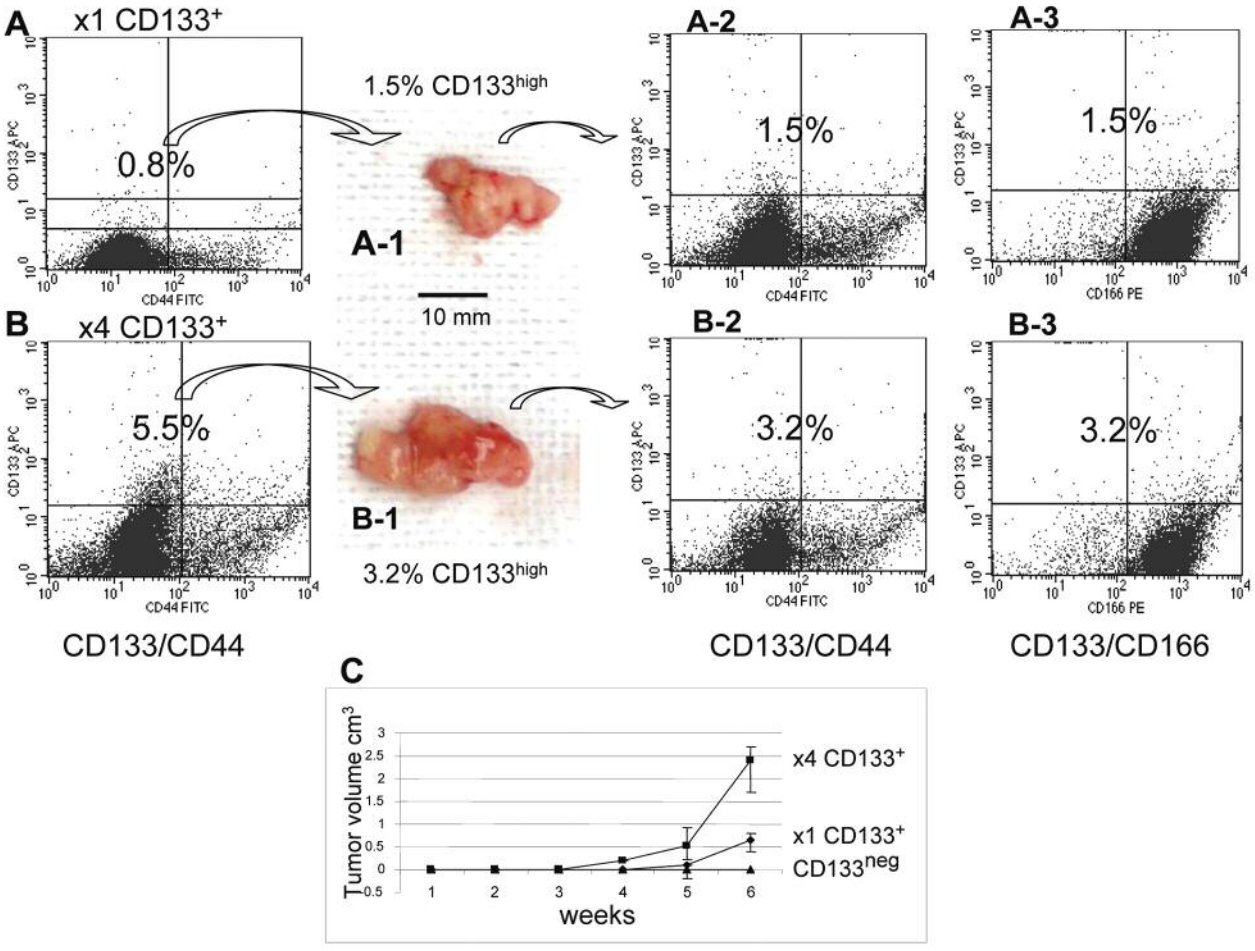


Figure 2. *Higher acquired proportion of CD133^{high} cells correlates with increased in vivo tumorigenic potential and is retained after transplantation to NOD/SCID mice. FACS analysis of single-time (A; x1 CD133^{+}) and repeatedly (B; x4 CD133^{+}) MACS sorted PC3MM2 cells shows significantly higher proportion of CD133^{high} cells (0.8% versus 5.5%) after repeated sorts. Transplantation of 1,500 x4 CD133^{+} MACS sorted cells induced significantly larger tumors in NOD/SCID mice with higher levels of vascularization and shorter latency compared to equal number of single-time sorted cells (A-1 versus B-1); no tumors were developed after transplantation of 5x105 negatively sorted cells (C). Dissociated cells from removed mice tumors (only cells expressing high levels of human epithelial antigen, hEpCAM, were analyzed) shows higher proportion of CD133^{high} cells compared to original cell types in general (A-2; B-2). The expression profiles of selected surface markers were analyzed in the same samples as a combination of CD133-APC/CD44-FITC (A, A-2; B, B-2), and CD133-APC/CD166-PE (A-3; B-3). In contrast to CD133, the majority of prostate PC3MM2 cells and corresponding mice tumor xenografts expressed high levels of CD44 and CD166 (lower right quadrants).*

higher ratio of CD133^{+} cells (Table I; Figure 2 A, B), but also to dramatic increase in their *in vivo* tumorigenic and *in vitro* clonogenic (sphere forming) capacities. Thus, transplantation of 1.5×10^{3} of the four-times sorted CD133^{+} cells to NOD/SCID mice induced tumor xenografts of significantly larger volumes, higher levels of vascularization and shorter latency compared to tumors induced by an equal number of single-time sorted CD133^{+} cells (Figure 2 A-1, B-1, C). In contrast to positively selected cells, transplantation of a much higher number of unsorted (bulk) PC3MM2 cells (500,000 cells) did not induce tumor growth by 6 weeks (Figure 2C). All removed mice tumors uniformly expressed high levels of the human epithelial antigen (hEpCAM). In general, the expression profiles for all studied surface markers were similar in original types of cells and corresponding mice tumor xenografts. However, xenograft cells possessed higher proportions of CD133^{high} cells, and to some extent, retained their increased ratio after transplantation of the four-time sorted cells (Figure 2 A-2; B-2). The highest expression of CD133 by a minority of prostate cancer cells overlapped with the highest expression of CD166 and CD44 (Figure 2 A-2, 3; B-2, 3; upper right quadrants). FACS analysis of CD44-FITC/CD166-PE revealed a distinctive cell population, much larger than the CD133^{high} population, which expressed the highest combined levels of these two markers (Figure 3; upper right quadrants). The ratio of this population also

Table I. *Correlation of the proportion of CD133+ cells with the density of cell culture and number of MACS sorting/culturing sets.*

Cells	High cell culture density*			Low cell culture density**		
	1st Isolation	2nd Isolation	4th Isolation	1st Isolation	2nd Isolation	4th Isolation
PC3-MM2	0.7±0.5	11.9±2.1	18.5±4.8	16.4±7.5	28.9±3.3	29.8±12.9

Before immunomagnertic sorting, cells were grown at standard conditions either till ≥75% confluency (*), or till 30-50% confluency (**).

correlated with the number of MACS-CD133 sorting sets and was higher in mice tumor xenografts compared to the original transplanted cells. After culturing at standard adherent conditions, the levels of CD133 expression and the number of cells with $CD133^{high}$ gradually decreased, reaching the basic pattern of expression characteristic for original unsorted PC3MM2 cells.

Formation of floating spheres in 3D cultures. Different phenotypic populations of PC3MM2 cells were examined to determine their ability to produce floating spheres in 3D soft agar or Matrigel matrix culture systems. A relatively low number of transplanted MACS-$CD133^+$ cells (1×10^3 cells per well in a 6-well plate) produced 50-60 large floating spheres in non-adherent culture conditions (Figure 4 A, B, upper panel), which can be serially passaged at least 6 times (C; late passage). In contrast, a much larger number (3×10^5 per well) of unsorted or bulk CD133-negative cells usually induced loose small colonies instead of well-formed large floating spheres by 3 weeks after transplantation (Figure 4 A, B, lower panel). Although some PC3MM2 spheres continued to grow in the same soft agar and Matrigel cultures after 5 weeks of culturing retaining an increased expression of CD133, by 6 weeks of culturing, the majority of floating spheres started to deteriorate, adhere and differentiate. FACS analysis of the dissociated sphere cells has shown that the majority of them expressed increased levels of CD133, CD44, CD166 and human EpCAM even after 5 weeks of culturing in non-adherent conditions (Figure 4 D; only CD133-APC/CD44-FITC is shown), also expressing higher ratio of cells with $CD133^{high}$ (Figure 4 D; area P4).

Prostate cells demonstrated an increasing sphere forming capacity and stability of floating spheroids after repeated MACS-CD133 sorts, transplantation to NOD/SCID mice and FACS selection of subpopulations with highest expression of CD133 or highest combined expression of CD44/CD166. To evaluate more accurately the difference in the efficiency of sphere forming, an equal number of cells of different type were plated on 96-well plates, cultured at non-adherent conditions for 3 weeks, 2 wells of each type were randomly chosen, all their contents dissociated and analyzed with FACS. Tumor xenograft cells after FACS selection of

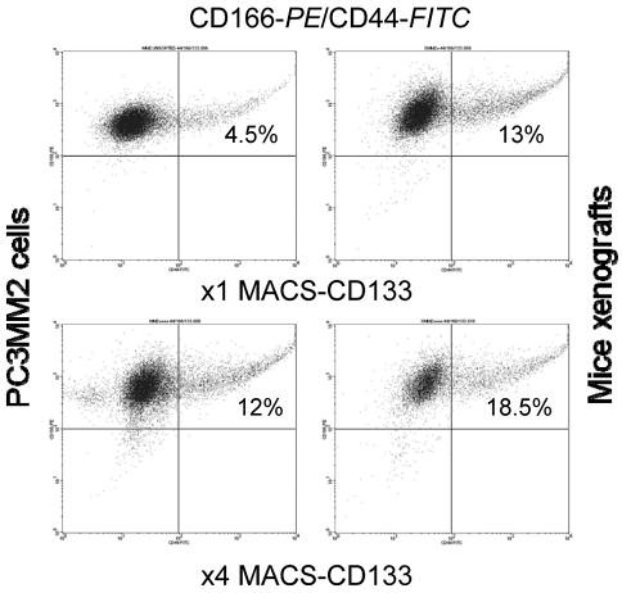


Figure 3. *The ratio of $CD44^{high}/CD166^{high}$ subpopulation of PC3MM2 cells correlates with the number of MACS-CD133 sorting sets and is higher in corresponding mice tumor xenografts.*

$CD133^{high}/CD44^{high}$ population produced at average 30% more sphere cells, which had also an increased number of $CD133^{high}$ cells compared to spheres induced by MACS-$CD133^+$ sorted cells. Taken together, these findings provide a basis for a working *in vitro* CSC model, which allows significant enrichment and relatively long-term propagation of highly tumorigenic and clonogenic $CD133^{high}$ cells needed for CSC-targeted drug development studies and research. Although, the $CD166^{high}/CD44^{high}$ cell population from both PC3MM2 line and corresponding mice tumor xenografts also possessed a high ability to form floating spheres, further experiments are needed to establish a functional significance of this subpopulation in prostate cancer.

Genome-wide gene expression analysis. To determine genomic differences of prostate CSC-enriched $CD133^{high}$ cells compared to their CD133-negative bulk counterparts, their

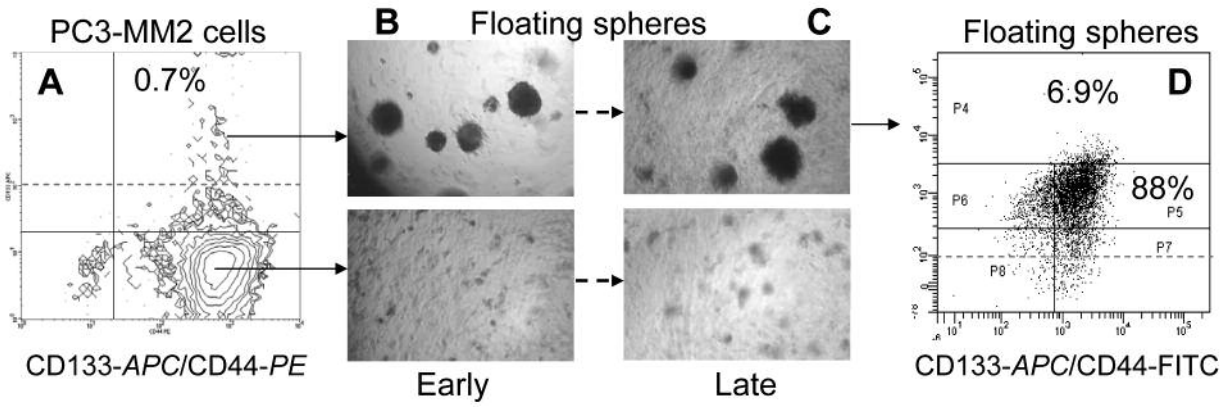
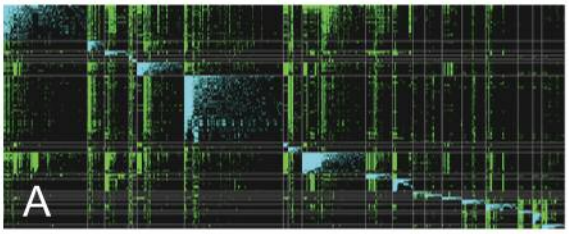


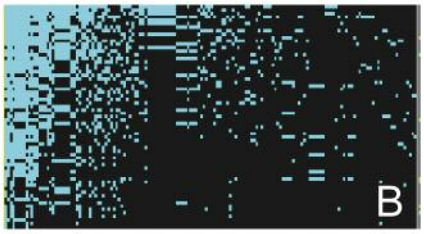
Figure 4. *Formation of floating spheres by different populations of PC3MM2 cells in 3D Matrigel culture. Transplantation of 1×10^3 PC3MM2 CD133+ cells (A, upper arrow) per each well of a 6-well plate induced 50-60 large floating spheres in non-adherent 3D culture conditions, which can be serially passaged at least 6 times (B, upper panels). In contrast, much larger number (3x105 per well) of unsorted or bulk CD133-negative cells usually induced loose small colonies instead of well-formed large floating spheres by 3 weeks after transplantation (A; B, lower panels). FACS analysis of floating sphere cells shows that the majority of them (area P6; 88%), in contrast to original PC3MM2 cells, expressed high levels of CD133, and 6.9% were $CD133^{high}$ (area P4).*

genome-wide gene expression profiles were studied using high-density oligonucleotide microarrays (Affymetrix Gene Chip HG-U133 Set). To increase the discriminating power of the gene microarray assay, repeatedly sorted CSC-enriched PC3MM2 cells with high expression of CD133 were studied compared to bulk tumor cells. Two hundred and thirteen genes with 10-100 fold increased activity were deter mined out of 8994 differentially expressed ones along with 87 genes with 5-50 fold decreased activity. Among the most up-regulated genes were anti-apoptotic genes, including *BIRC5* (survivin), *CDC2, TOP2A, MYBL2, HELLS, ANGPTL* and others. Another largest population of genes was related to the cell cycle regulation and proliferation, including cyclin B, *CCNB1, CDC2, CDCA 2, 3, 5* and *8, BUB1, ANLN, ATM, FOXM1, TACC3, PLK4, SHCBP1, GTSE1* and others. Several "stemness" genes involved in early development, including *MYBL* and *SOX4* were also significantly up-regulated. Among significantly down-regulated genes were those involved in regulation of apoptosis (*NUPR1, BCL2L1, TRIB3*), cell cycle/proliferation (*CDKN2B, TRIM13; SLC3A2*) and cell-cell and cell-matrix signaling (*S100A9, S100P, GDF15*). The microarray data and Gene Pathway analysis was carried out with DAVID (Database for Annotation, Visualization and Integrated Discovery) of the Advanced Biomedical Computing Center (National Cancer Institute, Frederick). The gene list with 2-fold cut-off was then further subdivided into functional categories with this bioinformatic analysis resource (Figure 5). All genes were grouped based on functional similarity using novel clustering algorithms. Global view of genes is shown on the fuzzy heat map (Figure 5 A); detailed view of several selected areas is shown on Figure 5 B-D. Summary of several functional gene clusters is shown on Figure 5E.

Discussion

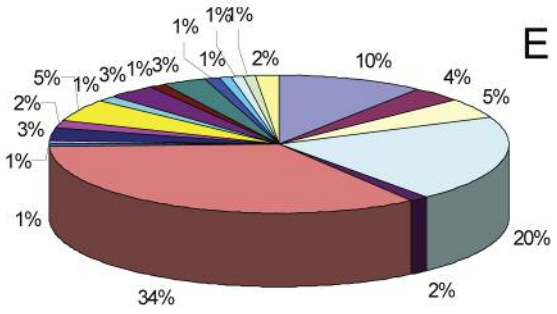
It is known that recurrent cancer is associated with more malignant phenotype, increased intrinsic or acquired drug resistance and high mortality rates (3). Therefore, it would be beneficial to develop drugs targeting CSCs from the most aggressive tumor types or cell lines, because such drugs can potentially have a larger spectrum of mechanisms of action, and therefore, broader anti-cancer implications. There is a growing body of evidence that tumorigenic cells with a $CD133^+$ phenotype are present in many human cancer types (reviewed above), and isolation of CSCs on the basis of CD133-positivity seems promising, since it was demonstrated that the expression of this marker correlates with both resistance to treatment (9-10, 33, 35-37, 40-41) and tumor aggressiveness and poor prognosis (37). However, primary tumors usually display a high variability in CD133, as well as in other marker content (8, 15-16, 42-43). The heterogeneity in the expression of CD44 was also demonstrated in several prostate cancer cell lines (32, 44), whereas it was reported that any primary prostate tumor has approximately 0.1 % of a $CD44^+/\alpha2\beta1/CD133^+$ phenotype (31). In addition, solid tumors contain both cancer stem cells and recruited normal stem cells, which share common cell surface markers, so obtaining the pure population of CSCs from primary tumors is difficult. Therefore, isolation of CSC from established cancer cell lines could be an attractive alternative approach providing a virtually unlimited source of CSCs necessary for CSC-targeted research and drug development. Since both $CD133^+$ and $CD44^+$ expressing cancer cells were associated with higher tumorigenicity and high drug resistance, the selection of





Gene Name	Function
ORC6	Cell cycle
Kinesin	Metastasis
Aurora kinase	Cell cycle
Cyclin B	Cell cycle
CDC6	Cell cycle

Gene Name	Function
CDC2	Apoptosis
BCL-2	Apoptosis
Angiopoietin	Cell Death
Rho	Motility
Rad	Motility
TNF	Proliferation
PP2B	Cell cycle



Gene Name	Function
ATM	Apoptosis
MAPK1	Cell Death
NOTCH	Cell cycle
Survivin	Apoptosis
SMAD 3	Cell cycle

Gene Function	%
Metabolic process	34
EGF-like binding	20
Cell differentiation	10
Stress response	5
Apoptosis	5
Transcriptional control	4
Protein degradation	3
Protein kinase	3
Cell adhesion	3
Rho signaling	2

Gene Function	%
Lipid & steroid synth	2
TGF-beta signaling	1
Ion binding	1
Gap junction	1
Protein secretion	1
NFkappaB cascade	1
Insulin signaling	1
Immune response	1
Chemotaxis	1
Protease inhibitor	1

Figure 5. *DAVID analysis of genome-wide gene expression profiling of CD133$^+$ versus bulk CD133-negative PC3MM2 cells with Affymetrix microarray assay.*

PC3MM2 cells with a constitutively higher ratio of tumorigenic cells expressing high levels of both markers would be useful for identification of the common mechanisms of drug resistance.

The suggested working prostate CSC model allows isolation and propagation of tumor-initiating cells with high *in vivo* tumorigenic potential and in sufficient quantities necessary for CSC-targeted research and drug development. The selected highly metastatic derivative of standard PC-3 cell line, PC3MM2, possesses a constitutively higher proportion of CD133^{high} cells, which results in higher intrinsic *in vivo* tumorigenic potential after transplantation to NOD/SCID mice and much higher *in vitro* clonogenic capacity in 3D culture systems compared to parental PC-3 cells. Several experimental conditions promoting an increased yield of tumorigenic CD133$^+$ cells and relatively long-term propagation without loss of CD133-positivity were employed. Such conditions include: a) culturing of original cells at low density, b) repeated cell sorting followed by culturing at low cell density; c) growing isolated CD133$^+$ cells in serum-free MSCB medium; d) growing floating spheres in 3D Matrigel or soft agar cultures by transplantation of cells with the

highest expression of CD133/CD44. This study is in line with the recently described culture of prostate telomerase-immortalized cell lines (27, 45-46) in non-adherent conditions; however, the PC3MM2 cell line represents a standard and largely available source of highly tumorigenic and metastatic prostate cancer cells.

Genome-wide differences have been determined in the pattern of gene expression in prostate CSC-enriched *versus* bulk cancer cells. DAVID analysis of the microarray data revealed several functional gene clusters related to anti-apoptotic and pro-apoptotic machinery, cell cycle regulation, proliferation, stemness, DNA repair, and cell-cell and cell-matrix signaling. These data support current knowledge that chemo- and radioresistance of CSCs should be attributed to up-regulation of anti-apoptotic genes, down-regulation of pro-apoptotic ones, active DNA repair, over-activation of ABC transporters and other mechanisms. Although further experiments are necessary to establish the functional significance of $CD44^{high}/CD166^{high}$ subpopulation in prostate cancer, recent findings suggest that targeting of CD44 can eradicate human acute myeloid leukemic stem cells (47). In conclusion, the suggested working prostate CSC model for the significant enrichment and relatively long-term propagation of highly tumorigenic and clonogenic $CD133^{high}$ or $CD133^{high}/CD44^{high}$ cells isolated from established aggressive prostate PC3MM2 cell line is potentially useful to model and study stem cell behavior and responses, since floating spheroids are enriched in cancer-specific stem cells (42, 48, 49), and is a more representative model compared to convenient monolayer cell cultures to study tissue hierarchy, the role of tumor microenvironment, metabolism, cell-cell and cell-matrix signaling and other aspects of tumor biology (50). It has potential practical implications in CSC-targeted drug development and may contribute to the identification of novel targets for CSC-targeted prostate cancer treatment.

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.

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Received October 22, 2008
Accepted October 30, 2008