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Prostate and Colon Cancer Stem Cells as a Target for Anti-Cancer Drug Development

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1. Introduction

With a worldwide cumulative incidence rate of 9.4%, colorectal cancer is the second leading cause of cancer deaths when both sexes are combined, and prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related death in men (Jemal et al., 2009). Standard anti-cancer drugs often fail to provide a long-term cure of epithelial tumors, which represent about 90% of human cancers. Thus, response rates in phase I oncology trials were as low as 2.5% over the last decade (Roberts et al., 04; Kamb et al., 07). Such limited effectiveness of standard anti-cancer therapies has been recently attributed to the existence of relatively rare, highly drug resistant, quiescent or slow proliferating tumor-driving cells - cancer stem cells (CSCs). Tumor cells with a stem cell-like properties, such as self-renewal and ability to differentiate into multiple cell types characteristic for particular tumor have recently been identified in all major human tumors, including prostate and colon cancers (reviewed in Dalerba et al., 07a; Mimeault et al., 07). Accumulated knoweledge suggests that majority, if not all tumors possess a minor subpopulation of stem cells and a major (or bulk) mass of progenitors at different stages of their maturation. Malignant stem-like subpopulation within the tumors possesses exclusive tumor-initiating capacity *in vivo* (after serial transplantation to the immunodeficient mice) and high potential to induce 3D cancer spheroids *in vitro* (after serial passaging). Since CSCs are responsible for tumor initiation, development and metastasis, and are highly resistant to standard anti-cancer therapies, they are likely to be the most crucial target in the treatment of cancer. This new concept of carcinogenesis and new paradigm in anti-cancer therapy requires significant reconsideration of previously accepted criteria of the drug effectiveness and development of novel, physiologically and clinically more relevant experimental models. Although isolation and purification of the cancer-specific CSCs reamain to be problematic due to lack of the unique CSC surface markers and insufficient knowledge of the CSC biology, several methodological approaches allow for prospective isolation, purification and reasonable propagation of these cells. Applying these approaches, we and others previously have shown that prostate and colon tumor-initiating cells are functionally, genomically and morphologically different from their bulk tumor counterparts. In this chapter we will discuss novel criteria of the anti-cancer drug efficacy, and present our data on the CSC-targeted activities of a new-generation taxoids.

2. Prostate and colon CSC phenotypes

It is increasingly recognized now that, similarly to normal mammalilan tissues, tumors are organized hierarchically, comprising a minor population of the long-lived self-renewing stem cells, which also give rise to all the heterogeneous cell phenotypes due to ongoing differentiation. CSCs share some basic features and signal transduction pathways, such as Wnt, Shh, Notch, Bmi-1 and others with normal stem cells (Pardal et al., 2003; Reya & Clevers, 2005), and most of the CSC types have been identified and isolated using common cell surface markers. Although none of the currently available cell surface markers can be considered as universal or highly specific for CSCs, several markers were successfully used for prospective isolation of the tumor-initiating cells from diverse tumor types. Among them are the two most commonly used, CD133 (also known as AC133 and prominin-1) and CD44. Thus, several human cancer types, including brain tumors (Singh et al. 2003), kidney (Bussolati et al., 2005), prostate (Collins et al., 2005), hepatocellular (Suetsugu et al., 2006; Yin et al., 2007), colon (O'Brien etal., 2007; Ricci-Vitiani et al., 2007), and pancreatic (Hermann et al., 2007; Li et al., 2007) carcinomas have minor population of CD133-positive cells which have much higher tumorigenic and clonogenic potentials compared to their CD133-negative counterparts or unsorted cells. Other markers, including CD166, Musashi-1, CD29, CD24 (Vermeulen et al., 2008), and leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5; Barker et al., 2007) were also suggested for isolation of CSCs.

CD133 is a cell-surface glycoprotein comprising five trans-membrane domains and two large glycosylated extracellular loops (Shmelkov et al., 2005). CD44 is also a multistructural and multifunctional cells surface adhesion molecule involved in cell-cell and cell-matrix interactions, stemness and tumour development, in part via β-catenin and Wnt signaling activation of the CD44 gene transcription (Ponta et al., 2003; Marhaba & Zoller, 2004). Although in many studies both CD44 and CD133 were used as a single cell surface markers and were reported as putative CSC markers, accumulated experimental data suggests that combination of several markers allows for better enrichment of cells with either exclusive or highly increased tumorigenicity in comparison to their bulk counterparts. Thus, the subfraction of prostate cancer cells with $CD44^{+}\alpha_2\beta_1^{hi}CD133^{+}$ phenotype was first described by Collins and colleagues (Collins et al., 2005) as possessing the highest *in vitro* proliferative potential, self-renewal, and the lack of androgen receptor expression. Of note, since normal prostate stem cells are also androgen independent (Isaaks, 1985; Collins et al., 2001; Richardson et al., 2004), it suggests they may be the cells of origin of prostate cancer. It remains to be established whether cancer-specific CSCs represent homogeneous or heterogeneous phenotypic populations. It is also unclear whether some commonly used markers, such as CD133 and CD44, are of equal functional importance. A recent study has demonstrated the unique role of CD133 in the normal and malignant colon, showing that $CD133^{+}$ normal stem cells at the base of crypts in the adult intestine (a stem cell niche) not only generate the entire intestinal epithelium, but give rise to all the neoplastic cells in mice colon tumors (Zhu et al., 2009a). However, another study has shown that only a knockdown of CD44, but not CD133, strongly prevented clonal formation and inhibited tumorigenicity in mice xenograft model (Du et al., 2008). Authors reported that $CD44^{+}$ did not colocalize with $CD133^{+}$ cells within colorectal cancer. Similar results reported by Horst and colleagues showed that the expression of CD133 correlates with that of CD166, while both do not correlate with CD44 (Horst et al., 2009). However, this data contradicts multiple reports showing not only the colocalization of the CD133 and CD44 in several types of human

cancer (Collins et al., 2005; Dalerba et al., 2007b; Haraguchi et al., 2008; Zhu et al., 2009b), but also suggesting their combined expression as the best CSC marker (Haraguchi et al., 2008; Zhu et al., 2009b). Since clinical specimens of solid tumors are highly heterogeneous, and membrane expression of CD133 and CD44 undergo a complex post-translational regulation, it may significantly contribute to controversial interpretation of experimental data obtained by diverse experimental approaches.

Although the tumorigenic subset of colon cancer cells was initially identified as CD133-positive (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), in several established cell lines and some clinical specimens both CD133 and CD44 are quite abundant and can not solely

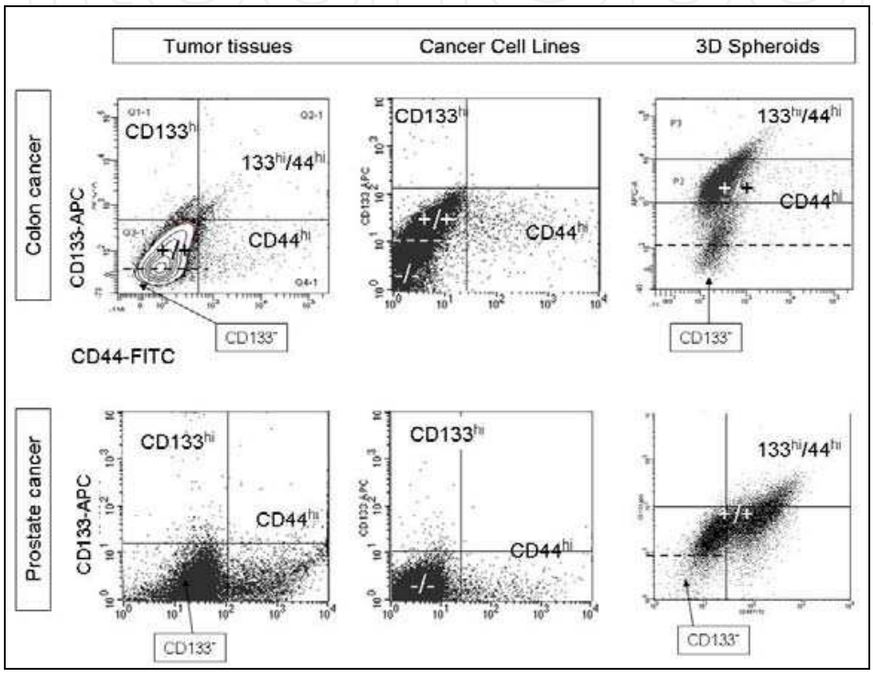

Fig. 1. Phenotypic analysis of colon and prostate cancer cells obtained from patient samples (left column), established cell lines (middle column) and 3D spheroids induced by $CD133^{hi}/CD44^{hi}$ subpopulations (right column). Majority of colon cancer cells of different origin are positive for CD133, CD44 (+/+). In contrast, bulk prostate cancer cells are negative for CD133, and negative or low positive for CD44. However, both cancer types possess minority subpopulations with high expression of each marker ($CD133^{hi}$; $CD44^{hi}$), or high combined expression ($CD133^{hi}/CD44^{hi}$). Both colon and prostate cancer spheroids induced by $CD133^{hi}/CD44^{hi}$ populations in general express much higher levels of these markers compared to parental cell lines, and much larger populations of cells with $CD133^{hi}/CD44^{hi}$.

demarcate the tumor-initiating cells. Clinical specimens often display highly variable levels of these markers, and in such cases combination of CD44 and CD166 with the epithelial-specific antigen (ESA; also known as the epithelial cell adhesion molecule, EpCAM) was suggested as more specific for colon CSCs (Dalerba et al., 2007b; Dylla et al., 2008). In addition, in some metastatic colon cancers and long-term maintained cell lines, such as HCT116, both $CD133^{+}$ and CD133-negative cell populations can induce tumors in NOD/SCID mice (Schmelkov et al., 2008; Botchkina et al., 2009). There is also some

misinterpretation of the terminology concerning colon cancer cells, which may be *positive* for particular CSC markers, but only minority populations enriched with CSCs can express *high levels* of these markers. Thus, majority of cells in invasive long-term maintained HCT116 cell lines is *positive* for CD133, CD44 and CD166 (*Figure 1*; upper row; marked as +/+), which were considered as a good single markers for isolation of the minor subpopulation of tumorigenic cell in multiple cancer types and established cell lines. In contrast to the colon cancer cell lines, majority of the prostate PC-3 cells and their metastatic derivatives are negative for CD133, and only minor subpopulations express high levels of both CD133 and CD44 (*Figure 1*; lower row; *Figure 2*, upper row). However, only cells with *highest* levels of CD133 and CD44 (marked as $CD133^{hi}$ and $CD44^{hi}$) grown under stem cell-promoting conditions (type I collagen-coated surfaces, serum-free medium, low cell number and repeated cell sorting) allows for significant enrichemnt of prostate and colon CSCs and increase of their tumor-initiating and clonogenic capacities (Rowehl et al., 2008; Botchkina et

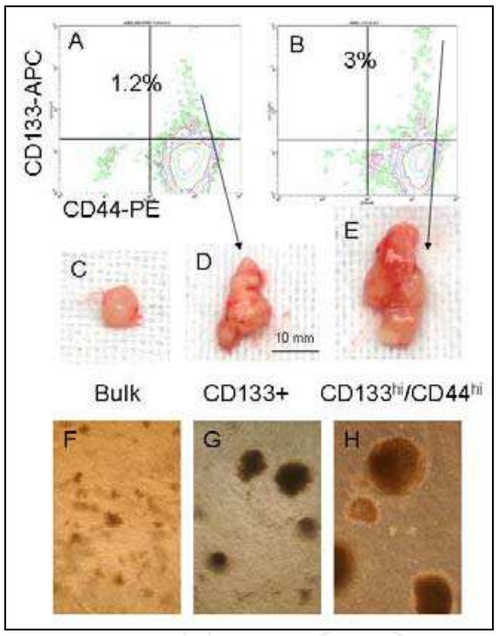

Fig. 2. Phenotypic (A, B), tumorigenic (C-E) and clonogenic (F-H) analyses of the prostate cancer cells. Subpopulation of $CD133^{hi}/CD44^{hi}$ cells is larger in repeatedly sorted cells (B; upper right). Mice tumor xenograrfts induced by $1x10^7$ of the unsorted (bulk) cancer cells (C), $1.5x10^3$ of one-time MACS sorted CD133+ cells (D), and by $1.5x10^3$ of repeatedly sorted and grown on type I collagen cells with higher ratio of $CD133^{hi}/CD44^{hi}$ phenotype (E). The FACS-sorted $CD133^{hi}/CD44^{hi}$ cells possess significantly higher sphere-forming capacity (H) in contrast to the unsorted (F) and MACS-sorted (G) cells.

al., 2009). Prostate and colon cancer spheroids induced by $CD133^{hi}/CD44^{hi}$ cell populations expressed much higher levels of these markers in general, and much more cells were highly positive for CD133 and CD44. Since cells with higher levels of expression of these cell surface markers (after repeated cell sorting and culturing under stemness-promoting conditions) possess significantly increased tumorigenic and clonogenic potentials (*Figure 2*; prostate cancer PC3MM2 cells are shown), it suggests that these cell populations are enriched with putative CSCs.

3. Cancer models

The relevance of the *in vivo* and *in vitro* cancer models to patient tumors remains to be a topic of controversy. Human cancers represent an extremely heterogeneous class of diseases, and each clinical case is unique pathologically and highly heterogeneous biologically in terms of gene expression patterns and levels, the tumor/host interactions, interecations between cells and the extracellular matrix, and many other. It is crucial to isolate tumorigenic cells from each cancer type and, ideally, from maximal possible number of clinico-pathologically different cases for their precise molecular characterization and designing of individual treatment strategies. In addition, cancer cells in general have high rates of genetic and epigenetic changes (Hill et al, 1984; Hill et al., 2006). It is established that long-term culturing can change a malignant phenotype of particular cell line, and same is true for *in vivo* passaging of human cancer xenografts as solid tumors in nonsyngenic host - immunodeficient mice. In this context, transplantation of the tumor cells directly derived from patients into immunodeficient mice (early passages without *in vitro* passaging) should recapitulate original tumors relatively closely. However, biopsy material is usually limited and contaminated with normal stem cells, therefore isolation of the rare putative CSCs is still problematic due to the lack of specific CSC markers. In contrast, established cancer cell lines do not have any normal stem cells, because they quickly loose their stemness and differentiate in standard culture condition. Therefore, cancer cell lines could be an attractive alternative source of cells for CSC research and drug development.

It is clear now that traditionally used monolayer of adherent cancer cells has a very limited relevance to the hierarchically organized *in vivo* tumors, because such cultures have unnatural cell-to-cell and cell-to-matrix contacts, which can significantly affect their phenotype, signal transduction pathways and drug response. Since monolayer cultures are directly exposed to medium content and are readily accessible to oxygen, which is an important signal for stem cell self-renewal, apoptosis, differentiation and migration (reviewed in Friedrich et al., 2009), biological and therapeutic studies on two-dimensional cancer cell cultures have limited clinical relevance and may lead to inaccurate conclusions. This model is even less suitable for stem cell-based studies, because even highly purified CSCs can undergo relatively fast differentiation after being placed in adherent culturing conditions. On the other hand, standard cancer cell lines represent virtually unlimited resource, therefore, it would be useful to have standardized experimental conditions for obtaining a highly tumorigenic and drug resistant CSCs in sufficient quantities, which is a prerequisite for preliminary screening/development of potentially effective CSC-targeted drugs, as well as for investigation of general properties of CSCs.

An alternative 3D model of free-floating cancer spheroids was established by Sutherland and colleagues long before the discovery of CSCs (Inch et al., 1970; Sutherland et al., 1971). This model is more closely related to original tumors with respect to cell morphology, metabolic and proliferative gradients, oxygen and drug penetration, cell-cell junctions, kinases activation and other parameters, compared to the cancer cell monolayers (Friedrich et al., 2009). Spheroid cells have an enhanced resistance to many of the commonly used anti-cancer drugs (Dessoize et al., 2000; Yoshida et al., 2008), showing dramatically lower cytotoxicity against 3D cancer spheroids compared to monolayer cultures, and exhibit chemoresistance which recapitulates this resistant phenotype *in vivo* (Dubessy et al., 2000; Durand et al., 2001; Friedrich et al., 2009). Increased resistance of spheroid cells to ionizing radiation was first demonstrated by Sutherland and colleagues (Inch et al., 1970; Sutherland

et al., 1971). The floating cancer spheroids are organized hierarchically, similarly to the *in vivo* tumors, containing relatively small (although usually increased compared to the parental tumor) population of the tumorigenic cells and a large spectrum of their progenitors, the bulk tumor cells at different stages of differentiation. They can be passaged for many generations, suggesting that they contain a population of cells with extensive self-renewal capacity. Thus, the cancer spheroids induced by primary colon carcinoma cells select for cells that coexpress multiple CSC markers, including CD133, CD166, CD44, CD24, CD29 (Vermeulen et al., 2008) and Lgr5 (Barker et al., 2007). We found that both mice tumor xenografts and 3D spheroids induced by more purified phenotypic populations of cancer-specific tumorigenic cells ($CD133^{high}/CD44^{high}$ for prostate and colon cancer) have higher tumorigenic and clonogenic potentials, and much higher ratio of cells with original phenotype, even after several weeks in 3D culture (Rowehl et al., 2008; Botchkina et al., 2009). Striking correlation between ability to form compact 3D spheroids and invasive potential was recently demonstrated on ovarian cancer cells (Sodek et al., 2009). Although 3D cancer cell cultures were developed several decades ago, earlier studies focused on analyses of drug responses were usually limited to the relatively short-term gross evaluation of the inhibition of spheroid growth and apoptosis, but specific stem cell-related responses of spheroid cells were not studied. Recently, several mechanisms were suggested as a mediators of the CSC drug resistance, including replication quiescence, high expression of ABC transporters, active DNA repair, activation of anti-apoptotic pathways, down-regulation of the apoptotic machinery and others (Dean et al., 2005; Donnenberg et al., 2005; Mimeault et al., 2007).

Therefore, taking into account all of the above, early passage cancer floating spheroids induced by purified cancer-specific CSCs and early passage patient-derived mice tumor xenografts can be suggested as relatively suitable models for studying CSC-targeted drug efficacy. Both mice tumors and spheroids induced by purified CSCs contain higher ratios of cells with original transplanted phenotypes compared to parental sources. Since CSCs represent a dynamic population with dual potential, self-renewal versus generation of the committed progenitors, which eventually will differentiate into all mature cell phenotypes, isolated CSC phenotypes should be cultured, tested and treated under conditions designed to retain their "stemness" and preclude differentiation to the bulk tumor cells. The isolated cell phenotypes should be functionally tested for at lest major stem cell properties, including self-renewal capacity *in vivo* (ability of the particular cell phenotype to induce tumors in NOD/SCID mice after serial transplantations of the low cell number); self-renewal capacity *in vitro* (ability of the particular cell phenotype to induce 3D colonospheres during serial passaging under non-adherent, serum-free culture conditions), and plasticity (ability to produce all the differentiated cell phenotypes characteristic for particular tumor under standard culture conditions). In addition to standard methods of analysis of cytotoxicity, CSC-targeted drug activities should be also evaluated by functional analyses of stem cell-related properties, as well as by comparative genomic and molecular analyses.

4. Genomic characteristics of the prostate and colon CSCs

Genome-wide Gene Expression Profiling

We studied the genome-wide gene expression profiles of prostate and colon CSCs using high-density oligonucleotide microarrays (Affymetrix Gene Chip HG-U133 Set). To increase

the discriminating power of the gene microarray assay, either repeatedly MACS-CD133 sorted and grown on type I collagen-coated surfaces at low density prostate PC3MM2 and colon HCT116 cells, or floating spheroids induced by $CD133^{high}/CD44^{high}$ phenotypes in serum-free MSCB medium were analyzed in comparison to their bulk adherent counterparts. In prostate tumorigenic cells, we have determined 213 genes with 10-100 fold increased activity out of 8994 differentially expressed ones, and 87 genes with 5-50 fold decreased activity (Rowehl et al., 2008). 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 developmental pathways, including *MYBL* and *SOX4* were also significantly upregulated. Of interest, the *ASPM* gene, which is responsible for accelerated human brain evolution and also is overexpressed in some human cancers (34) displayed 128-fold higher expression in prostate CSCs compared to the bulk tumor cells. Among significantly downregulated 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*).

In colon tumor-initiating cells, we have found that the microarray assay has much higher discriminating power in analysis of cells from floating spheres. Thus, we have determined more than 500 of significantly (3-120 fold) upregulated genes out of 4351 differentially expressed ones, and 436 genes which were downregulated by 3-1500 folds in colon CSCs grown as floating spheroids (Botchkina et al., 2009). For comparison, analysis of single-time MACS-$CD133^+$ cells versus unsorted cells has shown only 988 differentially expressed genes with 162 signifficantly up-regulated ones. It can be explained by constitutively high expression of CD133 by the majority of colon cancer HCT116 cells, which predominantly represent progenitor cells. We have determined that, similarly to the prostate CSCs, majority of the most upregulated genes were those related to anti-apoptosis (*APP*, Bcl3/NF*kappa* B2 complex, *BDNF, BIRC3, BIRC4, BTRC3, CBX4, CCAR1, CCPG1, CD74, DHCR24, FOXO3, HSPA1B, IGFBP3, IF16, NFKB1A, TBX3, TNFAIP3, TRIB3* and others); cell cycle/cell proliferation (*FOSB, IL-8, CCNG2, IGFBP3, TGFBP1, MXD1, INSIG1, EHF, CD74, CDC25A, HSMPP8*); and transcription factors (*ID2, ID2B, DENR, MXD1* and many others). Several stemness genes were also upregulated (NOTCH pathway; *APP, MIB1*; Wnt receptors *TGFB1I1, CSNK1D*). High number of genes regulating Ca^{2+} homeostatis and calmodulin binding also revealed significantly altered expression which is most likely connected with the altered induction and regulation of apoptosis in CSCs. The most significantly downregulated genes in HCT floating spheres were *HLI4* (1500-fold) which is responsible for heterophilic cell adhesion; apoptosis-related cytochrom *c, COX6A1* gene (300-fold), and *BCL2L1* which regulates the release of cytochrom *c* from mitochondria; *CXCL14* gene involved in cell-cell signaling (100-fold). Among other significantly downregulated genes were 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*); 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. This data is in line with current knowledge that chemo- and radioresistance of CSCs is attributed to up-

regulation of anti-apoptotic genes, down-regulation of pro-apoptotic ones, active DNA repair, reactivation of some developmental signaling cascades, and other mechanisms (Dean et al., 2005; Mimeault et al., 2007).

Stem Cell-Related Gene Expression Profiling

We analyzed the floating spheroids induced by CD133^{high}/CD44^{high} cell populations derived from the three independent colon cancer cell lines, including HCT116, HT29 and DLD-1 with the stem cell pathway-specific PCR Array assay (*SA*Biosciences). Each array contains SYBR Green-based real-time PCR gene-specific assays for a set of 84 genes. Using filtering criteria of a 1.5 or greater fold-change in expression, we have analyzed differentially expressed genes in these three types of floating colonospheres compared to their bulk differentiated adherent counterparts (Botchkina et al., 2010). The most profound differences were observed in HCT116 spheroids grown from CD133^{high}/CD44^{high} cells (*Figure 4 A;* left histogram), which is in line with their higher sphere-forming and tumor-initiating capacities compared to cells of the same phenotype isolated from HT29 and DLD-1 lines. About one-fourth of the analyzed stem cell-related genes, including Wnt and Notch pathway genes responsible for self-renew and cell cycle regulation, were commonly up-regulated in all types of spheroids, with significantly higher levels of expression in HCT116 ones. Thus, 6 of 6 analyzed genes responsible for stem cell self-renewal (*SOX1, SOX2, MYST1, MYST2, NEUROG2* and *HSPA9*), and 3 of 5 genes regulating symmetrical/asymmetricasl cell division (*NOTCH1, NOTCH2* and *PARD6A*) were significantly up-regulated in the HCT116 CD133/CD44-high colonospheres compared to their bulk counterparts. The most significantly up-regulated genes in HT29 spheroids were *ACAN, ALPI, APC, ASCL2, CCND2, CD3D, CD4, CD8A, CD8B, COL2A1, COL9A1, DHH, DLL3, DTX1 FGF1, GJA1, S100B,SOX2, T, TERT* and *WNT1;* and in DLD-1 spheroids - *ALDH1A1, ASCL2, CCND2, CD4, COL1A1, DLL1, DTX1, FGF1, GJA1, IGF1, JAG1, MME, NCAM1,* and *NOTCH1.*

In metastatic prostate cancer PC3MM2 cell line, majority of the analyzed stemness genes were also dramatically up-regulated in spheroids induced by CD133^{high}/CD44^{high} cells compared to their bulk counterparts (*Fig.4 B;* left histogram), wich is in line with the Affymetrix microarray data. Multiple developmental genes, including *NOTCH1, NOTCH2, NUMB, DTX2, DLL3, JAG1, WNT1, MYC, SOX1, SOX2,* and genes involved in general regulation of stem cells self-renewal and maintenance, including *NEUROG2, MYST1, MYST2, HSPA9B, DLL1, PPARD, FRAT, CD44, COL2A1, DVL1, TERT, ASCL2, BTRC* and others were overactivated. The ABC transporters-related gene, *ABCG2* was also up-regulated in prostate spheroids compared to the corresponding adherent cell cultures, which together with the upregulated anti-apoptotic and down-regulated pro-apoptotic genes might explain dramatic increase in the resistance to drug treatment of 3D spheroids versus adherent cancer cell cultures.

Accumulated data suggest that recently discovered transcription factors essential for stem cells self-renewal and maintenance of pluripotency, including OCT4, SOX2, c-Myc and Klf4 (Takahashi et al., 2006; 2007), are closely related to cancer invasion, metastasis and CSC maintenance. Thus, expression of the SOX2 and OCT4 was associated with less differentiated phenotype, distant recurrence and poor prognosis for colorectal cancer (Tsukamoto et al., 2005; Saigusa et al., 2009). It was shown that some prostate cancers overexpress several genes typically associated with stem cells, including *Bcl-2, OCT3/4, BMI1,* β-*CATENIN*, *SMOOTHENED* and others, which indicates that these tissues may contained some significant ratios of the CSCs (reviewed in Mimeault & Batra, 2006). We

have found that floating cancer spheroids contain a minority cell populations (about 3-4%of the spheroid cells) with high levels of expression of several transcription factors, including c-Myc, Oct4, Sox2 and NANOG. The flow cytometry data were confirmd with western blot analysis shown the presence of these proteins in total lysates of the spheroid cells, as well as in repeatedly sorted cells with $CD133^{high}/CD44^{high}$ phenotype.

5. CSC-targeted activities of the new-generation taxoids

It is largely accepted now that effective anti-cancer drugs should be targeted toward the cancer-specific tumor-initiating cells, not only the bulk tumor cells. For advanced prostate cancer, androgen deprivation therapy remains the most widely used treatment modality. However, although it induces remission in about 90% of patients, in ~18 months all patients relapse with a hormone-refractory drug resistant disease, which is invariably fatal (overall median survival is 23-37 months). Such resistance to hormonal therapy was associated with the lack of androgen receptors on the putative prostate CSCs (Isaaks, 1999; Taplin & Balk, 2004; Maitland & Collins, 2008). Colon cancer is inherently drug-resistant due to multiple mechanisms that are still poorly characterized, so both CSCs and the progenitor cells can potentially contribute to chemotherapy tolerance.

Numerous studies have demonstrated that both CD133- and CD44-positive fractions in many cancer types are exceptionally resistant to standard anti-cancer therapies (Frank et al., 2003; Frank et al., 2005; Bao et al., 2006; Liu et al., 2006; Hong et al., 09; Vlashi et al., 09). Moreover, there is growing evidence that conventional therapeutic modalities focused on the tumor debulking may actually *promote* cancer progression by stimulating quiescent CSCs to divide symmetrically (self-renewal) and repopulate the tumor mass with undifferrentiated cells (Bao et al, 2006; Dirks, 2006 ; Eramo et al., 2006; Woodward et al., 2007; Todaro et al, 2007; Bleau et al., 2009). Multiple evidence indicate that the ratio of $CD133^{+}$ cells correlates with tumor aggressiveness, histologic grade and clinical outcome (Al-Hajj et al, 2003; Liu et al., 2006; Zeppernick et al., 2008; Maeda et al., 2008; Horst et al., 2008; Wang et al., 2009). In colorecral cancer, elevated levels of CD133 expression were associated with distant recurrence (Yasuda et al., 2009) and resistance to chemo- and radiotherapy (Saigusa et al., 2010). The proportion of $CD133^{+}$ cells in colon cancer metastases is higher than in primary tumors (Puglisi et al., 2009). Similar data were reported for CD44-positive cells (Hong et al, 09). There is also growing data that CSCs, in particular CD133-positive cells, express several pluripotency markers (Chen et al., 2008), which was linked to their chemo- and radioresistant properties. The expression of CD133, Sox2 and Oct4, was increased after treatment with chemo- (Levina et al., 2008) and radiation therapy (Saigusa et al., 2009), and was also associated with an unfavorable clinical outcome (Wang et al., 2009). Taken together, it can explain the well known fact that metastatic lesions are more resistant to treatment compared to primary tumors. Since CSCs, similarly to other types of stem cells, have almost unlimited ability to self-renew, treatment strategies can be focused either to direct elimination of tumor-initiating cells, abrogation of their stemness, or promotion of their differentiation. This new paradigm of cancer treatment requires development of novel drug molecules and additional, stem cell-relevant criteria to assess CSC drug responses.

Paclitaxel (Taxol®, Bristol-Myers Squibb) and its semisynthetic analog Docetaxel (Taxotere®, Aventis) are the most commonly used anti-cancer drugs and standard chemotherapy of colon and hormone-resistant prostate cancers. These taxanes bind to the β-tubulin subunit, accelerate the polymerization of tubulin, thereby stabilizing the microtubules and inhibiting

their depolymerization, which results in the arrest of the cell division cycle and consequent apoptosis. Although both paclitaxel and docetaxel possess potent antitumor (debulking) activity, most treated patients ultimately manifest resistance to the drugs and recurrence of the disease, which is known to be associated with a more malignant phenotype and high mortality rates (Mimeault et al., 2007). Thus, two large phase III trials (TAX 327 and SWOG 9916; Southwest Oncology Group) have demonstrated that these drugs increased an overall survival in patients with hormone-refractory metastatic prostate cancer from 16-17 months to only 17.5-18.9 months (Roberts et al., 2004). To develop new taxane anticancer agents with fewer side effects, superior pharmacological properties, and improved activity against drug-resistant human cancers, extensive structure-activity relationship studies on taxol and its congeners have been performed in different laboratories. Several novel second- and third-generation taxoids with systematic modifications at the C2, C10, and C3'N positions were synthesized in Dr. Ojima's group (reviewed in Ojima & Das, 2009). It was determined that (i) the C3'-phenyl group was not an essential component for their potent activity and (ii) the modifications of the C10 position with certain acyl groups as well as the replacement of the phenyl group with an alkenyl or alkyl group at the C3' position made compounds 1–2 orders of magnitude more potent than the parent drugs (paclitaxel and docetaxel) against drug resistant human breast cancer cell lines. These highly potent taxoids were termed "second-generation taxoids". Furthermore, we found that introduction of a substituent (e.g., MeO, N_3, Cl, F, etc.) to the *meta* position of the C2-benzoyl group of the second-generation taxoids, enhanced the activities 2–3 orders of magnitude higher than the parent drugs against different types of the drug-resistant cancer cells (Ojima & Das, 2009).

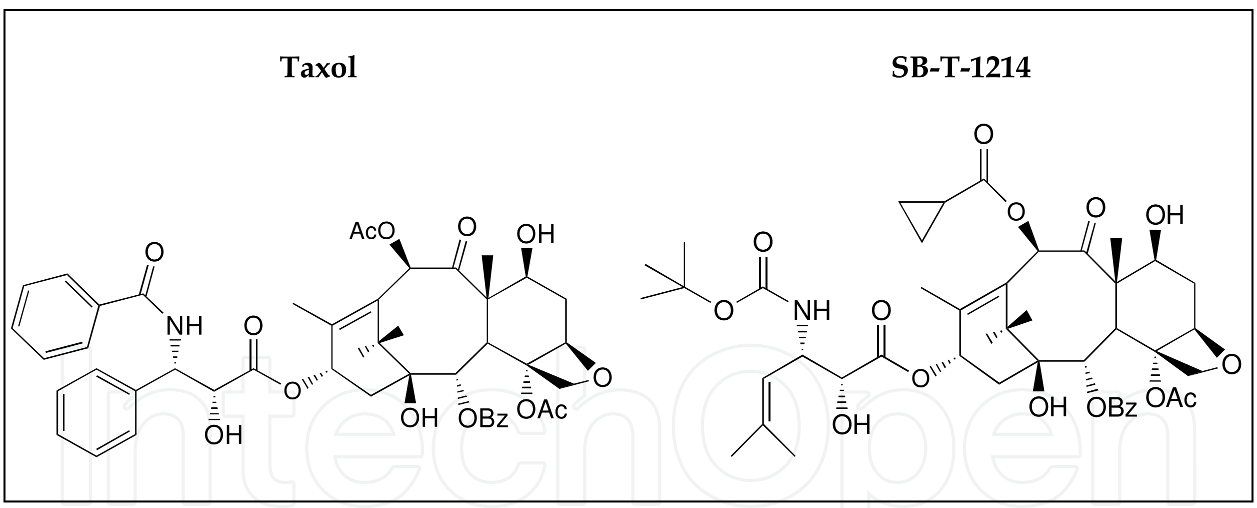

Fig. 3. Chemical structure of taxol (A) and new-generation taxoid, SB-T-1214 (B).

The antitumor activity of SB-T-1214 (*Figure 3*), one of the leading candidates among the new generation taxoids studied in our laboratory, was assayed *in vivo* against a Pgp+ DLD-1 human colon tumor xenograft in SCID mice, as well as against highly drug-resistant CFPAC-1 pancreatic tumor xenografts. The drug was administered intravenously in three doses 3 times using a 3-day regimen, starting from day 5 after DLD-1 subcutaneous tumor implantation. As anticipated, paclitaxel was ineffective against this highly drug-resistant (Pgp+) tumor at its optimal dose (60 mg/kg total dose). In contrast, SB-T-1214 has shown profound antitumor activity, with the best result at 60 mg/kg total dose, 20mg/kg x 3, wherein complete regression of the DLD-1 tumor was achieved in five of five mice (tumor

growth delay was >201 days). Systemic toxicity profile has shown that there was only a 3-5% weight loss during the period of day 15 to day 20, and the drug was well tolerated by animals (Kuznetsova et al., 2006). Histopathological analysis of the hematoxylin and eosin stained tissue sections of the tumor xenografts recovered from the control (vehicle treated) mice revealed a large tumor areas with densely packed tumor cells (Botchkina et al., 2010), which uniformly expressed membrane-bounded immunoreactivity for human epithelial cell adhesion molecule, *h*EpCAM. Several small clusters of cells with high levels of CD133 expression were found predominantly within the outer areas of the tumors corresponding to the tumor invasive front, whereas scattered CD133+ cells were detected across the entire tumor areas. Flow cytometry analysis of the dissociated and immunomagnetically (MACS-*h*EpCAM) sorted mice tumor xenografts confirmed the presence of a minor population (about 4%) of human cancer cells with high combined expression of the CD133 and CD44. After three consequent treatments with the SB-T-1214, we observed a complete reduction in tumor volume. Residual tissues showed multiple inflammatory infiltrates and fibrosis, and were negative for human EpCAM and CD133. Since tumor growth delay was comparable with the lifespan of SCID mice, we hypothesized that this compound could affect timorigenic cell populations by modulation of some stemness genes and signaling pathways. To test this hypothesis, the CSC-specific effects of SB-T-1214 were studied on previously characterized three independent invasive colon cancer cell lines (HCT116, HT29 and DLD-1), as well as on highly metastatic derivative of the prostate PC-3 cell line, PC3MM2, which was kindly provided by M. D. Anderson Cancer Center (USA). The tumor-initiating cells were first isolated and enriched with a fluorescence activated cell sorting (FACS) based on highest combined expression of the CD133 and CD44. We have found that majority of cells in all selected colon cancer cell lines grown at standard adherent conditions expressed moderate levels of CD133, CD44 and CD166. However, all three cell lines possessed minority cell populations with highest expression of CD133, which coincided with high expression of CD44 ($CD133^{high}/CD44^{high}$). Then selected cell subpopulations were subjected to further purification and propagation using several approaches, which include repeated cell sorting, short-term culturing at low cell density on type I collagen-coated surfaces, growing cells in serum-free stem cell medium and others. To confirm that selected cell phenotypes posess the stem cell-related characteristics, they were subjected to functional and genomic analyses as we previously described (Rowehl et al., 2008; Botchkina et al., 2009). We have determined that even without additional purification, the acutely isolated $CD133^{high}/CD44^{high}$ cells derived from all three colon cancer cell lines possessed relatively high efficiency in forming dense floating multicellular spheroids in non-adherent cultures with serum-free medium in contrast to their corresponding bulk counterparts, which produced a few loose flat colonies. Dissociated spheroid cells retained an original cell phenotype and expressed all the studied commonly used stem cell surface markers, including CD133, CD44, CD166, *h*EpCAM, CD49b, and CD117. Immunohistochemical analysis of spheroid cells revealed a minority cell population expressing high levels of nuclear β–catenin.

In our previous studies we have found that short-term culturing of repeatedly sorted cells on type I collagen-coated surfaces in serum-free stem cell medium led not only to the retaining, but to significant increase of the ratios of the tumor-initiating cell phenotypes. This data is in line with a recent study showing that human colorectal carcinoma cells grown on type I collagen in serum-free medium undergo an epithelial-mesenchymal-like transition and downregulation of E-cadherin and β-catenin at cell-cell junctions (Kirkland et al., 2009). Authors have found that collagen type I inhibited cell differentiation, increased

clonogenicity and promoted expression of CD133 and Bmi1, indicating that it promoted expression of a stem cell-like phenotype in colon cancer cells. Therefore, the CSC-targeted effects of the SB-T-1214 were tested under two experimental conditions: a) using purified CSCs grown adherent to the type I collagen, which promote stemness and retain selected cell phenotypes in undifferentiated state; and b) using 3D spheroid cultures induced by the purified CSCs, which also allow for enrichment of CSCs and retaining of the undifferentiated phenotype in major cell population. As we mentioned above, spheroid cells are highly resistant to standard treatment modalities, possess high tumorigenic and clonigenic potentials, and express many markers of stemness, including CD133, CD166, CD44, CD24, CD29 (Rowehl et al., 2008; Vermeulen et al., 2008; Botchkina et al., 2009) and Lgr5 (Barker et al., 2007). As discussed above, these features are characteristic for the most aggressive clinical cases with poor prognosis and, therefore, selected approach seems clinically relevant and adequate for search of drugs with the potential to eradicate cancer.

Administration of 0.1-1μM SB-T-1214 for 48 hours induced a loss of integrity of the floating spheroids and apoptosis in about 90% of the sphere cells (Botchkina et al., 2010), with higher rates of cell death in adherent type I collagen cultures. Although about 11% of cells survived this treatment regimen, such cells displayed multiple abnormalities, including a greatly enlarged size, multiple nuclei, a significant increase in the number of long and knobby projections, and severe vacuolization. Many cells displayed a clear sign of the mitotic catastrophe. Most importantly, viable cells which survived this treatment regimen significantly lost the ability to form secondary spheroids, which indicates that colon CSC population was critically affected. Thus, 1000 of untreated HCT116 primary spheroid cells induced 125±6 secondary spheroids, HT29 - 75±7, and DLD-1 gave rise to 93±6 secondary spheroids, whereas the SB-T-1214-treated dissociated spheroid cells produced only 1.5±0.3, 4±0.6, and 3±0.4 secondary spheroids, correspondently (P<0.01). After placement on type I collagen surfaces, cells that survived drug treatment, displayed profound morphological abnormalities similar to those described above.

The $CD133^{high}/CD44^{high}$-induced colon and prostate cancer spheroids were further tested for the expression of stem cell-related genes before and after treatment with SB-T-1214 using PCR array assay (*SA*Biosciences). Each array contains SYBR Green-based real-time PCR gene-specific assays for a set of 84 genes. Using filtering criteria of a 1.5 or greater fold-change in expression, we have analyzed differentially expressed genes in three types of floating colonospheres compared to their bulk differentiated adherent counterparts, as well as before and after treatment with SB-T-1214. The most profound differences were observed in HCT116 spheroids grown from $CD133^{high}/CD44^{high}$ cells (*Figure 4; left panel*), which is in line with their higher sphere-forming and tumor-initiating capacities. About one-fourth of the analyzed stem cell-related genes, including Wnt and Notch pathway genes responsible for self-renew and cell cycle regulation, were commonly up-regulated in all types of spheroids, with significantly higher levels of expression in HCT116 ones. Thus, 6 of 6 analyzed genes responsible for stem cell self-renewal (*SOX1, SOX2, MYST1, MYST2, NEUROG2* and *HSPA9*), and 3 of 5 genes regulating symmetrical/asymmetricasl cell division (*NOTCH1, NOTCH2* and *PARD6A*) were significantly up-regulated in the HCT116 CD133/CD44-high colonospheres compared to their bulk counterparts. The most significantly up-regulated genes in HT29 spheroids were *ACAN, ALPI, APC, ASCL2, CCND2, CD3D, CD4, CD8A, CD8B, COL2A1, COL9A1, DHH, DLL3, DTX1 FGF1, GJA1, S100B,SOX2, T, TERT* and *WNT1;* and in DLD-1 spheroids - *ALDH1A1, ASCL2, CCND2, CD4, COL1A1, DLL1, DTX1, FGF1, GJA1, IGF1, JAG1, MME, NCAM1,* and *NOTCH1.*

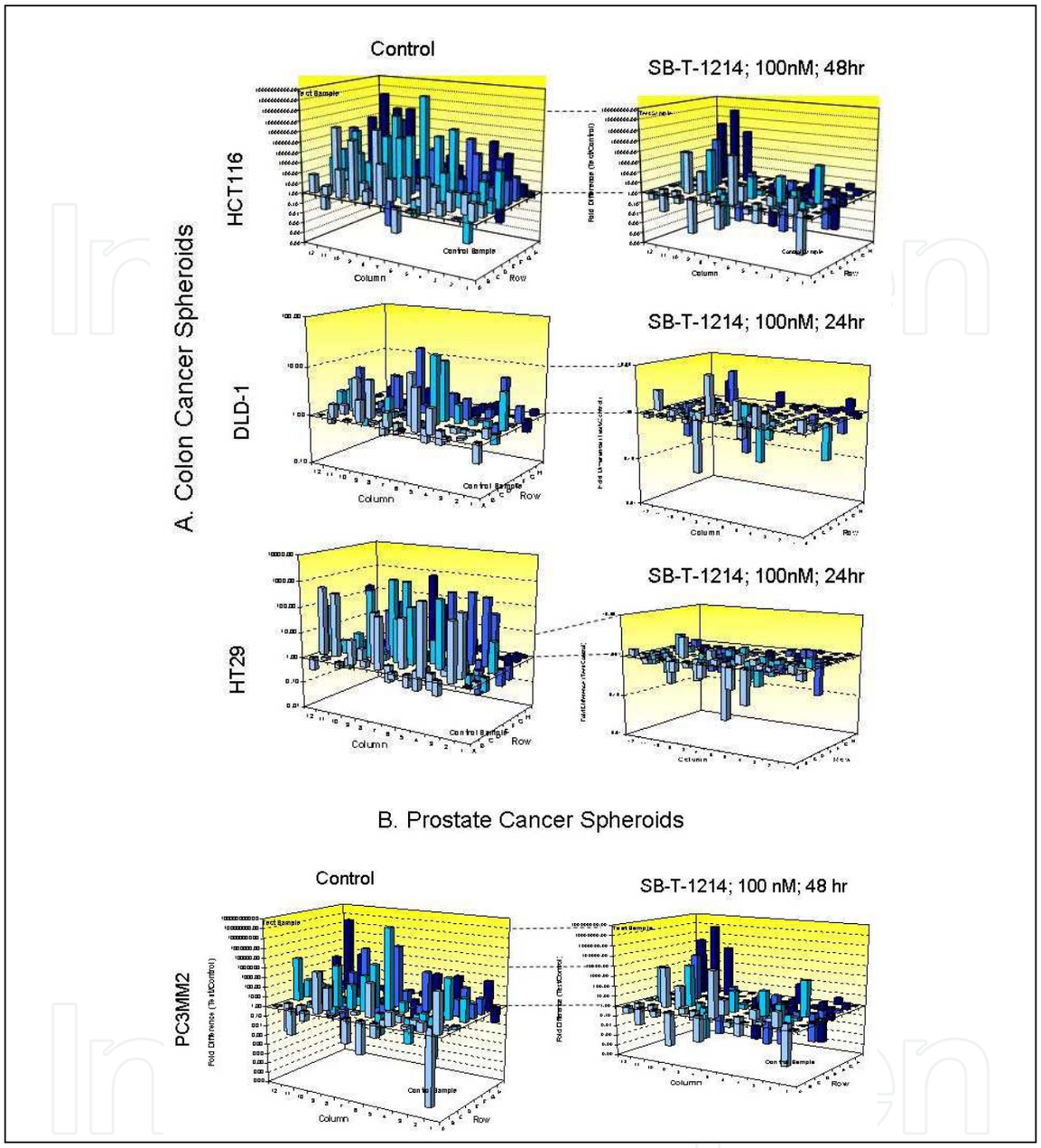

Fig. 4. Drug-induced alteration in the stem cell-related gene expression profiles (PCR Array assay) in colon and prostate cancer spheroids induced by $CD133^{high}/CD44^{high}$ cell populations. A majority of the stemness genes were up-regulated in floating spheroids grown from $CD133^{high}/CD44^{high}$ cells (upper half of each histogram) derived from colon HCT116, HT29and DLD-1 (A), as well as from PC3MM2 (B) cell lines in comparison with their corresponding bulk counterparts (lower half of each histogram). Treatment with 100nM SB-T-1214 for 24 or 48 hr induced down-regulation of a majority of the stem cell-related genes (right column). Importantly, relatively low concentrations of SB-T-1214 (100nM-1μM for 24 or 48 hr) induced dramatic down-regulation of the majority of stem cell-related genes in all three types of colonospheres, as well as in the prostate PC3MM2

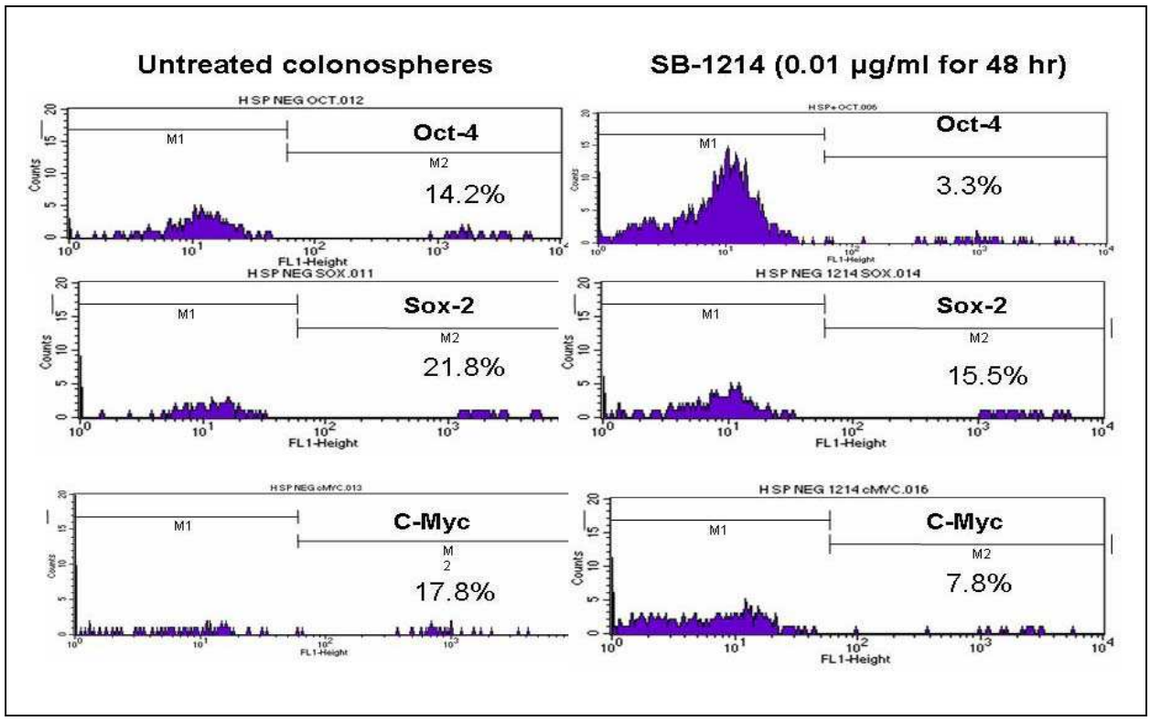

Fig. 5. Drug-induced alterations in the expression of the markers of pluripotency.
FACS analysis shows the presence of minor subpopulations of colon cancer cells within the 3D spheroids (left column), which express the three key pluripotency genes (Sox2, Oct4, and c-Myc). After treatment with SB-T-1214 percent of these cells was decreased (right column).

spheroids (*Figure 4, right panel*). The most significant drug-induced down-regulation of gene expression was detected: 1) in HCT116 colonospheres for *SOX1, RPL13A, BMP3, NEUROG2, GJB1, GJA1, ASCL2, CTNNA1, GDF2, ALPI, S100B, CD8B1, ACTB, CCND1, FGF1, PARD6A, DVL1, GDF3, ISL1, CD3D, MME, FGFR1, RB1, BMP1, AIN1, ALDH1A1, CD8A, PPARD, FZD1, NUMB, ABCG2*; 2) in HT29 colonospheres for *ACAN, ALPI, BMP3, CD3D, CD4, CD8A, CD8B, CDH2,COL2A1, COL9A1, DHH, DLL1, DLL3, DTX1, FGF1, FGF3, FZD1, GDF2, IGF1, MME, MYOD, NCAM1, NEUROG2, S100B, SOX2*, and *TERT*; 3) in DLD-1 colonospheres for *CD4, CDH2, COL1A1, DLL1, DTX1, IGF1, FGF3, FZD1, JAG1, KRT15, MSX1, NCAM1* and *NOTCH1*. Of note, many of these genes were related to the stem cells self-renewal, regulation of symmetric/asymmetric division and pluripotency.

We have found that the colonospheres induced by HCT116 cells with $CD133^{high}/CD44^{high}$ phenotype contained minority cell populations with high levels of expression of several markers, which are essential for pluripotency and self-renewal of embryonic stem cells (iPS-related genes) including c-MYC, SOX2, OCT3/4, LIN28, and NANOG (Botchkina et al., 2010). To analyze possible drug-induced alterations in the expression of these stem cell-specific transcription factors, which are low in abundance and present in a minority of colon cancer cell populations, we treated floating spheroids with 100nM of SB-T-1214 for 24 hours to induce such alterations, but avoid profound cell death. Importantly, both FACS and western blot analyses have shown that the expression of Oct-4, Sox-2, Nanog, Lin-28 and c-Myc was inhibited after a single treatment with relatively low drug concentration (*Figure 5*; FACS analysis is shown). These data are promising in light of a recent clinical study, which

has demonstrated that expression of several iPS-related genes, in particular, LIN28 and SOX2 is significantly associated with lymph node metastasis (Saiki et al., 2009). It was recently demonstrated that treatment with 5-FU and oxaliplatin, a standard therapy for metastatic colon cancer, induced up to 30-fold enrichment of CD133+ and up to 2-fold enrichment of CD44+ cells in HT29 cell line (Dallas et al., 2009). These data are in line with our observation that after a single treatment with 100μM Paclitaxel for 24 hours, the clonogenic potential of the dissociated HT29 and DLD-1 spheres cells was significantly increased, so we can assume that post-treatment spheroids contained a higher proportion of putative colon CSCs compared to untreated spheroids.

Therefore, SB-T-1214 efficiently suppressed the majority of stem cell-related genes, including Wnt and Notch, and in particular, several essential markers of pluripotent embryionic stem cells, including *SOX-2, Oct-4 and c-Myc,* on both transcriptional and protein levels. Importantly, WNT activity is known to regulate the self-renewal of prostate cancer cells with stem cell characteristics independently of androgen receptor activity (Bisson & Prowse, 2009); while c-*myc* gene (c-Myc is a Wnt target) amplification has been associated with the appearance of hormone-independent prostate cancer (Nupponen et al., 1998; Bernard et al., 2003), and a significant increase of c-*myc* amplification has been observed as a consequence of anti-androgen treatment (Kaltz-Wittmer et al., 2000). Of note, c-Myc is not essential for normal stem cells (Oskarsson et al., 2006), which makes it an even more attractive target for therapeutic intervention. Therefore, inhibition of WNT and NOTCH signaling by SB-T-1214 can reduce the self-renewal of prostate cancer stem cells and improve therapeutic outcomes. Since we have studied the SB-T-1214 induced alterations in the stemness gene expression profiles using total cell lysates (equal amounts of the total RNA for PCR arrays and total protein for western blot analyses), the significant inhibition of the stem cell-related genes induced by SB-T-1214 is promising.

6. Conclusions

Taken together, our data strongly support the suggestion that prostate and colon cancers cells with high combined expression of CD133 and CD44 represent stem-like cells with high tumorigenic and sphere-forming potentials, and significantly up-regulated multiple developmental pathways characteristic for pluripotent stem cells. Several mechanisms, including up-regulation of the anti-apoptotic and down-regulation of pro-apoptotic pathways, as well as high levels of expression of ABC transporters, active DNA repair and others, can contribute to the resistance of CSCs to standard treatment. Our findings provide first evidence that a new-generation taxoid, SB-T-1214, possesses significant activity against 3D colon and prostate cancer spheroids induced by, and enriched with, drug resistant tumorigenic $CD133^{high}/CD44^{high}$ cell populations, and efficiently inhibits the expression of a majority of stem cell-related genes, including several key regulators of pluripotency and self-renewal of embryonic stem cells. Therefore, our data indicate that the long-term efficacy of SB-T-1214 against drug resistant tumors *in vivo* (Kuznetsova et al., 2006; Ojima &Das, 2009) may be explained by down-regulation of multiple stem cell-related genes in tumorigenic cell populations, in addition to known efficacy of taxoids as a mitotic poisons due to their binding to microtubules (Jordan & Wilson, 2004) in the proliferating pool of cancer cells. These findings should be further tested across a large series of clinical specimens of primary and metastatic lesions of prostate and colon cancers.

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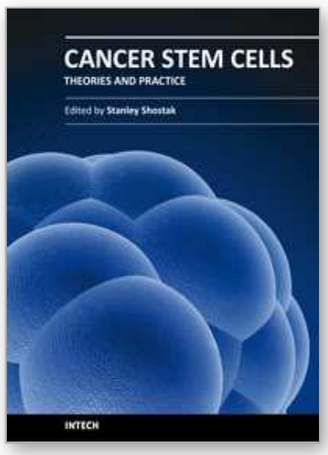

Cancer Stem Cells Theories and Practice

Edited by Prof. Stanley Shostak

ISBN 978-953-307-225-8
Hard cover, 442 pages
Publisher InTech
Published online 22, March, 2011
Published in print edition March, 2011

Cancer Stem Cells Theories and Practice does not 'boldly go where no one has gone before!' Rather, Cancer Stem Cells Theories and Practice boldly goes where the cutting edge of research theory meets the concrete challenges of clinical practice. Cancer Stem Cells Theories and Practice is firmly grounded in the latest results on cancer stem cells (CSCs) from world-class cancer research laboratories, but its twenty-two chapters also tease apart cancer's vulnerabilities and identify opportunities for early detection, targeted therapy, and reducing remission and resistance.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Galina Botchkina and Iwao Ojima (2011). Prostate and Colon Cancer Stem Cells as a Target for Anti-Cancer Drug Development, Cancer Stem Cells Theories and Practice, Prof. Stanley Shostak (Ed.), ISBN: 978-953-307-225-8, InTech, Available from: http://www.intechopen.com/books/cancer-stem-cells-theories-and-practice/prostate-and-colon-cancer-stem-cells-as-a-target-for-anti-cancer-drug-development

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