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ORIGINAL ARTICLE

WNT1 gene expression alters in heterogeneous population of prostate cancer cells; decreased expression pattern observed in CD133+/CD44+ prostate cancer stem cell spheroids

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Summary

Purpose: *Established cancer cell lines contain cancer stem cells (CSCs) which can propagate to form three dimensional (3D) tumor spheroids in vitro. Aberrant activation of WNT signaling is strongly implicated in the progression of cancer and controls CSCs properties. In this study we hypothesized that when cells were maintained as spheroids, the structure of CSCs could show differentiation between CSCs and non- CSCs.*

Methods: *CD133+/CD44+ cancer-initiating cells were isolated from DU-145 human prostate cancer cell line monolayer cultures, propagated as tumor spheroids and compared with the remaining heterogeneous cancer cells bulk population. The expression levels of WNT1, FZD1, ADAR, APC, AXIN, BTRC, FRAT1 and PPARD genes were measured by polymerase chain reaction (PCR) array assay and the protein expression levels of WNT1, FZD and AXIN by immunohistochemistry.*

Results: *The expression levels of WNT pathway-related molecules were found to increase in both CSCs and non-CSCs when CSCs were maintained as spheroids. However, different expression profiles were observed when CSCs and non-CSCs were compared. In spheroids, the expression levels of FZD1, APC, ADAR, WNT1, PPARD genes in CSCs decreased when compared to non-CSCs. Interestingly, when CSCs from spheroids were compared with CSCs from monolayers the most significant decrease was observed in FZD1 and increase in APC genes.*

Conclusion: *It is possible to assume that intracellular signaling of WNT-related molecules in the nucleus and/or cytoplasm might play an important role but it is independent from increased ligand expression and this expression strongly differentiate CSCs and non-CSCs population. This unexpected expression could be important for CSCs behavior and targeting this pathway could have therapeutic implications in cancer.*

Key words: *cancer stem cell, prostate, spheroid, WNT signaling*

Introduction

There is mounting evidence that tumors are initiated by a rare group of cells called CSCs. Normal stem cells and CSCs share significant properties like heterogeneity and plasticity. Maturation and differentiation play an important role in cancer cell heterogeneity, and tumor cell heterogeneity may result from clonal evolution driven by genetic instability of stem-like cells frequently called CSCs or tumor-initiating cells [1]. During early tumor development or in unperturbed tumor conditions, CSCs mainly undergo one-way maturation by developing into tumor progenitors and even differentiated tumor cells [2]. CSCs constitute the subpopulation most likely responsible of the tumor mass for treatment failure and cancer recurrence compared to bulk population of tumor cells (non-CSCs) that display low self-renewal capacity and a higher probability of terminal dif-

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ferentiation (i.e., transit-amplifying cancer progenitor cells) [3].

There is accumulating data suggesting that in vitro 3D tumor cell cultures more accurately reflect the complex in vivo microenvironment than simple two-dimensional monolayer, not least with respect to gene expression profiles, signaling pathway activity and drug sensitivity [4,5]. CSCs tend spontaneously to exist in spheroid formation as is seen in case of embryoid body formation during development [6,7]. Therefore, spheroids represent the differentiation properties of CSCs in serum contained medium in vitro and are used as a metastasis model in several studies [8,9].

CD133, also known as prominin-1 or AC133 (a glycoprotein comprising 5 trans-membrane domains), has been described as a marker of stem cells in several organs and appears to be the CSC marker for many tumor types [10]. CD44 is a member of the cell adhesion protein family and the expression of several CD44 proteins correlates with aggressive behaviors of various human cancers. A small subset of CD44+ cells in prostate cell cultures and xenograft tumors is more tumorigenic, proliferative, clonogenic and metastatic as compared to the CD44–subpopulation. This CD44+ subset expresses higher mRNA levels of several genes characteristic of embryonic stem cells [11,12]. Collins et al. have shown that prostate cancer tumorigenic cells have a CD44+/α2β1high/CD133+ phenotype [13].

WNT pathway is complex, with ligands, receptors, coreceptors and downstream molecules. This complexity has a crucial role in both embryonic development and cancer. Wnt family is an evolutionarily conserved pathway that regulates crucial aspects of all animal species, in the regeneration of tissues in adult organisms' factors, cell differentiation, and cell formation and defines the direction of polarization [14,16]. Dysregulation of WNT signaling plays a key role in the development of several types of cancer, including prostate cancer [17,18]. Activation of the WNT/β-catenin pathway has effects on prostate cell proliferation, differentiation and the epithelial-mesenchymal transition, which is thought to regulate the invasive behavior of tumors [19,20]. In this study we hypothesized that the structure of CSCs and/or non-CSCs could be different and differentiation of WNT signaling could be a clue in therapeutic strategies of cancer.

Methods

Cell culture conditions and reagents

The DU145 human prostate cancer cell line was supplied by the American Type Culture Collection (ATCC; Rockville, USA) and was grown in monolayer culture in Dulbecco's modified Eagle's medium-F12 (DMEM-F12; Biological Industries, Israel), supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, Missouri). Cells in semi-confluent flasks were harvested using 0.05% trypsin (Sigma Chemical Co., St Louis, Missouri), centrifuged after addition of DMEM-F12 for trypsin inactivation, and then re-suspended in culture medium.

Fluorescence-activated cell sorting and experimental groups

For fluorescence activated cell sorting (FACS), cells were detached using non-enzymatic cell dissociation solution (Sigma Aldrich, St Louis MO, USA) and approximately 56000 cells were incubated with antibody (diluted 1:100 in FACS wash [0.5% bovine serum albumin; 2 mM NaN3; 5 mM EDTA]) for 15 min at 4°C. An isotype - and concentration-matched phycoerythrin (PE) labeled control antibody (Miltenyi Biotec, UK) was used and samples were labeled with PE-labeled CD133/1 (clone AC133/1, Miltenyi Biotec, UK) and FITC-labeled CD44 (clone G44-26, BD Bioscience, San Jose, CA, USA). After three 5 min washes, the cells were re-suspended. Cells were sorted to be CD 133^{high}/$CD44^{high}$ population (sorting cells) and non-sorting counterparts. They were cultured in both 2D culture and 3D spheroid tissue cultures.

Constitution of spheroids and sphere formation assay

For spheroid culture, tumor cells grown as monolayer were re-suspended with trypsin and the clonogenic potential of different phenotypic populations was analyzed in 3D non-adherent culture condition coated with 3% Noble agar-coated (Difco, USA). Cells were counted, re-suspended and plated on 10^3 cells per well in a 6-well plate. Two weeks after initiation, the plates were inspected for colony (sphere) growth. The number of colonies within each well was counted. First passage floating spheres were removed, and gently disaggregated with a new 3% Noble agar-coated well.

PCR array assay

Total RNA was extracted from sorting cells and non-sorting counterparts (miRNeasy Kit Qiagen, Germany) and synthesis of cDNA was carried out using the SuperArray kit (SA Biosciences, C-03, USA). Stem cell-specific gene expression profiles were studied with a PCR array assay (Roche Custom Panel 384, UK) according to the manufacturer's recommendations. Briefly, total RNA was isolated from monolayer cell populations or whole floating spheroids. Up to 1 μg of total RNA was treated with DNase and cDNA was prepared using RT2 First Strand kit. For each analysis, pairs of

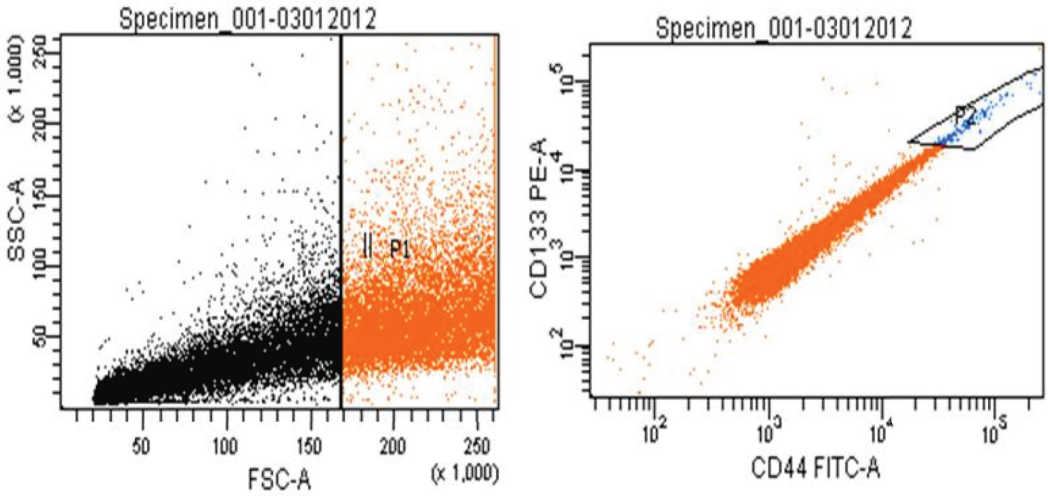

Figure 1. Prostate cancer stem cells sorted with FACS-Aria. $CD133^{high}/CD44^{high}$ populations are presented in P2 (CSCs). Aside from this population, the remaining cells were classified as non-CSCs.

the test and control cDNA samples were mixed with RT2 qPCR Master mix and distributed across the PCR array 96-well plates, each of which contained 84 stem cell-related probes and control housekeeping genes. After cycling with real-time PCR (LightCycler 480- LC 480, Roche Molecular Systems, UK), the obtained amplification data (fold-changes in threshold cycle (CT) values of all the genes) was analyzed with software and 1.5 or greater fold-change were used for filtering criteria. Detailed analysis of ADAR, APC, AXIN1, BTRC, CCND1, FRAT1, FZD1, MYC, PPARD, WNT1 genes were done.

Immunohistochemical analysis

Immunohistochemistry was adapted and modified from our published protocols [9]. Briefly, monolayer cells were maintained in 24-well plate and fixed with paraformaldehyde, and spheroids were processed in routine histological processing for embedding in paraffin wax. Cells were incubated with primary antibodies; Wnt1 (1:100 abcam-USA ab-15251), Fzd1(1:100 abcam-USA ab-71342), Axin1 (1:100 abcam-USA ab-79089), Gsk3β (1:100 abcam-USA ab-75745), β Catenin (1:100 abcam-USA ab-79089) overnight at 40°C in a humidity chamber and then a modified streptavidin-peroxidase technique was used. After incubation with DAB, (Invitrogen Ltd, UK) sections were counterstained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, Missouri, USA). Immunoreactivity of the molecules was assessed by light microscope equipped with a camera (Olympus BX-51 and Olympus C-5050 digital camera, Olympus Co., Tokyo, Japan) connected to a computer system running the software staining program (Image-Pro Express; Media-Cybernetics Inc., Bethesda MD) and was graded independently by 2 observers blinded to the groups, who evaluated the semiquantitative intensity immunostaining staining scores on the following scale: mild (1), moderate (2) and strong (3) and the percentage of total tumor cells with positive staining.

Statistics

All statistical analyses for semiquantitative evaluation of immunohistochemistry were performed using the DR SPSS II software (version 11.01 J; SPSS Japan Inc.,Tokyo, Japan). Statistical significance was determined using Mann-Whitney U test and p-value less than 0.05 was accepted as the level of statistical significance.

Results

Purity of CD 133^{high}/ $CD44^{high}$ sorted and non-sorted subpopulations and sorting rates

Before carrying out microarray, the purity of CSCs and non-CSCs samples was tested with CD133 and CD44. Sorting rate analysis and purity of cells were evaluated sequentially. Rates were 9.67 ±5.4 for sorting cells and 90.33 ±5.4 for non-sorting cells. In order to confirm the flow cytometry analysis, cells were re-evaluated after sorting and this analysis was repeated after one passage. Results showed that the cell purity after sorting was 85%. Immunofluorescence staining yielded cell purity of >85% in all samples.

Increased expression observed in monolayer CSCs when compared non-CSCs

After cell separation with the fluorescence activated cell sorted (FACS) (Figure 1), we analyzed the differentially expressed genes of the DU145

Table 1. Wnt pathway related genes ADAR, APC, AXIN1, BTRC, CCND1, FRAT1, FZD1, MYC, PPARD, WNT1 were investigated by CSCs as well as non-CSCs and increased expression levels of all those genes were observed in both groups. However, expression levels differed when groups were compared to each other. Fold changes are shown in the first column (monolayer CD $133^{high}/CD44^{high}$ vs monolayer non-sorting) and in the second column (spheroid CD 133^{high}/ $CD44^{high}$ vs spheroid non-sorting population)

Gene	*Monolayer CD $133^{high}/CD44^{high}$ versus monolayer non-sorting*	*Spheroid CD $133^{high}/CD44^{high}$ versus spheroid non-sorting*
PPARD	1.11	-1.03
APC	1.1	-2.72
AXIN1	1.1	1.09
BTRC	1.1	1.01
CCND1	1.12	1.01
FRAT1	1.1	1
FZD1	1.10	-5.97
MYC	1.1	1.08
ADAR	1.21	-1.07
WNT1	1	-1.04

Table 2. Spheroid forming cells originated from CD133+/CD44+ spheroids showed elevated expression of PPARD, APC, AXIN1, BTRC, CCND1, FRAT1, MYC, ADAR and WNT1 according to monolayer. However, significant FZD1 down-regulation was observed in CSCs spheroids while WNT1 and APC were the most up-regulated genes. In the second column comparison of fold changes was determined between monolayer non-sorting versus spheroid non-sorting cells

Gene	*Monolayer CD $133^{high}/CD44^{high}$ versus spheroid CD $133/CD44^{high}$*	*Monolayer non-sorting versus spheroid non-sorting*
PPARD	1.21	1.25
APC	3.98	1.33
AXIN1	1.25	1.17
BTRC	1.34	1.22
CCND1	1.28	1.15
FRAT1	1.29	1.17
FZD1	-6.81	1.34
MYC	1.14	1.11
ADAR	1.55	1.29
WNT1	1.35	1

human prostate cell line in both CD 133^{high}/ $CD44^{high}$ CSCs and their bulk counterpart (non-CSCs) cultured as monolayer cells or 3D spheroids. WNT pathway-related genes ADAR, APC, AXIN1, BTRC, CCND1, FRAT1, FZD1, MYC, PPARD, WNT1 were investigated by CSCs as well as non-CSCs and increased expression levels of all these genes were observed in both groups. On the other hand, these genes were significantly up-regulated in monolayer CD133^{high}/ CD44^{high} group when compared non-CSCs counterpart (Table 1). The mean fold change in expression of the target genes were PPARD (1.11), APC (1.1), AXIN1 (1.1), BTRC (1.1), CCND1 (1.12), FRAT1 (1.1), FZD1 (1.1), MYC (1.1), ADAR (1.21), WNT1 (1). According to statistical analysis immunohistochemistry revealed similar expression profiles with increased wnt1, fzd1, axin, gsk3β and β-catenin immunoreactivity in sorting monolayers (Figure 2) when compared to non-sorting counterpart ($p<0.05$; Figure 3).

Differential low expression pattern in FZD1, APC, ADAR, WNT1 and PPARD in CSCs spheroids when compared with non-CSCs

Spheroids showed different expression profile and FZD1, APC, ADAR, WNT1 and PPARD were significantly down-regulated in CSCs spheroids when compared to non-CSCs. Most significant decrease was observed in FZD1. The fold changes estimated after quantification of the level of expression using RT-PCR were FZD1 (-5.97), APC (-2.72), ADAR (-1.07), WNT1 (-1.04), and PPARD (-1.03), respectively. Therewithal (the canonical WNT pathway) increased expression profile was observed in AXIN (1.09), MUC (1.08), BTRC (1.01), CCND1 (1.01) and FRAT1 (1.00), respectively (Table 1). Immunohistochemistry showed statistically significant weak FZD1 and WNT1 expression in CSCs spheroids (Figure 4) when compared to non-CSCs spheroids ($p<0.05$; Figure 5). However, significantly increased immunoreactivity was found in axin1, gsk3β and β-catenin immunohistochemical analysis ($p<0.05$).

Distinct FZD1 down-regulation in CSCs spheroids when compared to monolayer

DU 145 tumor spheroids were cultured in low-adherence culture plates and maintained for 10 to 14 days to become spheroids. Spheroid-forming cells originating from CD133+/CD44+ spheroids showed elevated expression of PPARD (1.21), APC (3.98), AXIN1 (1.25), BTRC (1.34), CCND1 (1.28), FRAT1 (1.29), MYC (1.14), ADAR (1.55) and WNT1 (1.35) compared to monolayer. However, significant FZD1 down-regulation was observed in CSCs spheroids (-6.81) while WNT1 and APC were the most up-regulated genes (Table 2). Similar results were observed in the immunohistochemical analysis of these cells and Fzd1 showed

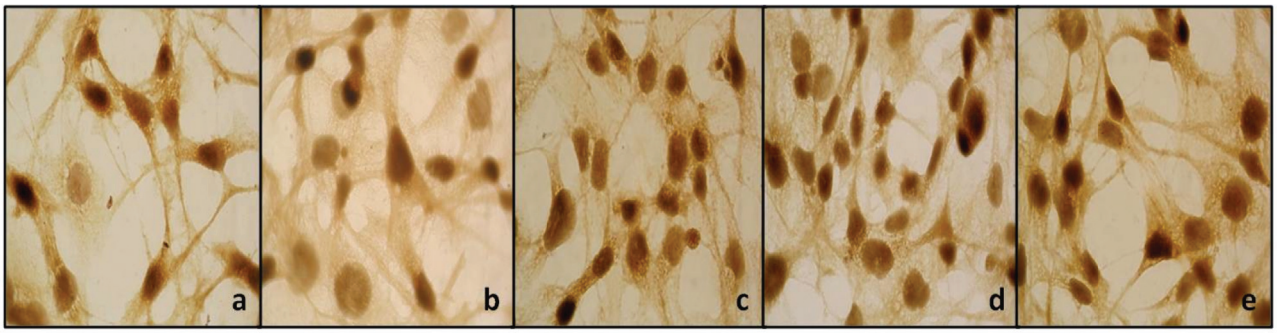

Figure 2. Immunohistochemistry of CD 133^{high}/$CD44^{high}$ monolayer cancer stem cells. Increased expression profiles were observed in wnt1 **(a)**, frizzled 1**(b)**, axin **(c)**, gsk3β **(d)** and β catenin **(e)** immunoreactivity.

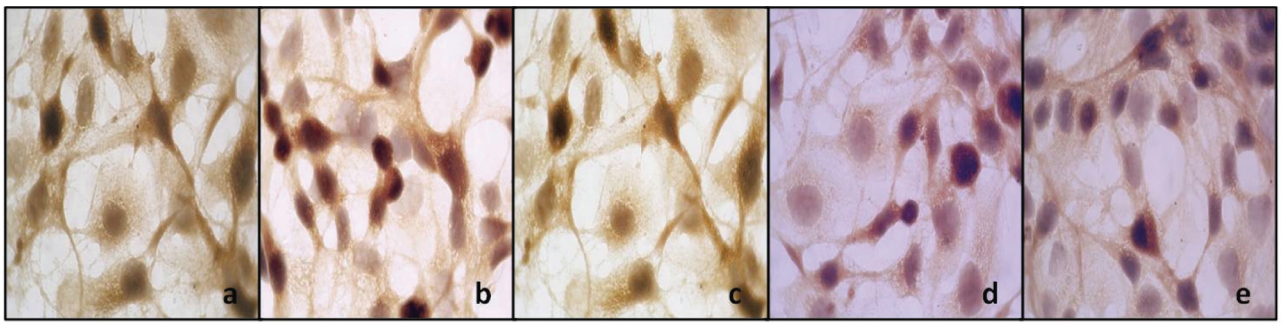

Figure 3. Monolayer non-cancer stem cells showed decreased immunoreactivity according to monolayer cancer stem cells. Immunoreactive cells were demonstrated in wnt1 **(a)**, frizzled 1**(b)**, axin **(c)**, gsk3β **(d)** and β catenin **(e)**.

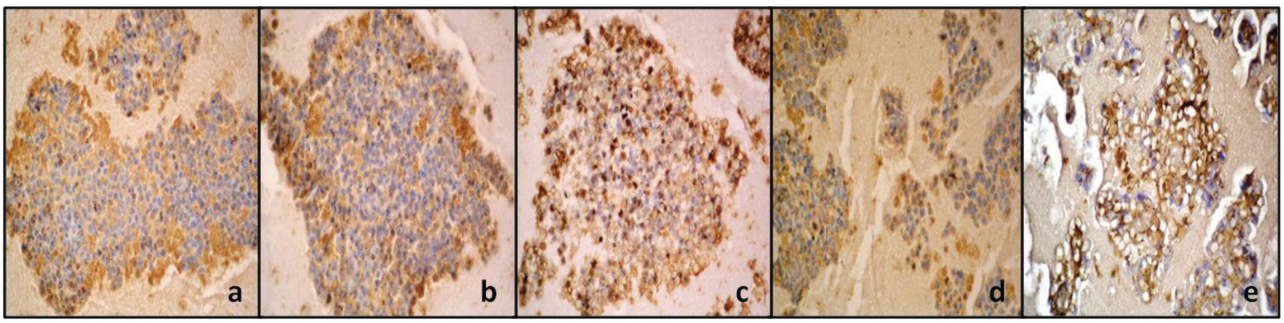

Figure 4. Immunohistochemistry showed weak wnt1 **(a)** and fzd1 **(b)** expression according to axin **(c)**, gsk3β **(d)** and β catenin **(e)** in CD 133^{high}/$CD44^{high}$ cancer stem cell spheroids. In this group cells were easily constitute spheroid formation and spheroids observed with wide diameters in plate.

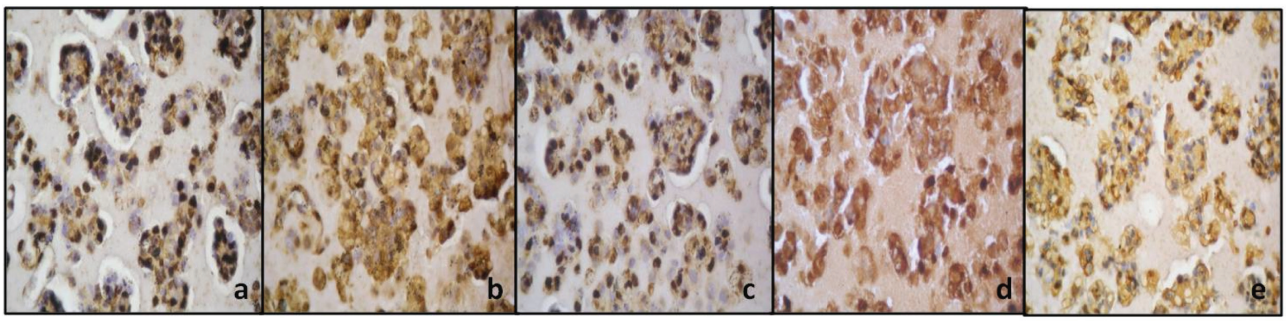

Figure 5. Increased immunoreactivity was observed in non- cancer stem cells spheroids in wnt1 **(a)**, frizzled1 **(b)**, axin **(c)**, gsk3β **(d)** and β catenin **(e)** according to CSCs spheroids. In this group cells showed straggle morphology in plate and exhibit weak cell to cell interactions. Approximately 5 or 6 cells contained cell clusters were occurred in plate but spheroid-like formation were observed in non-CSCs group.

statistically significant weak immunoreactivity ($p<0.05$; Figure 4).

WNT-related genes up-regulated in non-CSCs spheroids when compared to monolayer

PCR-array analysis was also performed in the cells sorted to be non-CSCs and maintained to become spheroids. The results showed that PPARD, APC, AXIN1, BTRC, CCND1, FRAT1, FZD1, MYC, ADAR, and WNT1 were significantly up-regulated. Comparison of fold changes was determined between non-CSCs spheroids and monolayer and showed PPARD (1.25), APC (1.33), AXIN1 (1.17), BTRC (1.22), CCND1 (1.15), FRAT1 (1.17), FZD1 (1.34), MYC (1.11), ADAR (1.29), and WNT1 (1) (Table 2). In these non-CSCs spheroids group significanlty increased immunoreactivity was determined in axin1, gsk3β and β-catenin immunohistochemical analysis ($p<0.05$; Figure 5).

Discussion

This study suggests that CD133+/CD44+ prostate CSCs effect their microenvironment and cellular signaling in the surrounding tissue and change their behavior with different expression profiles. When CSCs constitute a complex and organized formation, WNT1 and APC could be the highest up-regulated genes in the WNT pathway. Interestingly, in this sophisticated ($p=0.65$) tissue-like formation FZD1 was significantly down-regulated. FZD1 gene expression was also significantly down-regulated in CD133+/CD44+ prostate CSCs spheroids when compared to non-CSCs counterparts. In this study it is noteworthy to emphasize that FZD1 expression increased in both CSCs and non-CSCs. However, this down-regulation occurred when CSCs and non-CSCs were compared. Therewithal, as it is known the canonical WNT pathway, regulates cellular responses through β-catenin. In the absence of WNT, action of the destruction complex (casein kinase 1γ/CKI/2, Dishevelled/Dvl, scaffolding proteins glycogen synthase 3 beta/GSK3β, AXIN and APC protein) creates a hyperphosphorylated β-catenin, which is a target for ubiquitination and degradation by the proteasome. Binding of WNT ligand to a FZD/ LRP5-6 receptor complex leads to stabilization of the hypophosphorylated β-catenin and activates transcription. Wnt ligands are up-regulated in prostate cancer and this up-regulation often reflects more aggressive and metastatic behavior. Elevated expression levels of WNT1, WNT5A and WNT7B have also been correlated to prostate cancer aggressiveness [21,25]. Besides, WNT/β-catenin signaling pathway drives stem cell self-renewal and is involved in the pathogenesis of cancer. Increased activation of WNT-related molecules in normal stem cells can promote their transformation into CSCs [26,27]. This study showed that WNT was significantly up-regulated while FZD1 was down-regulated in CSCs maintained in 3D cell culture with serum contained medium. Human FZD1 was first cloned and mapped to chromosome 7q21 by Sagara et al. [26]. Previous studies have shown that FZD plays a crucial role in both canonical and non-canonical pathways and the expression of FZD has been reported to be up-regulated in some cancer tissues [28,29]. In our study we demonstrated increased FZD1 expression in CSCs monolayer cells, but on the contrary, significant down-regulation was observed in CSCs spheroids when compared to their bulk counterpart. Similarly, Ulivieri et al. have demonstrated that FZD1 expression was down-regulated and cell growth and invasion ability were decreased in follicular thyroid carcinoma cell lines [30]. This down-regulation is worth mentioning and the mechanism could be related with the miRNA. Overexpression of miR-204 decreased FZD1 mRNA expression in two primary human trabecular meshwork cell lines [31,32]. The role of the WNT-FZD pathway in cancer has been the subject of investigation by Nasse et al. after demonstration of the mouse mammary tumor virus retroviral integration events, resulting in inappropriate WNT1 expression which could result in murine mammary gland tumors [33]. APC is a crucial tumor suppressor gene. Loss of heterozygosity (23-40%), mutation (6-18%), and hypermethylation of the APC gene have been shown to result in loss of expression in almost 36-50% of breast tumors. According to our results, APC significantly increased and this APC up-regulation could occur in relation with the differentiation of CSCs during tumor progression [34]. Newly emerged evidence reveals that CSCs display significant phenotypic and functional heterogeneity and CSCs progeny can show diverse plasticity. Tang has shown that, similar with the induced plasticity in normal stem cell progeny, plasticity of non-CSCs probably occurs more prevalently under 'induced' conditions accompanying tumor progression in vivo or as a result of experimental manipulations *in vitro* or therapies *in vivo*. Therefore, cancer cell plasticity resembles fate reprogramming in differentiated normal cells [2]. The current study could be a model for 'induced' CSCs plasticity and differentiation in WNT-related molecules clearly revealed

with CSCs spheroids. In prostate cancer, as in other tumors, many seemingly divergent prostate CSC populations have been reported, and one of the main challenges is to delineate the interrelationship between various phenotypically different prostate CSCs [35].

Finally, this study demonstrated that isolated CSCs were found to possess multipotential differentiation capabilities through up-regulation and/or down-regulation of their markers, particularly WNT1 and FZD. We assume that CSCs must be engaged by one or more signaling cascades to differentiate and initiate tumor formation. New therapeutic strategies effectively targeting this critically important population of CSCs might terminate tumor progression.

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