Wnt/β-catenin signaling pathway in lung cancer stem cells is a potential target for the development of novel anticancer drugs

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Summary

Purpose: In the present study, we have analyzed the regulation of Wnt/β-catenin signaling in lung adenocarcinoma stem cells (CSCs), that are responsible for tumor recurrence.

Methods: Lung cancer samples were studied for the presence of cancer stem like cells and analyzed by flow cytometry. Then, the sorted cells were analyzed for the stem cell surface markers and Wnt/β-catenin signaling pathways. Moreover, the sorted side population (SP) and non-SP cells were also subjected to drug resistance assay.

Results: Western blot analysis showed that the protein level of β-catenin was highly upregulated in fluorescence activated cells (FACs) sorted SP cells which led to elevated expression of stem cell protein Oct-4 that is responsible for SP cells' self-renewal. RT-PCR revealed that the relative mRNA expression level of Wnt target gene cyclin D was significantly higher (p<0.01) in SP cells, enhancing thus the cell proliferation rate and clone formation efficiency. In addition, the matrigel invasion assay revealed that SP cells were highly invasive than non-SP cells.

Conclusion: In the present study we demonstrated that lung adenocarcinoma samples contain a small population of tumor-initiating SP cells which possess the characteristic features of CSCs. Wnt/β-catenin mediated increased expression of β-catenin, Oct-4 and cyclin D in SP cells but not in non-SP cells was also observed. FACs-purified SP cells are resistant to a number of chemotherapeutic drugs. Our data suggest that the use of novel anticancer drugs, targeting Wnt/β-catenin signaling pathways, may help eradicate the lung cancers stem cells.

Key words: cancer stem cells, cyclin D, drug resistance, self-renewal, Wnt/β-catenin

Introduction

Lung cancer is one of the most common malignancies and a leading cause of cancer-related deaths worldwide. The mean survival rate of lung cancer (including small-cell and non-small cell lung cancers) patients after diagnosis is 5 years [1]. Despite recent advances in chemo- and radiotherapy applications and strategies, the overall impact on patient survival rate remains poor. Accumulating evidence in several cancer types suggests that the presence of a small population of cells which are capable of initiating tumors, termed CSCs, are responsible for therapy failure and tumor relapse [2]. Recent data from mouse models and *in vitro* studies in lung cancer cell lines showed the presence of small populations of cancer initiating cells which possess stem-like characteristics [3,4]. CSCs have been isolated based on Hoechst 33342 dye exclusion and are named as Hoechst-stained SP cells [5]. Overexpression of ATP-binding cassette (ABC) transporter protein (ABCG2) in SP cells has

been shown to actively expel the dye or drug out of the cell and thus resulting in multidrug resistance [6]. These SP cells share the characteristic features of CSCs such as tumor initiation potential, self-renewal, drug and apoptosis resistance. The presence of cancer stem-like SP cells has been demonstrated in several solid tumors and SP cells are considered as enriched CSCs [7-10].

It was previously reported that the stem-like properties of CSCs and their maintenance are regulated by Wnt/β-catenin pathway in different cancers such as breast, liver and colon [4,7,8]. Hence, the isolation and characterization of SP cells may provide new insights into anticancer therapies to target the CSCs and therefore achieve complete elimination of the minimal residual disease (MRD).

In the present study, we have investigated the presence of cancer stem like SP cells from lung adenocarcinoma samples. These SP cells were also sorted with FACs technology. Furthermore, it was also observed that activation of Wnt/β-catenin mediates the upregulation of stem cell surface protein Oct-4, which may play a crucial role in self-renewal and the tumorigenic properties of SP cells.

Methods

Collection and processing of cancer samples

Lung adenocarcinoma samples were obtained in accordance with the hospital ethical rule. Patient details: N=20 (male 10; female 10), age range: 50-64 years (median 57); histology: adenocarcinoma (AC) (20 surgical specimens); stage and grade: pT2pN2pMX(IIIA)/G3 (N=12); pT2pN1pMX(IIB)/G2 (N=8). The obtained cancer tissues were washed several times and incubated in Dulbecco's modified Eagle's medium with Ham's nutrient medium F12 (DMEM-F12, Sigma-Aldrich, St.Luis, MO, USA), supplemented with 200 IU/ml penicillin (Gibco-BRL, Carlsbad, CA, USA), 200 μg/ml streptomycin (Gibco) and 20 μg/ml and amphotericin B (Gibco) overnight at 37 °C. Tissue dissociation was carried out by enzymatic digestion (20 mg/ml collagenase II) for 2 hrs at 37 °C. Cells were cultured in serum-free medium containing 50 mg/ml insulin, 100 mg/ml apo-transferrin, 10 mg/ml putrescine, 0.03 mM sodium selenite (all from Sigma-Aldrich), 2 mM progesterone (Pure Chemistry Scientific Inc., Sugarland, TX, USA), 0.6% glucose (LGM Pharma, Nashville, TN, USA), 5 mM HEPES (Nanjing Search Biotech Co., Ltd, Nanjing, China), 0.1% sodium bicarbonate (Nanjin), 0.4% bovine serum albumin (BSA;Wuhan Boster Bioengineering Co., Ltd, Wuhan, China), 2 mM glutamine (Amresco LLC, Solon, OH, USA) and 1% penicillin-streptomycin (Gibco) dissolved in DMEM–F12 medium and supplemented with 20 mg/

ml epidermal growth factor (EGF; PeproTech EC Ltd., London, UK) and 10 mg/ml basic fibroblast growth factor (bFGF; PeproTech).

FACS analysis

Cells were cultured and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco) at 37 °C in a humidified 5% CO_2 & 95% air atmosphere. The cells were grown to approximately 90% confluence in a T75 cell culture flask and then the monolayer of the cells was released from the flask by adding 1x Trypsin-EDTA (Sigma-Aldrich). Cells were washed twice with PBS (Sigma-Aldrich) and were resuspended in 10% DMEM. Cell count was performed using hemocytometer. *Study groups:* Control cells labeled with Hoechst 33342 dye alone (N=7); Drug-treated cells: cells treated with verapamil and Hoechst 33342 dye (N=7). Cells were counted by hemocytometer and approximately 10^6 cells/ml were labeled with Hoechst 33342 stock (Sigma-Aldrich)-bis-benzimide (5 μl/ml), either with dye alone or in combination with the ABC transporter inhibitor reserpine (50 μmol/L). Next, cells were counterstained with 2 μg/ml propidium iodide (PI) (Sigma-Aldrich). The cells were sorted using a flow cytom¬eter and the sorted cells were cultured and maintained in DMEM/ F-12 supplemented with 10% FBS. The Hoechst 33342 emission was first split by using a 610-nm dichroic short-pass filter, and the red and blue emissions were collected through 670/30- and 450/65-nm bandpass filters, respectively.

Chemoresistance assay

Approximately $1x10^3$ cells/plate were cultured in 96-well plates and treated with the chemotherapeutic drugs at the following concentrations: 5-fluorouracil (5-FU) with a concentration of 10 μg/ml, paclitaxel 30 ng/ml, cisplatin 5 mg/ml and docetaxel (2 μg/ml). The mean value of optical density $(OD)_{450}$ obtained was represented as a graph. Cell resistance was calculated using the following formula: Cell resistance rate (%) = (experimental group OD_{450} value/control group OD_{450} value) x100, as previously described [11].

RNA extraction and real-time PCR analysis

Total RNA was extracted and complementary DNA was prepared using Reverse Transcription system (Promega Corp., Madison, WI, USA) Real-time RT-PCR analysis was subsequently performed on an iCycler IQ real-time detection system (Bio-Rad, Hercules, CA, USA), using IQ Supermix with SYBR-Green (Bio-Rad). The sequences of human specific primers used were as follows (5'–3'): CCND1: (F – TGA TGC TGG GCA CTT CAT CTG and R - TCC AAT CAT CCC GAA TGA GAG TC); OCT-4 (F - GCA ATT TGC CAA GCT CCT GAA and R - GCA GAT GGT CGT TTG GCT GA); GAPDH: (F – GCA CCG TCA AGG CTG AGA AC and R - TGG TGA AGA CGC CAG TGG A); ABCG2: (F - TCA ATC AAA GTG CTT CTT

TTT TATG and R – TTG TGG AAG AAT CAC GTG GC) [12]. PCR conditions: 52°C for 2 min and 95°C for 6 min followed by 35 cycles of 15 sec at 95°C, 30 sec at 58°C, and 45 sec at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

Invasion assay

Cellular invasiveness of SP and non-SP cells was determined by using 6-well Matrigel invasion chambers (BD Biosciences Discovery Labware, Durham, NC, USA). Cells were seeded in serum-free medium at a density of 2×10^5 per insert. Outer wells were filled with DMEM containing 5% FBS as chemoattractant and incubated at 37°C for 48 hrs. Subsequently, the non-invading cells were washed by swabbing the top layer of Matrigel with Q-tip [13]. Membrane containing invading cells was stained with hematoxylin for 3 min, washed, and mounted on slides. The entire membrane with invading cells was counted under light microscope at 40× objective.

Western blot

Proteins were extracted from the SP and non-SP cells, and protein concentration was determined using the Bradford assay [14]. Following sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and transfer to a membrane, the gels were treated with the primary antibodies (rabbit anti-human β-catenin, Oct-4 and GAPDH), the secondary antibody (goat anti-rabbit IgG with alkaline phosphatase markers; all purchased from ABCAM, Cambridge, MA, USA) and a chemiluminescence reagent (SuperSignal, West Femto Substrate, Thermo Scientific, Wartham, MA, USA). Blots were detected and scanned by using a densitometer (Biorad GS-710, Biorad, Hercules, CA, USA).

In vitro proliferation activity

The sorted SP and non-SP cells were seeded in a 96-well plate at $2x10^6$ cells/well and then cultured in a CO_2 incubator. Each group was set up in triplicate. Cell proliferation activity was measured every day for 7 days. Each well was supplemented with CCK-8 solution (10 µl) and incubated in CO_2 incubator for 2-3 hrs. The OD was determined at 450 nm. These data were used to calculate cell growth graphs based on the mean value of OD_{450} and standard deviation values for each well.

Sphere formation assay

The sorted SP and non-SP were placed at a density of 1000 cells/ml, and resuspended in tumor sphere medium consisting of serum-free 1:1 mixture of Ham's F-12/DMEM, N2 supplement, 10 ng/ml human recombinant bFGF, and 10 ng/ml EGF, and subsequently cultured in ultra-low attachment plates for about 2 weeks. Sorted SP and non-SP cells were seeded at a low density of 20 cells/ml and the number of generated spheres (>100 µm) was counted after 7 days of culture.

Statistics

One-way analysis of variance (ANOVA) and Student's t-test were performed to determine significant difference between the SP and non-SP groups. A probability level of $p<0.05$ or 0.01 was considered as statistically significant.

Results

Presence of SP cells in lung adenocarcinoma

The lung cancer samples were investigated for the presence of SP cells by FACs-based Hoeschst 33342 dye exclusion method. We identified 1.9% of small population of SP cells (Figure 1A, gated region) whose presence was significantly reduced to 0.3% (Figure 1B, gated region) after treatment with reserpine. The activity of ABC transporter protein could be efficiently blocked by reserpine and thus SP population was significantly diminished.

Wnt/β-catenin mediated upregulation of Oct-4 in SP cells

We have evaluated the expression of Oct-4 and cyclin D1 in the FACs-sorted SP cells. As shown in the Figure 2A, the expression of β-catenin and Oct-4 protein level was relatively higher in SP cells than non-SP cells. Similarly, the expression of ABC transporter protein ABCG2 was significantly higher in SP cells. Furthermore, RT-PCR analysis revealed that the relative mRNA expression levels of Wnt target gene cyclin D1, ABCG2 and Oct-4 was highly elevated in SP than in non-SP cells (Figure 2B). Therefore, these data suggest that elevated levels of Wnt/β-catenin, ABCG2 and Oct-4 were involved in multidrug resistance and tumorigenic properties of lung cancer SP cells.

Chemoresistance and increased cell proliferation rate of SP cells

The sorted SP and non-SP cells were subjected to drug resistance and *in vitro* cell proliferation assays in order to determine the cell survival and growth rate. The SP cells showed increased resistance to chemotherapeutic drugs (docetaxel, 5-FU, cisplatin and paclitaxel). After treatment with the above-mentioned drugs, SP cells showed significantly higher survival rate than non-SP cells (Figure 3A). Subsequently, these SP cells underwent rapid cell proliferation starting from the third day
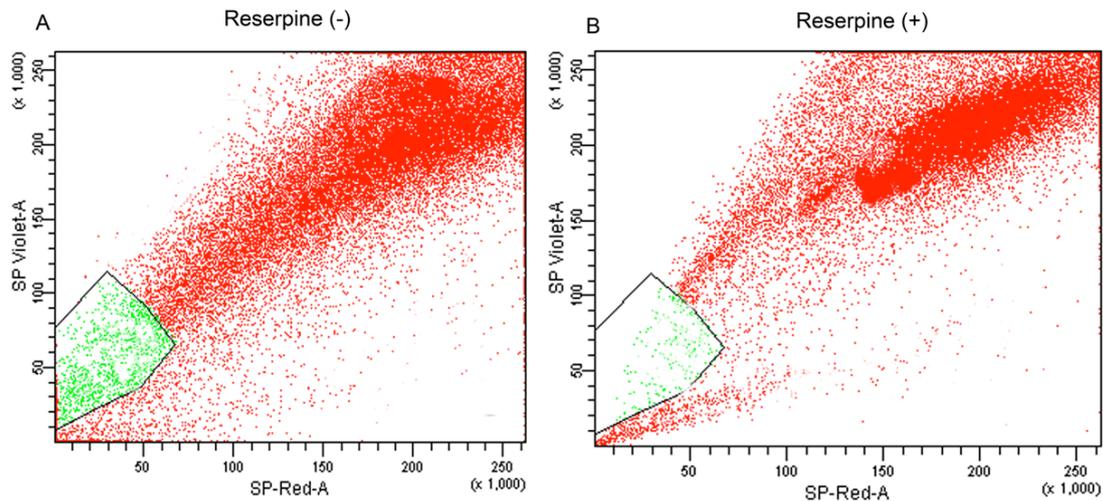
Figure 1. Analysis of SP cells in lung adeocarcinoma. **A:** Cells were stained using Hoechst 33342 dye and analyzed using flow cytometry. SP cells (1.90%) are outlined as gated population. **B:** The percentage of SP cells was significantly reduced to 0.3% in the presence of reserpine (p<0.001, t-test).
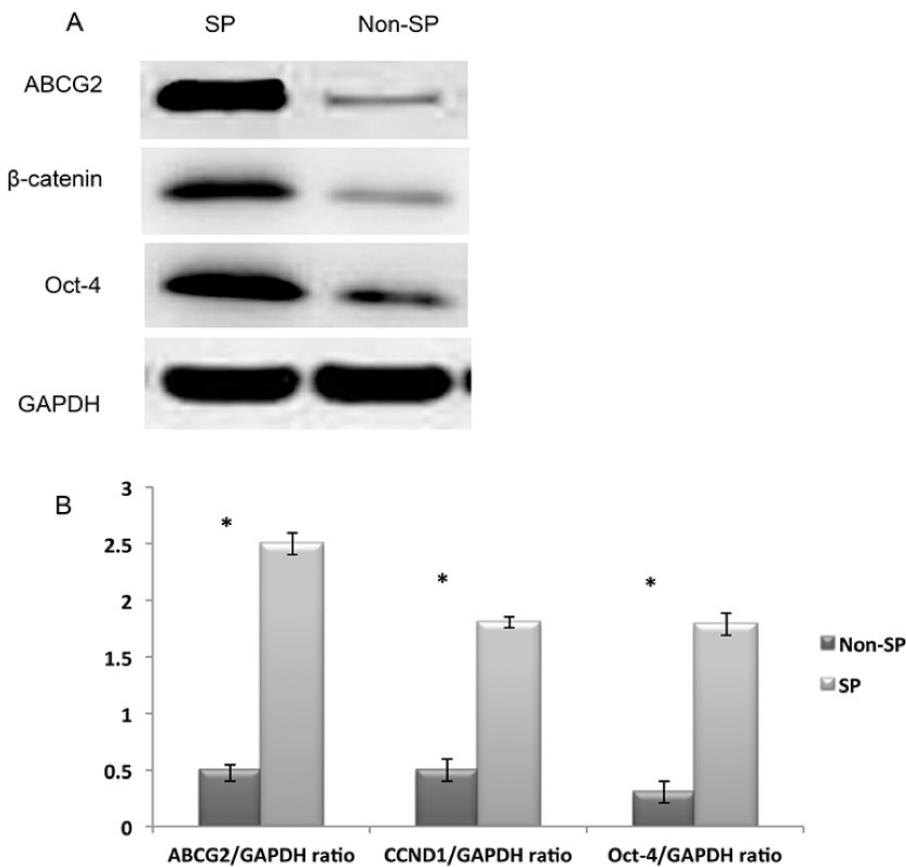

Figure 2. A: Western blot analysis of protein expression levels in SP and non-SP cells. Equal concentrations of protein were loaded per lane. **B:** The elevated expression of CCND1, Oct-4 and ABCG2 in SP cells was detected by RT-PCR. Quantification graph from the data of 3 separate independent experiments. GAPDH was used as a housekeeping gene. The bar represents the standard deviation. Results are representative of 3 independent experiments performed in triplicate (*p<0.001, t-test).
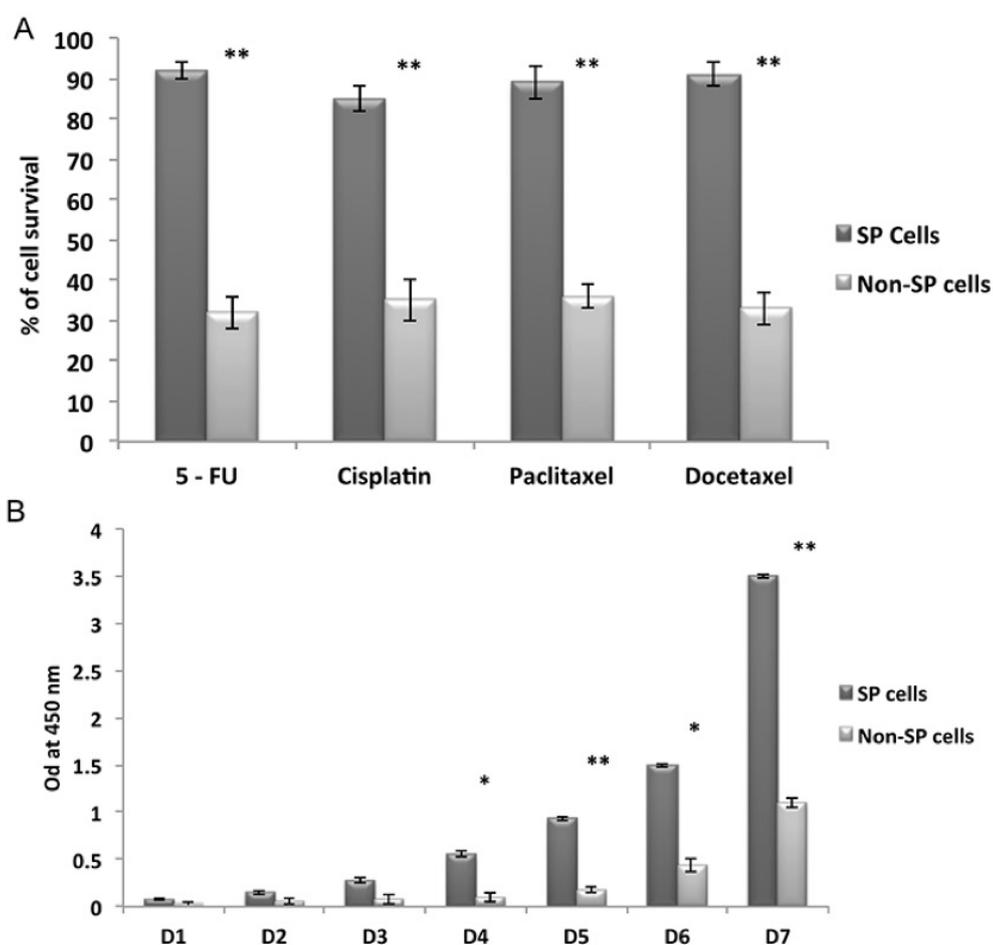
Figure 3. A: Comparison of cell survival rate of SP cells and non-SP cells after treatment with docetaxel, 5-FU, cisplatin, paclitaxel and oxaliplatin. SP cells were highly resistant and they had increased survival rate after treatment with these drugs. **B:** Cell proliferation rate for SP and non-SP cells. The x-axis represents time (days), while the y-axis indicates the corresponding optical density (OD) value at 450 nm. The bars represent standard deviation. Results are representative of 3 independent experiments performed in triplicate (*p<0.05; **p<0.01, t-test).

and became more confluent on day 8 (Figure 3B). Hence, these finding suggest that drug resistance and increased survival rate of the SP cells might be due to overexpression of ABCG2 and possibly to reduced apoptosis rate.

SP cells are highly self-renewal and invasive

In order to compare the regeneration capacity of SP and non-SP cells, sphere formation assay was performed. The total number of spheres generated by SP cells was significantly higher than by non-SP cells (Figure 4 A). *In vitro* matrigel invasion assay revealed that the sorted SP cells were significantly more invasive than the non-SP cells (Figure 4B). Taken together, it is clear that SP were highly potent for tumor initiation and involved in rapid tumor invasion.

Discussion

According to the recently proposed CSC theory, cancers are heterogeneous and they contain a small subpopulation of cells with indefinite repopulation potential, called CSCs. These CSCs escape from the current treatment strategies and efficiently regenerate cancer cell population, leading to treatment failure and tumor relapse. CSCs play a critical role in chemoradiation resistance, tumor initiation, progression, metastasis, and recurrence [10,15,16]. Therefore, cancer therapy targeting CSCs will lead to complete tumor eradication.

In the present study we demonstrated that lung adenocarcinoma samples contain a small population of tumor-initiating SP cells which possesses the characteristics features of CSCs. The
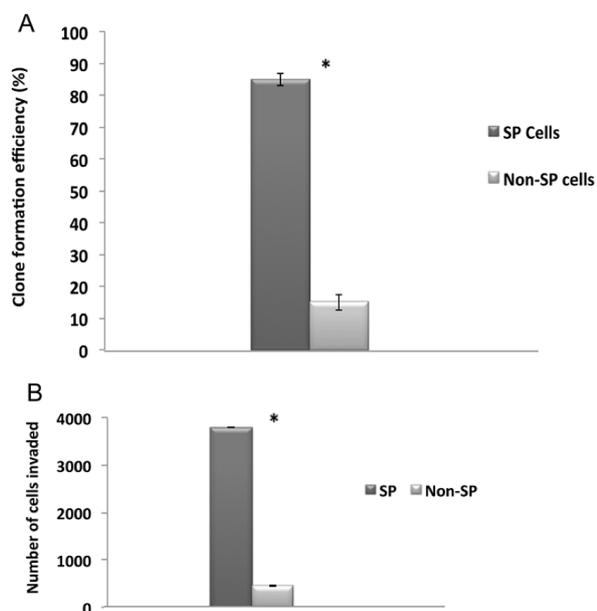
Figure 4. SP cells were more tumorigenic and invasive. **A:** Clone formation efficiency of SP cells. The total number tumor spheres generated by lung cancer SP cells was significantly higher compared to non-SP cells. **B:** SP cells invasiveness as measured by the Matrigel assay. SP or non-SP cells (4x10⁵) were seeded and incubated for 72 hrs. The bars represent standard deviation. Results are representative of 3 independent experiments performed in triplicate (*p<0.001, t-test).

of telomerase has been reported in early stage of disease which is the major cause for tumor invasion in both SCLC and NSCLC [17]. We speculate that SP cells might also have elevated expression of telomerase and thus prevent cancer cells from apoptosis during chemotherapy, however this should be studied in detail in future studies.

Recent studies have demonstrated that the Wnt/β-catenin is one of the most important signal transduction pathways involved in tumorigenesis, progression and maintenance of CSCs [18-20]. Studies in NSCLC cell line A549 showed an increased activation of Wnt/β-catenin pathway, leading to activation of Wnt target gene, cyclin D. Strikingly, the stem cell surface protein Oct-4 was highly elevated in these cells [12]. Oct-4 overexpression has been found to be crucial for the maintenance and self-renewal of CSCs in several tumors [21]. In line with these findings, we also observed increased expression of β-catenin, Oct-4 and cyclin D in SP cells than in non-SP. However, the precise molecular mechanism of Wnt/β-catenin mediated overexpression of Oct-4 is still unknown. We speculate that expression of other ABC transporter and stem cell genes (CD133, CD44, nestin) might be accelerated in SP cells and collectively contribute the chemoresistance and tumor recurrence properties. Therefore, novel anticancer drug(s), which could suppress the Wnt/β-catenin activation and their downstream signaling pathways, would be effective to reduce or even eliminate chemoresistance and tumorigenicity of SP cells.

In conclusion, lung adenocarcinoma SP cells are tumorigenic, invasive, and they also possess the properties of stem cells, escaping death and constituting the MRD with increased Wnt/β-catenin signaling pathways. Hence, the characterization of SP cells will be a valuable tool for improving treatment strategies for lung cancer and to design novel anticancer drugs for more effective therapies.

presence of SP cells is considered as a major obstacle for curative therapy and for prevention of tumor relapse [7,8]. The most peculiar phenotype of SP cells is overexpression of ABC transporters such as ABCG2, ABCA2, MDR1 and MRP1, which plays a crucial role in the export of chemotherapeutic drugs [9,10,15]. We also observed that FACs-purified SP cells are resistant to a number of chemotherapeutic drugs like docetaxel, 5-FU, cisplatin and paclitaxel as they have elevated expression of ABCG2. Previously, high expression

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