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Isolation of Liver Cancer Stem-Like Cells by Hoe33342 or Rhodamine123 Efflux

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1. Introduction

Already 150 years ago, the German pathologist Rudolf Virchow postulated in his theory of the cellular pathology that cancer initiates from immature cells. But it still took 100 years until Sajiro Makino introduced the term "tumor stem cells" for a small subpopulation of cells that were insensitive to chemotherapy and had chromosomal features different from the bulk of cells (Makino, 1959). Although the existence of cancer stem cells (CSCs) was proposed over decades ago, only in the past decade increasing evidence supports that cancers contain a small subset of their own stem-like cells (Dalerba et al., 2007; Pardal et al., 2003; Wicha et al., 2006). At present, these cells have been identified in human leukemias (Konopleva & Jordan, 2011), and more recently in solid tumors that include liver (Ma et al., 2007), breast (Dontu et al., 2003), prostate (Lukacs et al., 2010), brain (Huang et al., 2010), colon (Kemper et al., 2010) and lung cancers (Sullivan & Minna, 2010). These studies suggest that, like normal stem cells, CSCs should be rare, quiescent, and capable of self-renewing and maintaining tumor growth and heterogeneity. Therefore, development of effective therapies that will specifically target CSCs may become a promising therapeutic option to fight cancer.

The researches of CSCs are based on successful viable CSCs sorting. Universally accepted markers of CSCs are very important to cell sorting. In the last decade several molecular properties have been utilized to identify and characterize CSCs from different hematopoietic and solid tumors. The first markers used were cell surface proteins already known to define stem and progenitor cells, e.g. CD133 and CD166. Meanwhile, some surface markers that are associated with human and mouse stem cells are also found on cancer stem cells, such as CD34, CD117, Sca-1, and other markers, such as CD44, CD24, CD20 CD105, and CD326 (EpCAM) have been found on cancer stem cells (Keysar & Jimeno, 2010). Furthermore, molecules that facilitate drug resistance in cancer cells like ABCB1 and ABCG2 were added to the list of putative CSC markers (Calcagno et al., 2010) as well as proteins for which no

involvement in stemness or cancerogenesis was known, e.g. CD20. Although CSCs have been isolated by cell surface markers such as CD24, CD44, and CD133 as well as on the basis of sphere formation after in vitro cultivation (Jordan et al., 2006), the identification of a putative cancer stem cell subpopulation with validated methods and markers for each tumor entity remains controversial. Because of this, suitable cells are needed for the analysis of CSCs biology.

While the cells are stained with Hoe33342 (Hoe) vital dye, a cell-permeable DNA-specific bisbenzimidazole dye, the display of Hoe fluorescence simultaneously at two emission wavelengths localizes a distinct, small, non-stained cell population that is designated as side population (SP) cells. Because SP cells are known to have highly efficient pumps for the dyes, they are considered to be resistant to multi-chemotherapeutic drugs and to confer malignant phenotypes to tumors (Wu & Alman, 2008). More importantly, the SP cells express high levels of stem cell markers and low levels of differentiating markers (Haraguchi et al., 2006). Hence, the characterization of SP cells might be a useful tool for analysis of CSCs, especially when specific CSC surface markers are unknown.

As an alternative approach, the SP phenomenon has been used to identify and isolate stem cell populations from a variety of tissues including bone marrow (Goodell, 2002), mammary gland (Welm et al., 2003), skin (Montanaro et al., 2003), liver (Wulf et al., 2003), lung (Majka et al., 2005), skeletal muscle (Meeson et al., 2004), limb (Umemoto et al., 2006), heart (Martin et al., 2004), and brain (Kim & Morshead, 2003). This approach overcomes the barrier of phenotypical markers and replaces it by more direct functional markers. The most widely accepted assays to obtain SP cells are efflux analysis of Hoe as well as further detection of known stem cell markers in cancer cells and verification by xenotransplantations. Such specific dye can only be excluded out of cells by the ATP-Binding Cassette (ABC) transporters, which is a stem-like characteristic. That is to say, only the cells with ABC transporters expression can exclude such dye. Thus, dye exclusion is a valuable technique to indirectly identify stem-like cells.

The dye Hoe exclusion has been successfully applied in many tumors and corresponding cell lines. The blue fluorescent Hoe is a cell permeable bisbenzimidazole derivative that binds to the minor groove of DNA. After excitation of Hoe its emission can be measured simultaneously in the blue and red spectrum. But although Hoe enters viable cells, it is also actively pumped out by ABC transporters of the cell membrane (Scharenberg et al., 2002). There is mounting speculation that ABC transporters repress the maturation and differentiation of stem cells (Spangrude & Johnson, 1990). Although several subfamilies of genes coding for these transporters have been identified (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG), the best-known are: members of the ABCB and ABCG subfamilies, such as ABCB1 (MDR1, P-gp) and ABCG2 (Bcrp1) in humans (Choi, 2005). It has been demonstrated that the exclusion of Hoe by SP cells is an active process mainly involving Bcrp1 (Uchida et al., 2002). To determine the size of the SP, Verapamil, an L-type calcium channel blocking agent serves as an important control. Blocking the calcium channels inhibits the efflux of Hoe-dye from these cells, so it is then possible to gate for the side population, which is suspected to consist of cancer stem cells. However, the toxicity of Hoe should be addressed and always kept in mind when applying this dye to isolate putative CSCs. As Hoe binds to DNA, it can disrupt DNA replication during cell division. Consequently, it is potentially mutagenic and carcinogenic. This toxicity suggests a potentially severe limitation for the use of Hoe dye in combination with fluorescence activated cell sorting (FACS).

Rhodamine123 (Rho) is always used as a substrate of ABCB1/P-gp transporter to evaluate the toxicity of drugs (Vautier et al., 2008) and to examine the functional activity of P-gp in cultured cells (He & Ji, 2008). Like Hoe, it is actively pumped out of the cells by ABC transporters, e.g. ABCB1/P-gp protein (Zhou et al., 2001). Our study found that Rho was shown to be non-toxic to cells even at high concentrations, it can be an alternative to the use of Hoe.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third most frequent cause of cancer deaths worldwide. It has been ranked the second leading cause of cancer death in China since the 1990s. The prognosis of HCC patients remains extremely poor, with a 5-year survival rate of less than 5%. HCC respond poorly to standard chemotherapy treatment regimens. The reasons for this clinical phenotype are believed to be based on cellular heterogeneity of the tumor and the presence of multidrug resistance genes, which encode for pumps that actively expel the cytotoxic substances. Because the Hoe and Rho efflux capacity of SP cells is also dependent on the presence of membrane pumps, we hypothesized that identification of the SP cells in HCC could potentially represent a suitable isolation method to evaluate stem cell-like tumor characteristics in HCC. It is widely accepted that the MHCC97 cell line has heterogeneity, but there are few methods to separate SP cells from this cell line. Thus, we investigate the method to isolate liver cancer stem like cells by Hoe or Rho efflux from MHCC97 cell line and detect the stem cell properties of the obtained SP cells.

In this chapter, we will present the method that isolation of liver cancer stem like cells by Hoe or Rho efflux and discuss the advantages and disadvantages of the two methods.

2. Methods and analysis

We firstly examined in vitro cytotoxicity of Hoe and Rho, and then used both Hoe/FACS and Rho/FACS to enrich CSCs from the MHCC97 cell line (Key Laboratory for Carcinogenesis and Cancer Invasion, Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China). We tried different conditions detailed in methods to determine the optimal dye concentration. After cells were successfully sorted by the two methods, we obtained four typed cells: SP cells (low staining with Hoe) and NSP cells (high staining with Hoe), Rho^{low} subpopulation cells (low Rho fluorescence and Verapamil negative) and Rho^{high} subpopulation cells (high Rho fluorescence). Among them, SP and Rho^{low} cells were CSC like cells. We compared the effects of Hoe and Rho on CSCs sorting and found the ability of Rho staining to separate subpopulations from the MHCC97 cell line is similar to Hoe staining. Based on the analysis of several CSCs' characteristics, we found the Rho^{low} cells had similar characters as the SP cells, such as high proliferative ability, high expression of stem cell markers (early hepatic marker AFP and CSCs marker CD133) and strong tumorigenicity in vivo. Specific methods of operation are as follows.

2.1 Agents test

Each compound was screened for possible cytotoxic effect with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). This is a colorimetric assay for the quantification of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant. Cytotoxicity was calculated as a percentage of the effect obtained

with the positive control (total cell lysis) (Storch et al., 2007). To guarantee the specificity of subpopulations, Verapamil (Sigma Chemical Co., St. Louis, MO) was used for second check. The influence of Verapamil on the fluorescence of the ABC transporter substrates was evaluated by adding increasing concentrations to Rho or Hoe solution. Among the tested compounds, only 8 μg/ml Hoe caused cytotoxic effect >50%. The cytotoxic effect caused by 6 μg/ml Hoe was 30.8%. Besides, when the concentration of Hoe was<4 μg/ml, it caused cytotoxic effect <30%. Through statistical analysis, the IC50 of Hoe was 7.52 μg/ml. For all Rho patterns (the concentrations ranged from 0.05 to 1 μg/ml), no cytotoxic effect was found. There was no quenching effect caused by Verapamil to both Hoe and Rho solutions.

2.2 Cells culture and grouping

The cells from the MHCC97 cell line were maintained in DMEM culture (Invitrogen, Karlsruhe, Germany), which was supplemented with 10% FCS, 2 mM glutamine, 1 mM HEPES, 100 U/ml penicillin G and 100 g/ml streptomycin (Invitrogen, Karlsruhe, Germany). These cells (at logarithmic growth phase) were randomly divided into four groups: group A (Hoe trial group), group B (Hoe control group), group C (Rho trial group) and group D (Rho control group). All the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2 Hoe/FACS

2.2.1 Cells sorting by Hoe/FACS

As a standard control, the cultured cells were sorted by Hoe/FACS. The single-cell suspension was prepared by passing through a 50 μm mesh filter and diluted to a working concentration of 1×10^6 cells/ml with ice-cold PBS containing 2% FCS. In group A the cells were stained with the Hoe dye (Sigma Chemical Co., St. Louis, MO) at a concentration of 6 μg/ml (37 °C for 90 min) under mild shaking. In group B the cells were incubated in the presence of Verapamil (50 μM) and Hoe for 90 min at 37 °C. The Varapamil, which blocks the transporters responsible for Hoe exclusion, is applied to check the purity of the SP cells. After washed twice by PBS/2%FBS, the cells were incubated with 10 μg/ml propidiumiodide (PI) (Sigma Chemical Co., St. Louis, MO) for exclusion of dead cells. After excitation of the Hoe dye at 350 nmby ultra-violet (UV) laser on MoFlow (Cytomation Inc., Fort Collins CO., USA) and measurement of the fluorescence profile in two wave-lengths analysis (450 and 695 nm), both SP and non side population (NSP) cells were collected. SP cells showed low staining with Hoe and NSP cells were more brightly stained. A second 488 nm argon laser (100 mW) was used to excite PI fluorescence for excluding dead cells. Results were analyzed using the Summit v3.1 software (Cytomation, Dako, Glostrup, Denmark).

2.2.2 Profiles of isolated subpopulations by Hoe/FACS

SP cells should be present in the low forward scatter (FSC) and the low side scatter (SSC) fractions. There are two scatter plots reflected the distribution of cells, and a very low profile of SP (Fig. 1A) cells was shown. According to PI tests, there were about 30.8% dead cells in the Hoe stained cells (Fig. 1C). In group A, the percentage of SP was 1.4 ± 0.01% (Fig. 1B). When exclusion of the dye was inhibited by Verapamil in group B, the SP cells almost could not be discriminated from the MHCC97 cell line (Fig. 1D).

2.3 Rho/FACS

2.3.1 Cells sorting by Rho/FACS

The cells in group C were stained with Rho (Sigma Chemical Co., St. Louis, MO) at a concentration of 0.1 μg/ml (37 °C for 30 min) and washed with PBS/2%FBS twice. The cells in group D, which were used to define fluorescence threshold, were co-incubated in the presence of Verapamil (50 μM) and Rho for 30 min at 37 °C. All the above cells were centrifuged and suspended in PBS/2%FBS containing 10 μg/ml PI to extrude dead cells. Flow cytometric analysis of Rho fluorescence at 485 nm was carried out with Aria flow cytometer (Becton Dickinson cooperation, Mountain View, CA). Similar to Hoe/FACS, two filters were selected to discriminate cell subpopulations. As the Rho dye is activated through the FITC filter (Wang et al., 2006), the combination of FITC and PerCP-Cy5.5 filters was found optimal. The low Rho fluorescence and Verapamil negative was designated as Rho^{low} subpopulation, and the remaining cells were Rho^{high} subpopulation.

2.3.2 Profiles of isolated subpopulations by Rho/FACS

Rho^{low} cells should be present in the low FSC and the low SSC fractions. There are two scatter plots reflected the distribution of cells, and a very low profile of Rho^{low} (Fig. 1E) cells was shown. According to PI tests, there were about 30.8% dead cells in the Rho stained cells (Fig. 1G), much fewer dead cells than in the Hoe stained cells. In group C, the percentage of Rho^{low} in total cells was 2.1 ± 0.02% (Fig. 1F). When the exclusion of Rho dye was inhibited by Verapamil, the Rho^{low} cells could not be discriminated from the MHCC97 cell line either (Fig. 1H).

2.4 Cross detection of cell phenotypes

To further compare the effects of Hoe and Rho on CSCs sorting, the cross detection of SP and Rho^{low} cells was done. After the SP cells were isolated, they were immediately incubated with 0.1 μg/ml Rho (at 37 °C for 30 min) for Rho fluorescence analyzing. Meanwhile, the freshly isolated Rho^{low} cells were also evaluated by Hoe dyeing. The Rho^{low} (Fig. 1I) and SP (Fig. 1K) cells contained much fewer cells than the unsorted cells. The SP cells in Rho^{low} subpopulation was 52.5 ± 0.29% (Fig. 1J), in contrast, the Rho^{low} cells in SP subpopulation was 72.7 ± 0.36% (Fig. 1L). Based on the above results, the ability of Rho staining to separate subpopulations from the MHCC97 cell line is similar to Hoe staining.

2.5 Comparison of the proliferative and self-renewal ability of SP and Rho^{low} cells

The cells were adjusted to 1×10^7/ml and seeded in six-well plates (5×10^5 cells per well). Each kind of cells had 7 parallel samples. The culture media was changed every two days. During 7 days period, in each group, the cells of one parallel sample were trypsinized at 8 o'clock each day. Then the cells were counted under an inverted microscope (BX50-32E01, Olympus, Tokyo, Japan). As Fig. 2A shows the Rho^{low} cells with stronger ability of self-renewal and shorter doubling time than the Rho^{high} cells ($P < 0.05$). The Rho^{low} cells doubled their number in 16.9 ± 0.24 h; the Rho^{high} cells doubled in 25.4 ± 0.28 h. Besides, the Rho^{low} cells had a bigger multiplication number than the Rho^{high} cells ($P < 0.05$). The biggest multiple of the Rho^{low} cells was 15.2 ± 0.15 and that of the Rho^{high} cells was 12.3 ± 0.18. Thus, the Rho^{low} (Fig. 2B) and Rho^{high} (Fig. 2C) cells had greatly different densities 3 days after cultivation. Through statistical assay, the SP cells had little shorter doubling time (16.2 ± 0.23

h) and a slightly bigger multiplication number (15.7 ± 0.21) than the Rho^{low} cells ($P > 0.05$). Taken together, the Rho^{low} cells had similar ability to the SP cells for self -renewal.

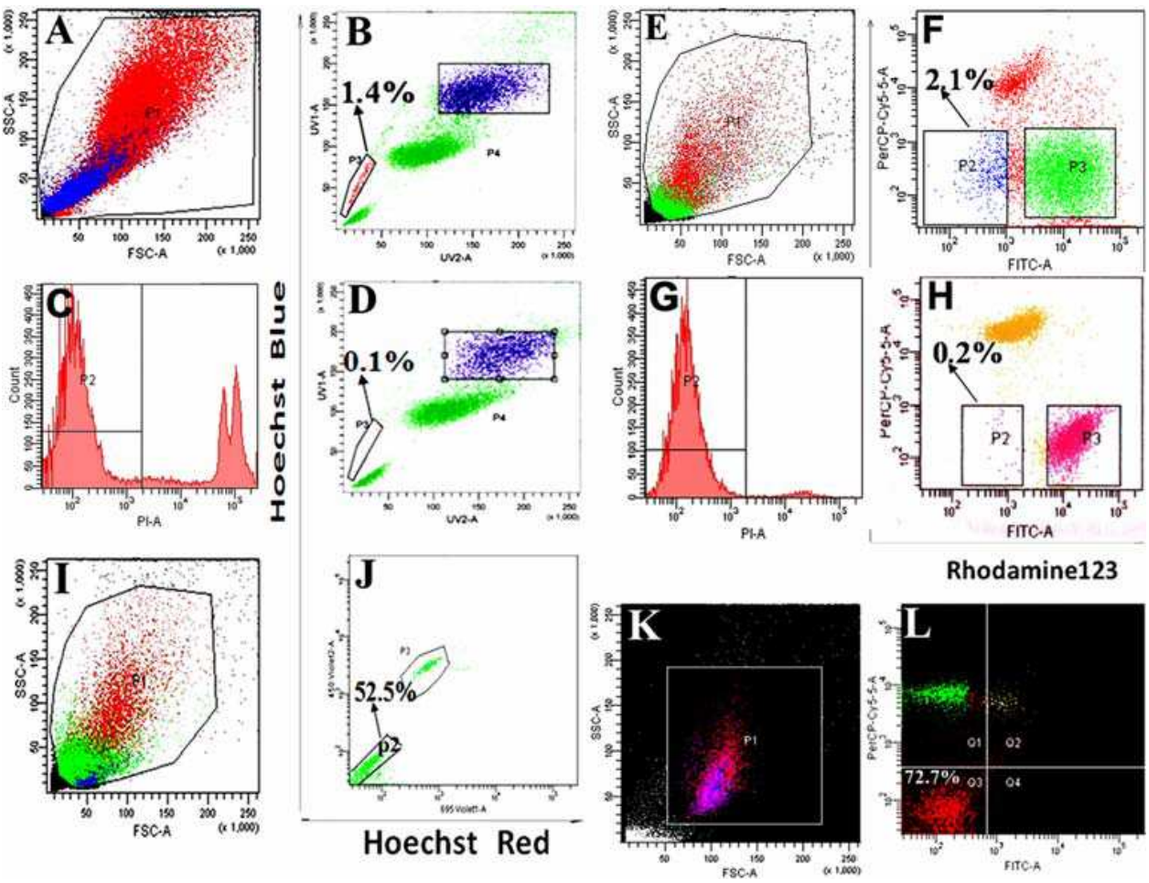

Fig. 1. The profiles of cell subpopulations in the MHCC97 cell line

(A), when total cells were stained with Hoe (p1), the blue population indicated possible SP cells with low fluorescence. (B), parts of them were demonstrated to be true SP cells (p3), parts were non SP cells (p4). (C), the viable and dead cells were determined by PI. (D), the profile of SP population (p3) decreased in the presence of Verapamil. (E), the distribution of total cells stained with Rho. (F), the Rho^{low} cells were shown as a low Rho fluorescence (p2) and the Rho^{high} cells were with high fluorescence (p3). (G), the most cells were viable. (H), when the cells were treated with Verapamil, the fluorescence of the Rho^{low} cells was shifted to a higher level (p3). The distributions of (I) the Rho^{low} cells stained with Hoe (p1) and (K) the SP cells stained with Rho (p1). (J), the percentage of the SP cells in Rho^{low} (p2). (L), the percentage of Rho^{low} cells in SP (Q3).

We also designed soft agar clone formation test to compare the self-renewal capacity of cells from each subpopulation. The 2×DMEM (containing 200 ml/L FCS) were mixed with equal volume agarose (12 mg/ml) (Sigma Chemical Co., St. Louis, MO) as the bottom-layer agar. The cells from SP, NSP, Rho^{low} and Rho^{high} were diluted to 1×10^4 cells/ml and put on the bottom-layer agars, respectively. Immediately, the cells were covered with the top-layer

agars, which consisted of agarose (7 mg/ml) and equal volume 2×DMEM. Culture for 14 days, the number of cell clones was counted. After culture for 14 days, most of the SP and Rho^{low} cells had formed cell clones. The rate of clone formation in the SP cells was 73.5 ± 0.12%, and that in the Rho^{low} cells (Fig. 2D) was 70 ± 0.11%. In contrast with the SP and Rho^{low} cells, the NSP and Rho^{high} cells had much lower clone formation rates ($P < 0.01$). Such as the clone formation rate in the NSP cells was 25.2 ± 0.06%, and that in the Rho^{high} cells (Fig. 2E) was 30.4 ± 0.07%.

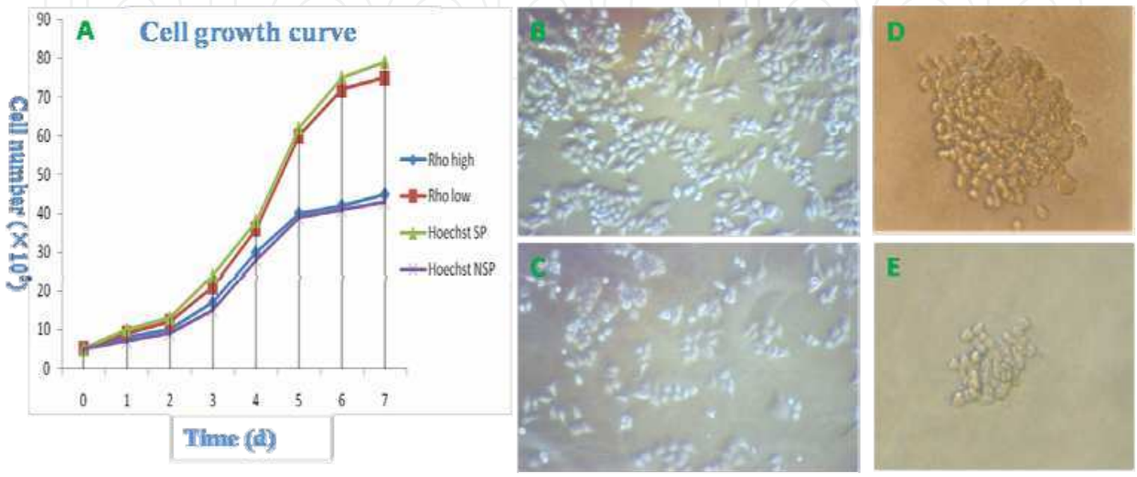

Fig. 2. The proliferative abilities of different typed cells

(A), cell growth curve. (B) the Rho^{low} and SP cells from the MHCC97 cell line had a stronger capacity to proliferate than (C) the Rho^{high} and NSP cells, respectively. The cell clones formed by (D) the Rho^{low} cells was much bigger than that by (E) the Rho^{high} cells. Magnification: panels B-D, ×100.

2.6 Cell marker expression

2.6.1 Immunocytochemical assay

One early hepatic marker was selected to evaluate the maturity of the cells from different subpopulations. First-anti AFP mAb (Zhong-Shan Co., Beijing, China) (1μl) was diluted with PBS (99 μl) to a working density. The cells from different subpopulations ($1×10^4$/ml) were incubated with mAb, coloured by DAB (Zhong-Shan Co., Beijing, China), and stained by hematoxylin. In negative control groups, the PBS was adopted instead of first antibody. These cells were periodically viewed under an inverted microscope. When the appearance of Buffy colour particles showed in the cytoplasm, the result was identified positive. The positive cells were counted and their percentage was calculated. The percent of AFP positive cells<10% was defined as negative (-), 10–25% as positive (+), 25–50% as moderate positive (++), >50% as strong positive (+++). We collected and incorporated the same immunocytochemical results. The detailed data have been enumerated in Table 1. The Rho^{low} (Fig. 3A) and SP cells (Fig. 3C) expressed higher AFP than the Rho^{high} (Fig. 3B) and NSP (Fig. 3D) cells, respectively ($P < 0.01$). Besides, the SP cells had similar expression level of AFP to the Rho^{low} cells ($P > 0.05$) (Fig. 3I). These results demonstrated the cells were immature and from hepatic carcinoma.

cell subpopulation	sample number	AFP-	AFP+	AFP++	AFP+++
Rho^{low} a	35	1±0.14	6±0.28	20±0.86	8±0.28
Rho^{high}	35	10±0.43	16±0.56	6 ±0.28	3±0.14
SP b	35	1±0.14	4±0.14	21±0.86	9±0.43
NSP	35	12±0.43	15±0.56	5±0.14	3±0.14

Table 1. The expression of AFP in cell subpopulation

All AFP immnocytochemical data were viewed by three different researchers and expressed as mean±SD. a Rho^{low} **VS** Rho^{high} , N=35, $P<0.05$; b SP **VS** Rho^{low}, N=35, $P>0.05$.

2.6.2 Immuno-flow cytometric analysis

One CSCs marker was applied to identify the characters of CSCs in each subpopulation. The freshly isolated Rho^{low}, SP, NSP and Rho^{high} cells were prepared as single-cell suspensions at a density of 1×10^6 cells/ml, incubated with 0.075 ml CD133 mAb (Zhong-Shan Co., Beijing, China) for 30 min at 4 °C, washed in PBS twice, and fixed in 0.1% formaldehyde for flow cytometric analysis. Irrelevant isotype matched mAb was used as negative controls. Dead cells were excluded by PI gating. The percentage of CD133 positive cells in Rho^{low} (Fig. 3E) was 51.84 ± 0.33%, in Rho^{high} (Fig. 3F) was 13.36 ± 0.11%, in SP (Fig. 3G) was 53.24 ±0.42%, and in NSP (Fig. 3H) was 12.70 ± 0.10%. The CD133 expressed significantly different among these subpopulations ($P < 0.01$) (Fig. 3J).

AFP expressed in (A) the Rho^{low} cells was strongly higher than that in (B) the Rho^{high} cells; AFP expressed higher in (C) the SP cells than in (D) the NSP cells. For figures (E)-(H), the black histogram was the isotype control, and the red histogram was the trial. A fluorescent value was set as the regional marker (brunneus bar), which must be just bigger than the fluorescent values of cells in control group. Then the fluorescent value was remembered and used as the regional marker (brunneus bar) in trial group. The positive region was where the fluorescent values were bigger than the set fluorescent value in trial group. The percentages of the CD133 positive cells in (E) Rho^{low} and (G) SP were also higher than those in (F) Rho^{high} and (H) NSP, respectively. The detailed expressions of (I) AFP and (J) CD133 are reflected by column charts. Magnification: panels A-D, ×400.

2.7 Tumor formation in nude mice

The gold standard for measuring tumor initiating potential is to assess the capacity of cells to form tumors when injected into immune-deficient mice. Using this model, we tended to check the differentiating ability of the cells from each subpopulation. Thirty-two male BALB/C nude mice (Fourth Military Medical University, Xi'an, China) were maintained under standard conditions according to the College's guidelines. The freshly isolated SP, NSP, Rho^{low} and Rho^{high} cells (1×10^6) were injected into the right back of mice, respectively. When the xenograft tumors grew to proper size, the mice were sacrificed and the tumor tissues were collected, fixed in 4% formaldehyde, and embedded in paraffin for H&E staining to assess tumors histology. To avoid subjective bias, each chip was marked with

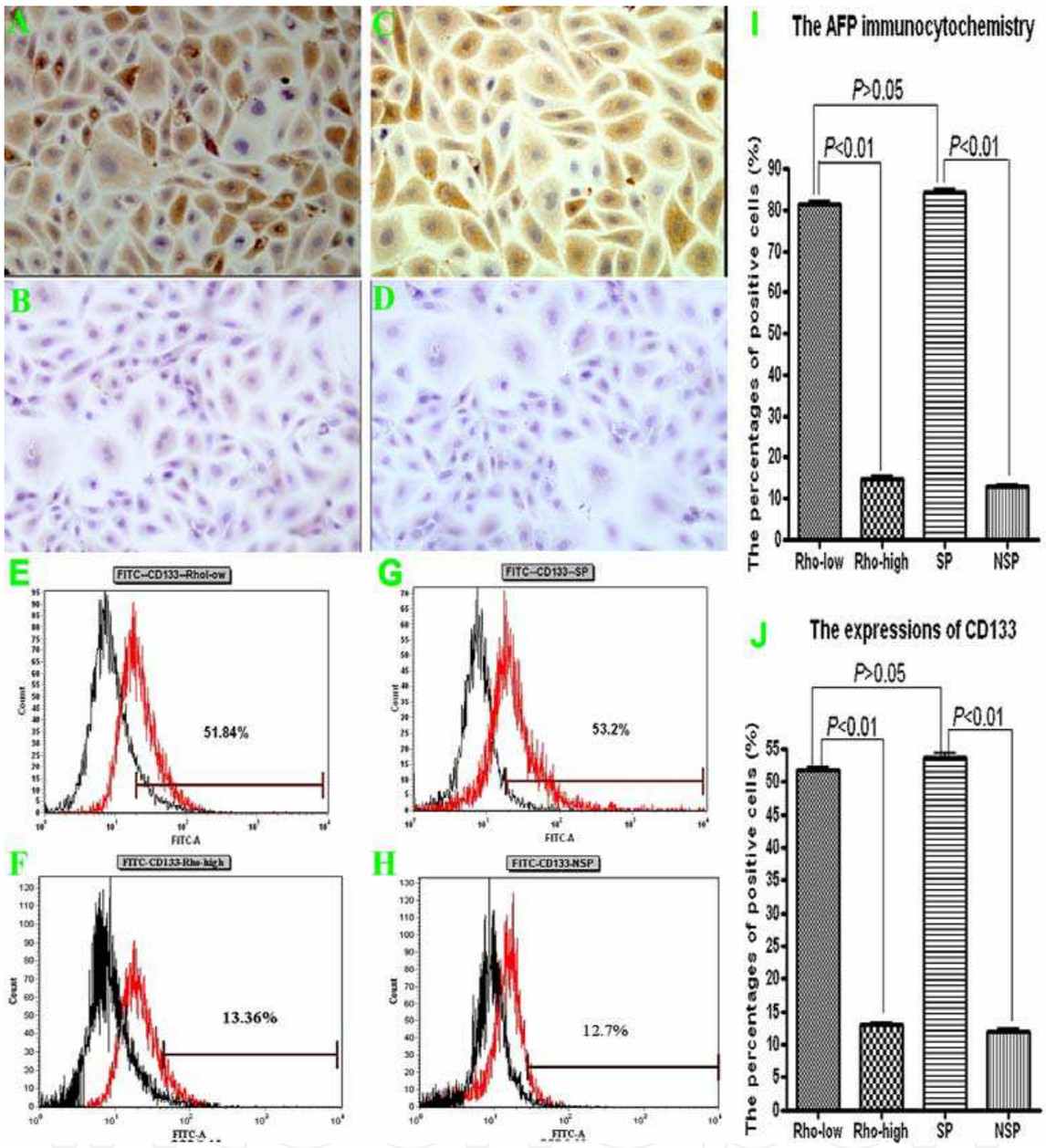

Fig. 3. The expressions of CSCs markers

one number within 1–1 to 1–7 (SP), 2–1 to 2–7 (NSP), 3–1 to 3–7 (Rho^{low}) and 4–1 to 4–7 (Rho^{high}), and viewed by three different pathologists. Based on the tumor size, no macroscopic tumor was defined as negative (-), the diameter of tumor <0.2 cm as positive (+), 0.2–0.5 cm as moderate positive (++), >0.5 cm as strong positive (+++).Since the mice were injected with cells from different subpopulations, they were checked each day. The xenograft tumors grew to proper size 5 weeks later, within the mice which were injected with SP or Rho^{low} cells, however, we couldn't find obvious tumor in some mice injected with NSP or Rho^{high} cells. We counted the number of tumors in each mouse, measured the size of each tumor and summarized those data in Table 2. The tumor formation rate in mice with

SP cells injected was 78.0 ± 1.21%, and that with Rho^{low} cells injected was 66.0 ± 1.03%. In addition, the rate in mice with Rho^{high} cells injected was <15.0%. This indicates that the Rho^{low} cells had stronger ability to form tumors in vivo than the Rho^{high} cells ($P < 0.01$); however, the Rho^{low} cells (Fig. 4A) had slightly lower ability to form tumors than the SP cells ($P > 0.05$) (Fig. 4B). Part of each tumor was made into chips and stained by H&E (Fig. 4C–F) to assess tumor pathology. According to three different pathologists' judgements, the tumors were all hepatic tumor genesis, which could help us discriminate the neoplasms from other kinds of tumors. After the cells from some tumors were isolated and cultured, we used flow cytometry to analyze the phenotype of those cells. Through our analysis, the tumors generated by the injected cells, were mainly from the SP or Rho^{low} cells.

cell subpopulation	sample number	tumor-	tumor+	tumor++	tumor+++
Rho^{low} a	28	1±0.11	12±0.51	11±0.28	4±0.14
Rho^{high}	28	17±0.36	7±0.21	4±0.15	0±0.00
SP b	28	1±0.11	10±0.23	8±0.24	9±0.21
NSP	28	19±0.36	6±0.20	3 ±0.12	0±0.11

Table 2. The tumor formation rate of cell subpopulation in nude mice

All the mice were checked carefully and wholly for tumors, and the diameter of each tumor was measured. a Rho^{low} VS Rho^{high}, N=28, $P<0.01$; b SP VS Rho^{low}, N=28, $P<0.05$.

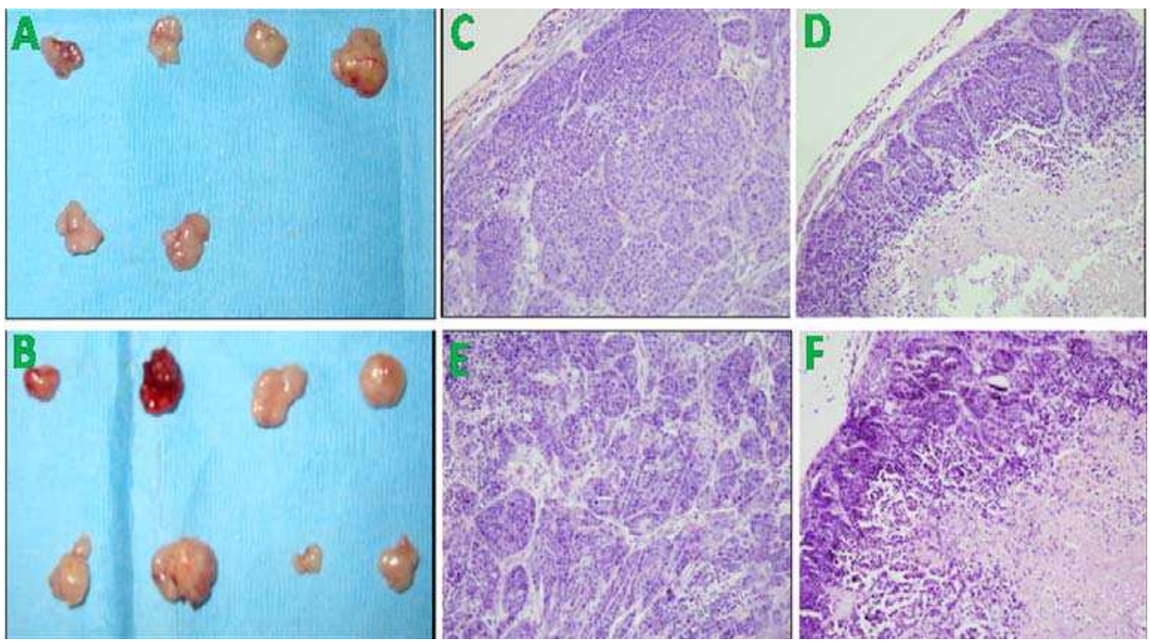

Fig. 4. Tumor formation capacity of each typed cells

Both (A) the Rho^{low} and (B) SP cells formed different sized tumors. H & E dyeing revealed the tumors formed by (C) the Rho^{low}, (D) Rho^{high}, (E) SP and (F) NSP cells were all hepatic carcinoma genesis. Magnification: C-F,×100.

2.3 Comparison of two methods

The cells that exclude Hoe dye are SP cells. Because the SP cells express high levels of stem cell markers and low levels of differentiating markers (Haraguchi et al., 2006), the SP subpopulation is widely used to enrich stem cells. Early in 1993, with a sequential Hoe/Rho sorting system, the subfractions of cells that stained most weakly with both dyes were demonstrated to be long-term repopulating primitive hematopoietic cells (Wolf et al., 1993). Again in 2004, Bertoncello and Williams found that the supravital dyes Rho and Hoe have proven to be remarkably powerful probes for the characterization, resolution, isolation and purification of primitive hematopoietic stem cells (PHSCs) (Bertoncello & Williams, 2004). However, since the cytotoxicity of Hoe is confirmed in recent years (after 2004) (Adamski et al., 2007), the combination of the two dyes is rare.

There are two major limitations in the SP research. First one is the toxicity of Hoe (Kondo et al., 2004). It is demonstrated that Hoe interferes with C2C12 cell fusion as long as the dye is present in the nucleus (Adamski et al., 2007), more and more researchers have found that Hoe staining can affect on cell differentiation. For each cell line, the Hoe concentration should be optimised to minimize cytotoxicity. Second one is the requirement of an UV laser to excite the Hoe dye. The flow cytometer equipped with UV sources is expensive to own and operate and is not readily available to many laboratories or institutions. To solve this problem, some researchers (Cabana et al., 2006) tried to design a minimum UV excitation implement for measuring Hoe. At present, the related endeavor is still undergoing. However, the major toxic drawback of Hoe remains unsolved. Thus, it may be better to find a replacement of Hoe in isolating stem-like cells. Like Hoe, Rho is actively pumped out of the cells by ABC transporters. Some researches have also shown that no toxicity on cells is found by Rho, even at large dose (1–10 mM) (Ribou et al., 2003). In our study, we tested the cytotoxic effects of the two dyes and found Hoe was much more harmful to cells than Rho. That is to say, the Rho dye avoided one main disadvantage of the Hoe dye for CSCs isolation. Besides, to perform Rho/FACS, it only needs a common flow cytometer, in which the fluorescence is activated by argon-ion or helium–cadmiumlaser. The common flow cytometer is a lower cost (both in terms of purchase and maintenance) than cytometers offering UV excitation. Our FACS results indicate the percentage of SP was slightly lower than that of Rho^{low} in the MHCC97 cell line. It does not mean that Rho can accumulate more CSCs than Hoe. The reason may be that the purity of CSCs in Rho^{low} is lower. Based on the cross detections of SP and Rho^{low} cells, the percentage of CSCs in total cells may be 1% (72.7% Rho^{low} cells in 1.4% SP subpopulation; 52.5% SP cells in 2.1% Rho^{low} subpopulation). In other words, the combination of Rho/FACS and Hoe/FACS may enrich much purer CSCs, which is consistent with others.

Based on the analysis of several CSCs characteristics, we found the Rho^{low} cells had similar characters as the SP cells, such as high proliferative ability and high expression of CD133. The use of CD133 has proven to be very successful in the identification of CSCs in both human brain and colon carcinomas (O'Brien et al et al., 2007). Interestingly, about 5 days in culture, Rho^{high} and NSP subpopulations reached a plateau in the growth curve. The reasons

maybe parts of the cells in NSP or Rho^{high} became older and older and finally died. Meanwhile, a few of stem cells gave birth to new cells. The number of dead cells was similar to that of the new-born cells. However, the cells from SP and Rho^{low} were mostly stem cells, which were long lived and could continually differentiate into different typed cells. These cells would continue proliferating for a long time. In addition, we found that the Rho^{low} and SP cells had similar tumor formation rates and the H&E staining confirmed the tissues hepatic genesis. In one word, the Rho^{low} and SP cells shared similar characters both in vivo an in vitro. That is to say, although Rho/FACS is not as effective as Hoe/FACS to isolate stem cells, given its low cytotoxicity and low cost, it may replace Hoe/FACS in isolating some typed CSCs.

2.4 Limitations

Although many findings support the isolation of SP cells via Hoe and Rho staining as an identification method for CSCs, some controversial results were obtained regarding the expressions of specific stem cell/CSC markers on SP cells. Burkert et al found that SP cells of several gastrointestinal cancer cell lines showed no increased expressions of stem cell markers like CD133, CD44, Musashi-1, Oct-4 and CD117 compared to non-SP cells. Both fractions were similarly clonogenic in vitro, tumorigenic in vivo, and displayed similar differentiation potential in vitro and in vivo (Burkert et al., 2008). Due to these controversial findings, dyes efflux and isolation of SP cells can not be applied to identify and isolate CSCs, at least for some tumor entities. Various cell types needs different optimized protocols, such as single cell suspension levels, optimal dye concentration, concentration of Verapamil, the density of cells in culture and so on. Optimized and standardized protocols for each cell type as well as stringent cell culture and isolation settings are required to eliminate the risk of analyzing different SP cells. These standards will help to abolish skepticism and uncertainty about the general validity of the technique and potential of SP cells.

3. Conclusion

Based on the results, we get these conclusions: firstly, to isolate CSCs from the MHCC97 cell line, the effectiveness of Rho/FACS in enriching CSCs is similar to that of Hoe/FACS. Secondly, the Rho^{low} cells had similar characters as the SP cells; lastly, the Rho/FACS is less toxic and expensive than the Hoe/FACS. To conclude, when the Hoe/FACS is too toxic or expensive to do biological researches of some typed stem cells, the Rho/FACS is still another elective method. To conclude, in circumstances where suspected cancer stem-like cells may be susceptible to Hoe toxicity and/or where the cost of UV-FACS is prohibitive, the application of Rho/FACS is a credible method of isolation.

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5. References

Adamski, D., Mayol, J.F., Platet, N., Berger, F., Hérodin, F., & Wion, D. (2007). Effects of Hoechst 33342 on C2C12 and PC12 cell differentiation. *FEBS Lett*, Vol.581, No.16, (June 2007), pp.3076-3080, ISSN 0014-5793

Bertoncello, I., Williams, B. (2004). Hematopoietic stem cell characterization by Hoe 33342 and rhodamine 123 staining. *Methods Mol Biol*, Vol.263, pp.181-200, ISSN 1064-3745

Burkert, J., Otto, W.R., & Wright, N.A. (2008). Side populations of gastrointestinal cancers are not enriched in stem cells. *J Pathol*, Vol.214, No.5, (April 2008), pp.564-573, ISSN 0022-3417

Cabana, R., Frolova, E.G., Kapoor, V., Thomas, R.A., Krishan, A., & Telford, W.G. (2006). The minimal instrumentation requirements for Hoechst side population analysis: stem cell analysis on low-cost flow cytometry platforms. *Stem Cells*, Vol.24, No.11, (November 2006), pp.2573-2581, ISSN 1066-5099

Calcagno, A.M., Salcido, C.D., Gillet, J.P., Wu, C.P., Fostel, J.M., Mumau, M.D., Gottesman, M.M., Varticovski, L., & Ambudkar, S.V. (2010). Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. *J Natl Cancer Inst*, Vol.102, No.21, (November 2010), pp.1637-1652, ISSN 0027-8874

Choi, C.H. (2005). ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. *Cancer Cell Int*, Vol.5, (October 2005), pp.30, ISSN 1475-2867

Dalerba, P., Cho, R.W., & Clarke M.F. (2007). Cancer stem cells: models and concepts. *Annu Rev Med*, Vol.58, pp. 267-284, ISSN 0066-4219

Dontu, G., Al-Hajj, M., Abdallah, W.M., Clarke, M.F., & Wicha, M.S. (2003). Stem cells in normal breast development and breast cancer. *Cell Prolif*, Vol.36, Suppl 1, (October 2003), pp.59-72, ISSN 0960-7722

Goodell, M.A. (2002). Multipotential stem cells and 'side population' cells. *Cytotherapy*, Vol.4, No.6, pp.507-508, ISSN 1465-3249

Haraguchi, N., Utsunomiya, T., Inoue, H., Tanaka, F., Mimori, K., Barnard, G.F., & Mori, M. (2006). *Stem Cells*, Vol.24, No.3, (March 2006), pp.506-513, ISSN 1066-5099

He, L., Ji, B.S. (2008). In vitro and in vivo study of CJY, an isoflavone, on p-glycoprotein function in rats. *J Chemother*, Vol.20, No.3, (June 2008), pp.361-367, ISSN 1973-9478

Huang, Z., Cheng, L., Guryanova, O.A., Wu, Q., & Bao, S. (2010). Cancer stem cells in glioblastoma--molecular signaling and therapeutic targeting. *Protein Cell*, Vol.1, No.7, (July 2010), pp.638-655, ISSN 1674-8018

Jordan, C. T., Guzman, M. L., & Noble, M. (2006). Cancer stem cells. N Engl J Med, Vol.355, No.12, (September 2006), pp.1253-1261, ISSN 0028-4793

Kemper, K., Grandela, C., & Medema, J.P. (2010). Molecular identification and targeting of colorectal cancer stem cells. *Oncotarget*, Vol.1, No.6, (October 2010), pp.387-95, ISSN 1949-2553

Keysar SB, Jimeno A. (2010). More than markers: biological significance of cancer stem cell-defining molecules. *Mol Cancer Ther*, Vol.9, No.9, (September 2010), pp.2450-2457, ISSN 1535-7163

Kim, M., Morshead, C.M. (2003). Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J Neurosci*, Vol.23, No.33, (November 2003), pp.10703-10709, ISSN 0270-6474

Kondo, T., Setoguchi, T., & Taga, T. (2004). Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A*, Vol.101, No.3, (*January 2004), pp.781-786, ISSN* 1091-6490

Konopleva, M,Y., Jordan, C.T. (2011). Leukemia stem cells and microenvironment: biology and therapeutic targeting. *J Clin Oncol*, Vol.29, No.5, (February 2011), pp.591-599 ISSN 0732-183X

Lukacs, R.U., Memarzadeh, S., Wu, H., & Witte, O.N. (2010). Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation. *Cell Stem Cell*, Vol.7, No.6, (December 2010), pp.682-693, ISSN 1934-5909

Ma, S., Chan, K.W., Hu, L., Lee, T.K, Wo J.Y., Ng, I.O., Zheng, B.J, & Guan, X.Y. (2007). Identification and characterization of tumorigenic liver cancer stem/ progenitor cells. *Gastroenterology*, Vol.132, No.7, (June 2007), pp.2542-2556, ISSN 0016-5085

Majka, S.M., Beutz, M.A., Hagen, M., Izzo, A.A., Voelkel, N., & Helm, K.M. (2005). Identification of novel resident pulmonary stem cells: form and function of the lung side population. *Stem Cells*, Vol.23, No.8, (September 2005), pp.1073-1081, ISSN 1066-5099

Makino, S. (1959). The role of tumor stem-cells in regrowth of the tumor following drastic applications. *Acta Unio Int Contra Cancrum*, Vol.15, Suppl 1, pp. 196-198, ISSN 0365-3056

Martin, C.M., Meeson, A.P., Robertson, S.M., Hawke, T.J., Richardson, J.A., Bates, S., Goetsch, S.C., Gallardo, T.D., & Garry, D.J. (2004). Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Dev Biol*, Vol.265, No.1, (January 2004), pp.262-275, ISSN 0012-1606

Meeson, A.P., Hawke, T.J., Graham, S., Jiang, N., Elterman, J., Hutcheson, K., Dimaio, J.M., Gallardo, T.D., & Garry, D.J. (2004). Cellular and molecular regulation of skeletal muscle side population cells. *Stem Cells*, Vol.22, No.7, pp.1305-1320, ISSN 1066-5099

Montanaro, F., Liadaki, K., Volinski, J., Flint, A., & Kunkel, L.M. (2003). Skeletal muscle engraftment potential of adult mouse skin side population cells. *Proc Natl Acad Sci U S A*, Vol.100, No.16, (Augest 2003), pp.9336-9341, ISSN 1091-6490

O'Brien, C.A., Pollett, A., Gallinger, S., & Dick, J.E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*, Vol.445, No.7123, (*January 2007), pp.106-110, ISSN 0028-0836*

Pardal, R., Clarke, M.F., & Morrison, S.J. (2003). Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer*, Vol.3, No.3, (December 2003), pp. 895-902, ISSN 1474-175X

Ribou, A.C., Vigo, J., Kohen, E., & Salmon, J.M. (2003). Microfluorometric study of oxygen dependence of (1"-pyrene butyl)-2-rhodamine ester probe in mitochondria of living cells. J *Photochem Photobiol B*, Vol.70, No.2, (May 2003), pp.107-115, ISSN 1011-1344

Scharenberg, C.W., Harkey, M.A., & Torok-Storb, B. (2002). The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood*, Vol. 99, No.2, (January 2002), PP.507-512, ISSN 0006-4971

Spangrude, G.J., Johnson, G.R.(1990). Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc Natl Acad Sci U S A*, Vol.87, No.19, (October 1990), pp. 7433-7477, ISSN 1091-6490

Storch, C.H., Theile, D., Lindenmaier, H., Haefeli, W.E., & Weiss, J. (2007). Comparison of the inhibitory activity of anti-HIV drugs on P-glycoprotein. *Biochem Pharmacol*, Vol.73, No.10, (May 2007), pp.1573-1581, ISSN 0006-2952

Sullivan, J.P., Minna, J.D. (2010). Tumor oncogenotypes and lung cancer stem cell identity. *Cell Stem Cell*, Vol.7, No.1, (July 2010), pp.2-4, ISSN 1934-5909

Uchida, N., Leung, F.Y., & Eaves, C.J. (2002). Liver and marrow of adult mdr-1a/1b(-/-) mice show normal generation, function, and multi-tissue trafficking of primitive hematopoietic cells. *Exp Hematol*, Vol.30, No.8, (Augest 2002), pp.862-869, ISSN 0301-472X

Umemoto, T., Yamato, M., Nishida, K., Yang, J., Tano, Y., & Okano, T. (2006). Limbal epithelial side-population cells have stem cell-like properties, including quiescent state. *Stem Cells*, Vol.24, No.1, (January 2006), pp.86-94, ISSN 1066-5099

Vautier, S., Milane, A., Fernandez, C., Buyse, M., Chacun, H., & Farinotti, R. (2008). Interactions between antiparkinsonian drugs and ABCB1/P-glycoprotein at the blood-brain barrier in a rat brain endothelial cell model. *Neurosci Lett*, Vol.442, No.1, (September 2008), pp.19-23, ISSN 0304-3940

Wang, Y., Hao, D., Stein, W.D., Yang, L. (2006). A kinetic study of Rhodamine123 pumping by P-glycoprotein. *Biochim Biophys Acta*, Vol.1758, No.10, (October 2006), pp.1671-1676, ISSN 0006-3002

Welm, B., Behbod, F., Goodell, M.A., & Rosen, J.M. (2003). Isolation and characterization of functional mammary gland stem cells. *Cell Prolif*, Vol.36, Suppl 1, (October 2003), pp.17-32, ISSN 0960-7722

Wicha, M.S., Liu, S., & Dontu, G. (2006). Cancer stem cells: an old idea--a paradigm shift. *Cancer Res*, Vol.66, No.4, (February 2006), pp.1883-1890, ISSN 0008-5472

Wolf, N.S., Koné, A., Priestley, G.V., & Bartelmez, S.H. (1993). In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. *Exp Hematol*, Vol.21, No.5, (May 1993), pp.614-622, ISSN 0301-472X

Wu, C., Alman, B.A. (2008). Side population cells in human cancers. *Cancer Lett*, Vol.268, No.1, (September 2008), pp.1-9, ISSN 0304-3835

Wulf, G.G., Luo, K.L., Jackson, K.A., Brenner, M.K., & Goodell, M.A. (2003). Cells of the hepatic side population contribute to liver regeneration and can be replenished with bone marrow stem cells. *Haematologica*, Vol.88, No.4, (April 2003), pp.368-378, ISSN 0390-6078

Zhou, S., Schuetz, J.D., Bunting, K.D., Colapietro, A.M., Sampath, J., Morris, J.J., Lagutina, I., Grosveld, G.C., Osawa, M., Nakauchi, H., & Sorrentino, B.P. (2001). The ABC

transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med*, Vol.7, No.9, (September 2001), pp.1028-1034, ISSN 1078-8956

Liu, W.H., Qian, N.S., Li, R., & Dou, K.F. (2010). Replacing Hoechst with rhodamine123 in isolation of cancer stem-like cells from the MHCC97 cell line. *Toxicol In Vitro*, Vol. 24, No. 2, (March 2010), pp.538-545, ISSN 0887-2333

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