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**Fluctuation of the SP/non-SP phenotype in the C6 glioma
cell line.**

**Nadine Platet*, Jean-François Mayol*[□], François Berger, Francis Hérodin[□]
and Didier Wion[‡].**

**INSERM U318, CHU Michallon, 38043 Grenoble, France,[□] Centre de
Recherches du Service de Santé des Armées, PO Box 87, 38702 La Tronche,
France.**

* N.P. and JF. M. contributed equally to this work.

‡ corresponding author didier.wion@ujf-grenoble.fr

Abstract:

Using the C6 glioma cell as a paradigm, we found that i) the clonogenicity of C6 cells is several orders of magnitude higher than the percentage of SP cells; ii) non-SP cells are able to generate SP cells, and conversely SP cells generate non-SP cells; iii) non-SP sorted cells behave as tumorigenic cells. Hence, in C6 cells cultured in serum-containing medium, SP cells can be generated from non-SP cells. This dynamic equilibrium explains in C6 cells the maintenance of the SP phenotype with cell passaging and demonstrates the existence of tumorigenic non-SP cells.

Keywords: Cancer stem cell; side population; Hoechst 33342; glioma.

Introduction

In the hematopoietic system, stem cells (HSC) have been enriched as a side population (SP) on their ability to exclude the fluorescent vital dye Hoechst 33342 [1]. This Hoechst efflux phenomenon, initially described as a reliable tool for HSC enrichment, has received particular attention when it became apparent that this property is shared with stem cells found in several other tissues [2]. Afterwards, several studies have provided evidence for the existence of a distinct SP in tumor cell lines such as C6, U373, MCF7, HeLa and many other cancer cell lines [3-11]. Moreover, SP cells have also been found in primary neuroblastoma tumor cell samples [4], and leukemic SP cells have been detected in the bone marrow from patients with acute myeloid leukemia [12]. According to the cancer stem cell paradigm, these SP cells generate both SP and non-SP cells, whereas non-SP cells produce non-SP cells only [3,4,10-11]. Moreover, because of their higher tumorigenicity or proliferative potential these SP cells are assimilated to stem-like cancer cells [3-11]. Hence, the SP phenotype may prove invaluable for the identification and characterization of cancer stem cells from cell lines and tumors, a point of crucial concern if these cells are the only cells capable of maintaining the growth of the tumor [13]. However, even if in a given cell line SP cells are described to be more tumorigenic than non-SP cells, there is no correlation between the presence of SP and tumorigenicity in general. Thus, SP is undetectable in the highly tumorigenic prostate tumor cells PPC-1, but is detected in the prostatic LAPC9 cell line which behaves intermediate tumorigenicity [5]. Likewise, SP cells are found in the low tumorigenic breast cancer cell line MCF7, but remain undetectable in the highly tumorigenic MDA-MB231 and MDA-MB435 breast cancer cells [5]. Another intriguing point is that the little percentage of SP cells found in cancer cell lines harbouring a detectable SP (0.05%-0.4%) seems low compared to their expected clonogenicity. In an attempt to elucidate these paradoxical findings, we decided to

reinvestigate the relationship existing between SP cells and cancer stem-like cells using the C6 glioma cell as a paradigm.

Material and methods.

Cell culture :

Rat glioma C6 cells (ATCC CCL107) [14] and the clonal derivative C6.9 cell line [15] were cultured in complete medium consisting in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and pyruvate, and supplemented with 10 % foetal bovine serum (FBS), and 2mM L-glutamine, all purchased from Gibco-Invitrogen. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Establishment of clonal population of C6 :

A suspension of C6 cells (passage 48) was obtained from subconfluent culture by trypsinisation. Cells were isolated by the mean of microscopic examination and micromanipulation, then distributed in 96-wells plate containing complete medium supplemented with conditioned medium of C6 cells. Each well was then checked for the presence of a solitary cell. After amplification, cells were tested for long-term proliferation and tumorigenicity.

Flow cytometry :

For side population analysis and isolation, cells were retrieved from subconfluent culture by trypsinisation, resuspended at 10⁶ cells/ml in prewarmed DMEM containing 2% FBS, 10mM Hepes, then incubated for 90 minutes at 37°C with Hoechst 33342 (2.5 µg/ml final concentration, Sigma) with intermittent mixing. Control cells were given Verapamil (50µM final concentration, Sigma) to block pumps that exclude the Hoechst. At the end of incubation, cells were spun down in the cold and resuspended in cold HBSS (purchased from Gibco-Invitrogen) containing 2% FBS and 10mM Hepes.

Cytometric analysis was performed on FACS Vantage SE. Hoechst dye was excited at 351 nm by UV laser and its fluorescence detected at two wavelengths using 424/44 (Hoechst blue) and 630/22 (Hoechst red) band pass filters. Dead cells were excluded from the plots based on propidium iodide staining. Data acquisition was achieved on 10^6 cells for SP analyses and 10^5 cells for cell sorting. Data were reanalyzed with FCS Express 2 software (De Novo Software, Thornhill, Canada).

For FACS analyses, the gates were placed using the verapamil control tube. Hence, only cells with Hoechst efflux potential were included in the SP gate. Conversely, the non-SP gate only included cells without efflux potential. Non-SP cell population included cycling and non-cycling cells. For cell sorting, the non-SP gate was slightly shifted to the right side of the histogram to ensure that SP cells were not included in this cell population (see Fig 2). Immediately after purification, sorted cells were either plated in complete medium for determination of in vitro growth potential and subsequent SP analysis, or either resuspended in DMEM for injection into nude mice.

Tumorigenicity:

For tumor experiments, various numbers of cells resuspended in 100 μ l of DMEM were injected subcutaneously into female nude mice (8-12 weeks old, Harlan). Tumorigenicity was evaluated by tumor incidence (i.e. number of tumor-bearing animals / number of animals injected) and latency (i.e. time of apparition of a palpable tumor). Animals without tumor uptake were sacrificed 4 months after cell injection.

RT-PCR analysis.

Total RNA from either SP or non-SP cells were amplified using Fast Lane cell cDNA and One step RT-PCR kits from Qiagen. RT-PCR were performed using the following primers:

mdr1: 5'-GCAAAGCTGGAGAGATCCTCACCAAG-3' and 5'-CAACATTTTCATTTC AACC ACTCCTGC- 3'; *ABCG2/bcrp1*: 5'-CCAGTTCCATGGCCCTGGCCATAG-3' and 5'- CAGGGCCACATGATTCCTCCACAG-3'; *gapdh*: 5'- CATGACCACAGTCCATGCCATCAC-3' and 5'-CCCTCAGATGCCTGCTTCGCCTTC-3'. PCR parameters were 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Numbers of cycles were 40 for *mdr1* and *ABCG2/bcrp1*, and 38 for *gapdh*. Genomic DNA contamination was controlled by PCR of non-reverse transcribed RNA (data not shown).

Results.

SP analysis of C6 and C6.9 glioma cells.

We first investigated the presence of SP cells in the C6 glioma cell line cultured in serum containing medium. As previously reported by others [3], a SP was detected in C6 cells (passage 50) cultured in serum-containing medium (Fig 1A). Cell passaging increases the percentage of SP cells (Fig 1B and table 1). Interestingly, when we defined SP cells by their verapamil sensitivity to efflux Hoechst 33342, we also detected an additional cell population whose staining is affected by verapamil. This cell population, we named “upper-SP” (Fig 1), represents the existence of SP cells with a higher DNA content (aneuploid cells, or cells in S and G2 phases). Since normal stem cells are largely quiescent and euploid, this upper-SP may not have been detected previously and could be a cancer cell line feature. Surprisingly, no SP nor upper-SP was detected in the C6.9 cells (Fig 1C and table 1), a subclone obtained by limited dilution of the original C6 cell line and previously used to investigate the effect of vitamin D and cannabinoids on glioma cell growth [15,16].

Tumorigenicity of C6.9 cells.

According to the assumption that SP cells are more tumorigenic than non-SP cells, we reasoned that C6.9 cells should be less tumorigenic than the C6 parental cells. To test this point we investigate the tumorigenicity of C6.9 cells, and found that 500 injected C6.9 cells or C6 cells were sufficient to induce tumor growth in nude mice (table 2A). Hence, C6.9 was as tumorigenic as the parental C6. Thus, this data raised again the paradox of the existence of a tumorigenic cell line with undetectable SP.

C6 SP and non SP cells are similarly tumorigenic.

In a first set of experiments we considered, according to the data presented in Fig 1A, that the percentage of SP cells in low passage C6 cell cultures was less than 1%. Hence, assuming that non-SP cells were mainly non-tumorigenic cancer cells unable to give rise to “cancer stem cells”, we expected that the probability for one isolated single C6 cell to establish a tumorigenic clonal culture should be low. Therefore we addressed the clonogenicity of C6 cells (passage 48; SP \approx 0.2%). In these experiments, single C6 cells were plated at a density of one cell per well in conditioned culture medium as described in the material and methods section. Using these experimental conditions, cultures were consistently established for almost each single plated cell (28/29). All these clones can be maintained in culture for at least 4 months and are tumorigenic when injected in nude mice (data not shown). To further investigate the relationship between SP and tumorigenicity, SP and non-SP cells were sorted from C6 glioma cells (passage 50). Injection of 500 of these SP or non-SP sorted cells in nude mouse induced tumor formation (table 2B). These data confirms that non-SP C6 cells behave as tumorigenic cells.

Non-SP C6 cells generate SP cells.

We next challenged the assumption that non-SP cells cannot generate SP cells. For this purpose non-SP C6 cells were sorted. After three consecutive rounds of cell sorting, SP cells were undetectable in the non-SP isolated cell population (Fig. 2A). These non-SP cells were then expanded for 2 weeks, and FACS analyzed. Data presented in Fig. 2B demonstrate that the non-SP C6 cells generated SP cells.

C6 SP cells express ABCG2/bcrp1.

ABCG2/bcrp1 is the major drug transporter conferring the SP phenotype [17]. It is expressed in a wide variety of normal and cancer stem cells [1, 3-5, 17]. Results presented in Fig. 3 demonstrate that the expression of this gene is detected in SP cells only. In addition, both SP and non-SP cells expressed *mdr1*, a gene coding for another ATP-binding cassette (ABC) transporter involved in drug resistance (Fig. 3) [3].

Discussion

Our results confirm the presence of SP cells in the C6 glioma cell line and the expression of the *ABCG2/bcrp1* drug resistance gene in SP cells only [3]. In addition to being a molecular determinant of the side population phenotype [17], *ABCG2/bcrp1* has been shown to efflux over 20 different chemotherapeutic agents such as methotrexate, topotecan, mitoxantrone and doxorubicin [18]. This suggests that C6 SP cells should have an increased resistance to these chemotherapeutic agents. Interestingly, we also demonstrate that, in the presence of fetal bovine serum, non-SP C6 cells proliferate and do generate SP. Moreover, we found no difference in tumorigenicity between SP and non-SP cells. These results may appear at odds with previous findings showing that culture of non-SP C6 cells do not generate SP cells [3] and that SP cells are more tumorigenic than non-SP cells [3,5,10,11]. However, several explanations can be provided to account for this discrepancy. For example, it is known that SP profiles from different laboratories can vary and the SP gating is quite arbitrary [19]. On the other hand, differences between early and late passage C6 cell cultures have been repeatedly reported [20-22]. All our experiments have been initiated between passage 42 and 50. Unfortunately, the cell passage number of C6 cells used in previous cancer stem cell studies is rarely indicated [3]. A second possible reason for the observed discrepancy concerns the cell culture conditions. It is noteworthy that all of our experiments have been performed in the presence of fetal bovine serum instead of the serum-free medium used by Kondo et al. [3]. Thus, a possible explanation is that, in the C6 cell line, the culture medium used by Kondo et al. allows both the growth of SP cells and the generation of non-SP cells, but is suboptimal for the generation of SP cells from non-SP cells. It has been previously reported that C6 cells are transformed glial stem/progenitor cells [23,24]. Therefore, extracellular signals provided by fetal bovine serum could induce non-SP cells to revert to SP cells, and this potential could be

a feature of transformed glial stem/early progenitor cells. Interestingly, this fluctuating phenotype has been described for oligodendrocyte precursor cells that can be reprogrammed to become multipotent CNS stem cells [25], and for the muscle cell line C2C12 and a thyroid cancer cell line in which SP cells arise from non-SP cultures [26,27]. Hence, our results confirm the possibility for non-SP cells to regenerate SP cells, and demonstrate the existence in the C6 cell line of a dynamic equilibrium between these two cell populations (Fig. 4). These data do not necessarily challenge previous results demonstrating that SP cell analysis can be used to isolate cancer stem cells, but confirm that in some experimental models, non-SP cells can generate SP cells [26,27]. Thus, it is possible that the fluctuating phenotype observed here is a feature of highly tumorigenic and proliferative cancer cells such as C6 cells when they are cultured in serum-containing medium. In the C6 cell line, cell passaging or subcloning, genomic instability as well as epigenetic events can lead to the selection of mutants such as C6.9 cells that have lost this fluctuating phenotype in serum-containing medium. These non-SP cells can therefore be viewed as “secondary” tumorigenic cells and are indistinguishable from cancer stem cells on the basis of clonogenicity and tumorigenicity assays. Whether or not these “secondary” tumorigenic cells mimic the progression of the tumor *in vivo*, or are *in vitro* artefacts remains an open question. Overgrowth of these “secondary” tumorigenic cells in cell culture could lead to the depletion of the original cancer stem cells pool and explain the existence of tumorigenic cancer cell lines with undetectable SP cells.

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Legends to figures.

Fig. 1. Side population (SP) analysis of C6 and C6.9 cells.

Cells were stained with Hoechst 33342. The SP and “upper-SP” regions are indicated on each panel. Addition of verapamil inhibits Hoechst efflux from both SP and “upper-SP” cells. FACS profiles are representative of at least three independent experiments.

(A) C6 cells, passage 50; (B) C6 cells, passage 57; (C) C6.9 cells.

Fig.2. Generation of SP cells from non-SP C6 glioma cells.

Fluctuation of the SP/non-SP phenotype in the C6 glioma cell line.

C6 glioma cells were incubated with Hoechst 33343 and FACS analysed. The gates shown were used to isolate SP or non-SP cells.

- A) After three sorting rounds SP cells were undetectable in the non-SP cell population whereas the SP sorted cell population still contained very few non-SP cells (0.14%).
- B) Cell cultures from C6 non-SP or SP sorted cells were expanded for two weeks, stained with Hoechst and FACS analyzed. Non-SP cells from C6 cell line generate non-SP and SP cells. Likewise, cell cultures expanded from C6 SP sorted cells were stained with Hoechst and FACS analyzed and contained both SP and non-SP cells.

Fig. 3. Expression of *mdr1* and *ABCG2/bcrp1* in SP and non-SP C6 cells.

RNA from SP (lane 1) and non-SP (lane 2) cells were reverse transcribed and resulting cDNAs amplified as described in “Material and Methods”. *ABCG2/bcrp1* expression correlates with SP phenotype.

Fig. 4. SP and non-SP in cell cultures: Two hypothetical models.

(A) According to the prevalent model, only a small subset of cancer cells are SP cells. They self-renew, proliferate extensively, form new tumors and generate low tumorigenic non-SP cancer cells.

(B) Our results suggest that in serum-containing medium, cell populations SP cell and non-SP cell populations exist in a dynamic equilibrium. Consequently both have the capacity to proliferate extensively and to form new tumors.

We suggest the origin of the primitive cancer initiating cell (stem cell, early progenitor, more mature progenitor, terminally differentiated cell) will determine which model is relevant for a given cell line. Moreover, in the (B) model, microenvironment cues could shift the equilibrium and generate a differentiation process leading to the appearance of non-

tumorigenic, non-SP cells. In both models non-SP tumorigenic cells may appear as a consequence of continual selection for genetic or epigenetic variants.

Table 1 : Percentage of SP cells in low and high passages C6.

cells and in C6.9 cells. (mean % of side population detected in five independent experiments. UD= undetectable)

Table 2: Tumor incidence indicates the number of tumor-bearing animals /number of animals injected. Latency refers to the time of apparition of a palpable tumor. ND= not determined.

Table(s)

	% SP cells in total population (n=5)
C6 low passage (p42 to p50)	0.076 +/-0.045
C6 high passage (p53 to p76)	1.95 +/- 0.68
C6.9	UD

A

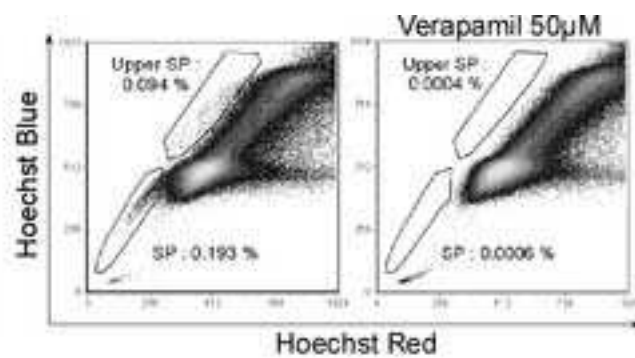
Number of cells injected	Tumor incidence (latency)
500 C6.9	4/4 (13-16 days)
500 C6	4/4 (13-18 days)

B

Number of C6 sorted cells injected	Tumor incidence (latency) for SP cells	Tumor incidence (latency) for NSP cells
50000	ND	3/3 (6 days)
5000	ND	3/3 (11-13 days)
500	2/3 (11-15 days) 4/4 (10-19 days)	3/3 (13-18 days) 4/4 (14-19 days)

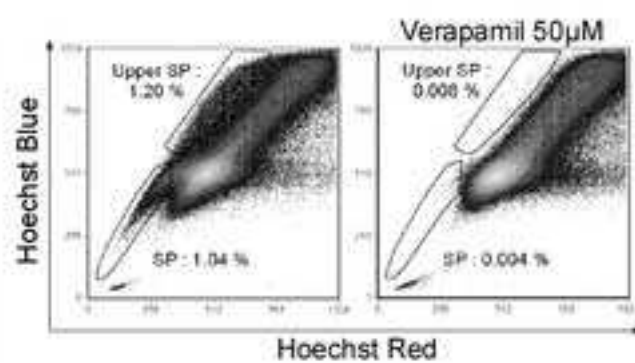
C6
(passage 50)

A



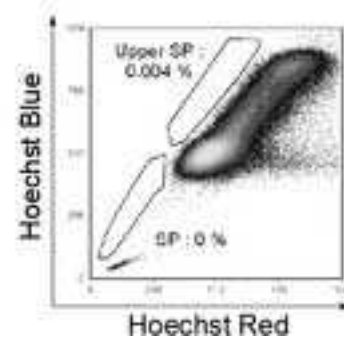
C6
(passage 57)

B



C6.9

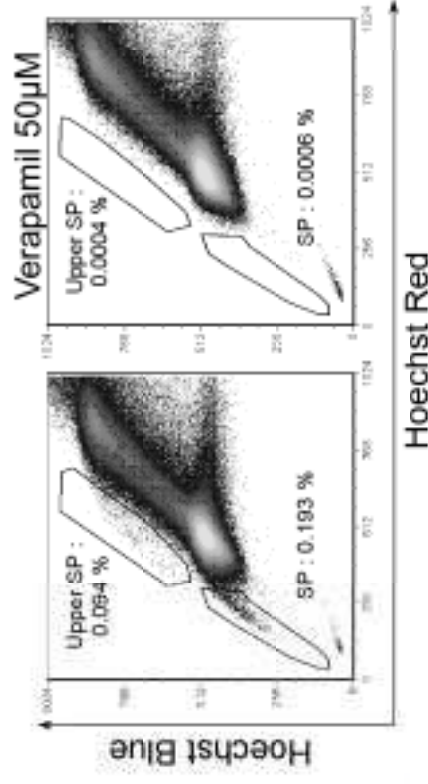
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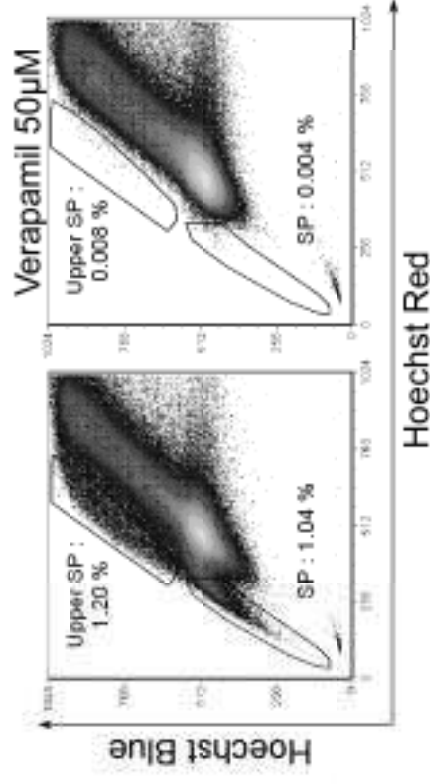
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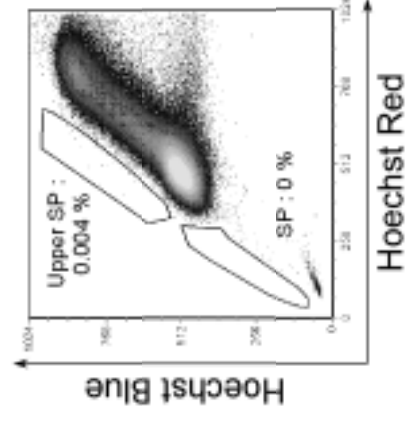
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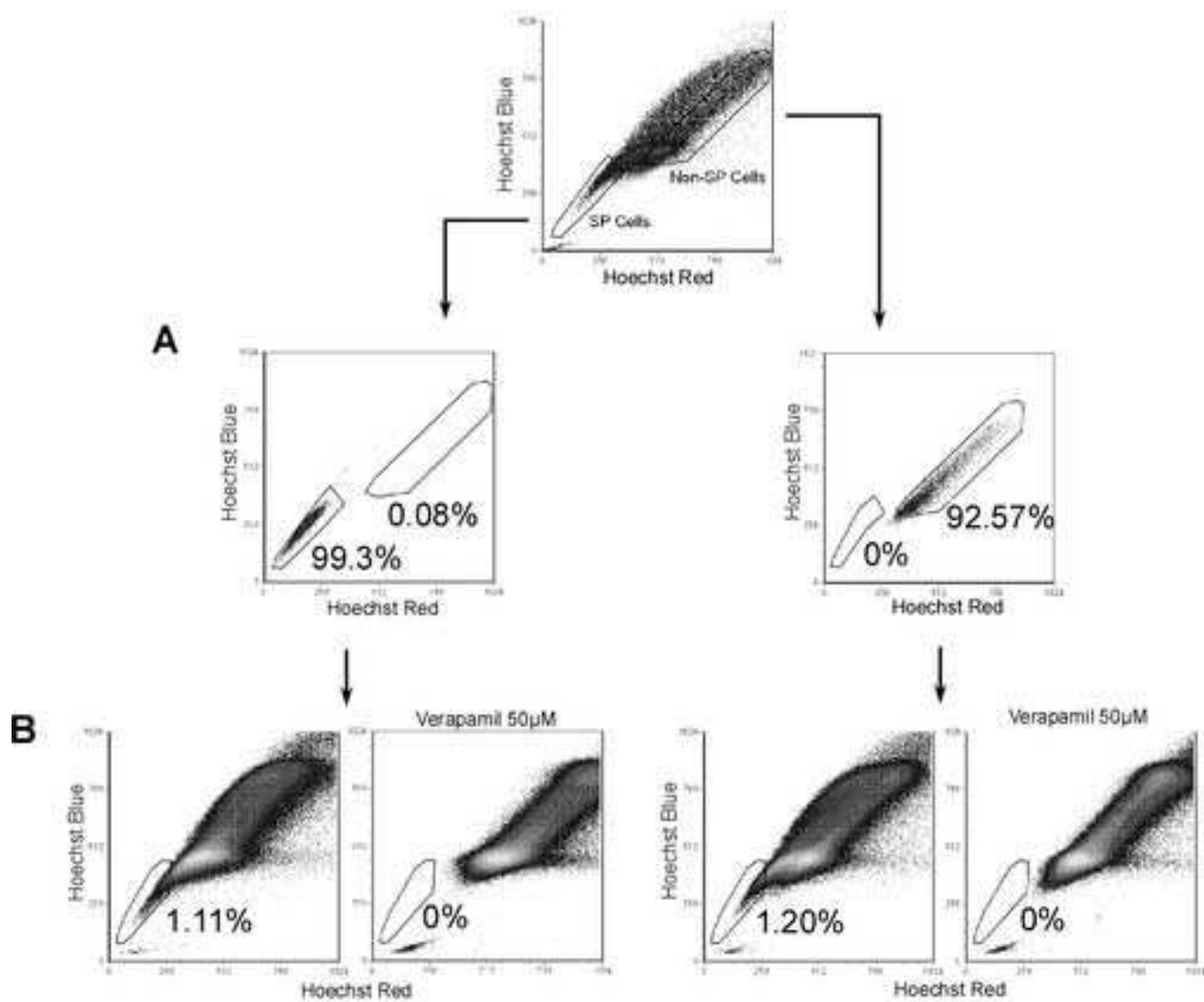
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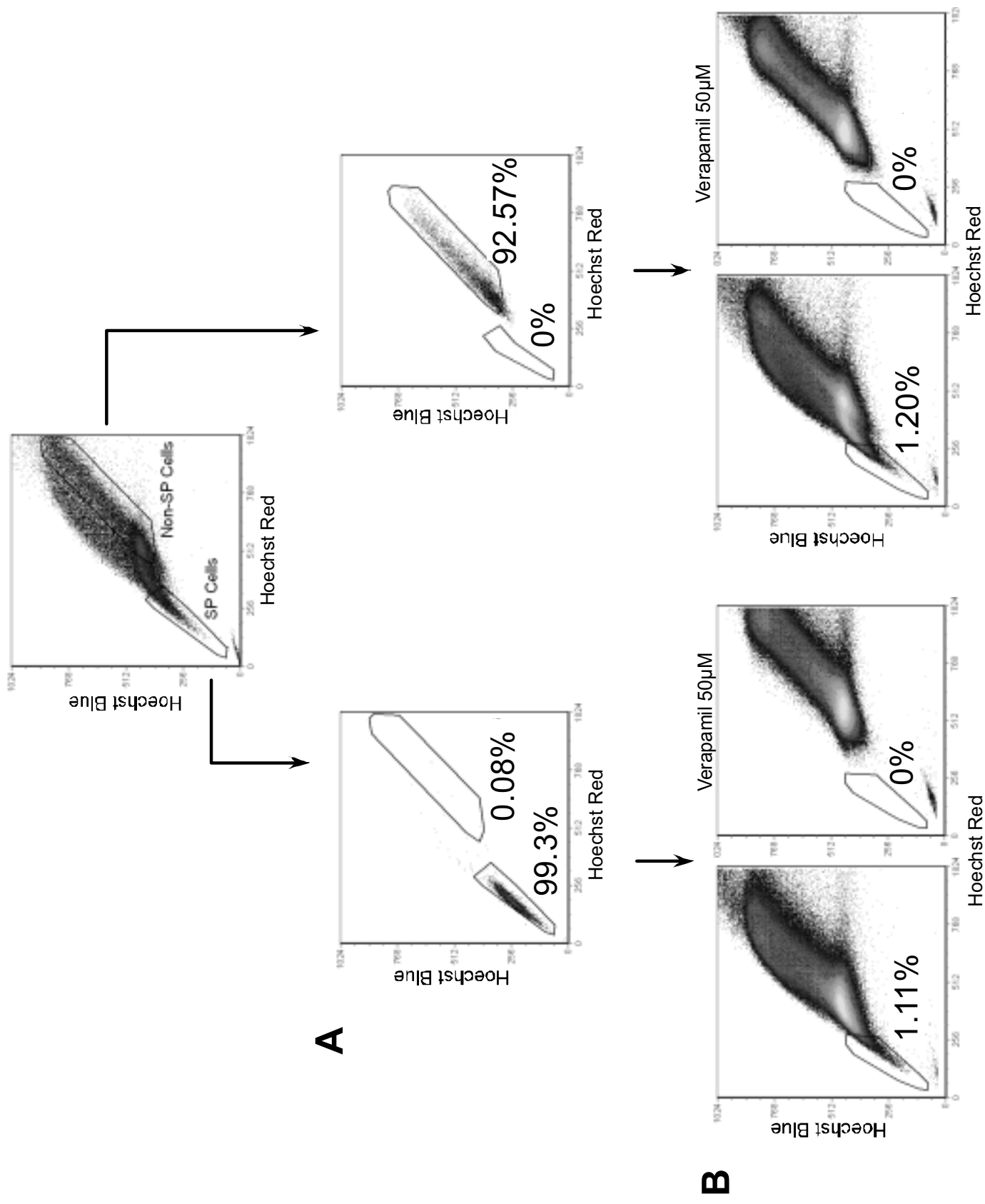


C6.9

C







Figure(s)

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