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# **Effects of Hoechst 33342 on C2C12 and PC12 cell differentiation.**

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## **Introduction.**

In the past few years, Hoechst 33342 staining has been widely used for identifying normal as well as cancer stem cells. This technique is based on the observation that following staining with Hoechst 33342, a small subset of cells actively effluxes this fluorescent vital dye. Hence, when analyzed simultaneously on a flow cytometer at two emission wavelengths (red and blue), some low fluorescent cells may appear as a “side population” (SP) well separated from the main population of high fluorescent cells (MP). The finding that in murine bone marrow SP cells are enriched in haematopoietic stem cells (HSC) [1] leads to use Hoechst 33342 staining for the purification of stem cells from other tissues. Thus, for example, the SP phenotype has been used to identify stem cells from muscle [2], liver [3], brain [4], testis [5], epidermis [6], dental pulp tissue [7] and bone [8]. Likewise, SP analysis has been used to identify cancer stem cell populations [9-11]. Therefore, even if some conflicting results have been reported [12-14], Hoechst exclusion is commonly viewed as a stem cell feature [15]. However, a major question regarding the association of SP phenotype to stem cells is the existence of a functional relationship between Hoechst exclusion and stemness. Indeed, even if the molecular determinant of the SP phenotype is identified as the ABC transporter Bcrp1/ABCG2 [16], its physiological role in the maintenance of the stem cell phenotype remains elusive. Thus, *Bcrp1*<sup>-/-</sup> mice are fertile and do not show any physiological or anatomical abnormalities [17]. Moreover, the reduced number of SP cells found in these mice, does not correlate with a reduced number of haematopoietic stem cells or with any stem cell-associated disease [17]. Therefore, we cannot formally exclude that the association of SP phenotype with haematopoietic stem cell, even if valuable, is coincidental.

Although the use of Hoechst 33342 for stem cell analysis is relatively recent, it has long been known that Hoechst 33342 is a non-intercalating AT base pair-specific ligand which binds to

cellular DNA by non-covalent association [18]. Consequently, this specific vital stain for DNA has been used since many years in conjunction with fluorescence activated cell sorting in cell cycle analysis [19,20]. Thus, it is during a cell cycle analysis with Hoechst 33342 that the serendipitous discovery of SP cells was done [1].

A prerequisite for a general use of Hoechst 33342 in stem cell sorting is that this fluorescent dye does not interfere with cell differentiation. In this regard, it is noteworthy that even if Hoechst 33342 is usually considered as non-toxic, it has been demonstrated, more than one decade ago, that it induces teratocarcinoma cell differentiation [21]. However, this report published well before the use of Hoechst 33342 for stem cell sorting retained little attention (two citations according to the Scopus Citation Tracker). Here, we demonstrate using two cell lines distinct from teratocarcinoma cells that Hoechst treatment, as it is performed during staining for SP analysis, can affect cell differentiation.

## **Materials and Methods.**

### ***Cell culture.***

The C2C12 cell line (ATCC product number CRL-1772 ) was established from adult C3H mouse leg muscle. It has been widely used for studying myogenesis since its differentiation produces myotubes. The PC12 cell line (ATCC number CRL-1721) was derived from a transplantable rat pheochromocytoma [22]. Because NGF-PC12 treated cells extend neurites this cell line has been considered as a model system for neuronal differentiation studies. PC12 cells were cultured at 37°C in growth medium (RPMI with 5% fetal bovine serum and 10% horse serum). C2C12 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). For experiments C2C12 cells were plated at  $2.5 \times 10^3$  cells/cm<sup>2</sup> (low density) or  $2 \times 10^5$  cells/cm<sup>2</sup> (high density). PC12 cells were plated at  $5 \times 10^3$  cells/cm<sup>2</sup>. For quantification of cell proliferation, control cells and Hoechst-treated cells were detached by trypsinization, and the number of viable cells was determined using trypan blue exclusion and a hemocytometer.

### ***Hoechst labelling and FACS analysis.***

Hoechst concentrations used for SP sorting in published data vary from 2.5 µg/ml to 12.5 µg/ml, 5 µg/ml being the concentration most often used. Therefore a concentration of 5 µg/ml was used for all the studies.

For Hoechst staining and side population analysis , cells were retrieved from subconfluent culture by trypsinisation, resuspended at  $10^6$  cells/ml in prewarmed DMEM containing 2% FBS, 10mM HEPES, then incubated for 90 minutes at 37°C with Hoechst 33342 (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 washed twice in cold HBSS (purchased from Gibco-Invitrogen) containing 2% FBS and 10mM Hepes. Then cells were either seeded on culture dishes or FACS analyzed.

Cytometric analyses were performed on a FACS Vantage SE option DiVa and  $1.10^6$  cells were analyzed for each sample. Dead cells were excluded from the plots based on propidium iodide staining (5 $\mu$ g/mL). Hoechst staining intensity was evaluated by measuring the fluorescence mean intensity of the entire cell population. Data were reanalysed with FCS Express 2 software (De Novo Software, Thornhill, Canada).

### **Differentiation index**

To quantify the fusion of C2C12 cells, cells were fixed with methanol, processed for immunofluorescence using an anti-myosin heavy chain (MHC) monoclonal antibody and stained with propidium iodide. The differentiation index was calculated as the percentage of nuclei in multinucleated MHC-positive cells relative to the total number of nuclei.

## **Results and Discussion.**

In a first set of experiments exponentially growing C2C12 cells were treated for 90 minutes with 5  $\mu\text{g/ml}$  Hoechst 33342, as described in Materials and Methods. Control cells were treated similarly without Hoechst addition. After washing, cells were then either FACS analyzed or replated at a cell density of  $2.5 \times 10^3$  cells/cm<sup>2</sup>. Using these experimental conditions, and as previously described [23], SP cells were detected in C2C12 cells (Fig 1A). Moreover Hoechst treatment did not decrease cell viability (data not shown) or proliferation (Fig 2). Likewise the treatment had no apparent effect on cell myogenic differentiation, since both control and Hoechst-treated cell culture displayed extensive myotubes formation eight days after plating (Fig. 3A and 3B). According to this, fluorescence was no longer observable in the nuclei of treated cells (Fig 4A and B). However, when C2C12 were plated at high density ( $2 \times 10^5$  cells/cm<sup>2</sup>) cell fusion was dramatically reduced in Hoechst-treated cells only (Fig 3C and D). Moreover, Hoechst staining was readily observable eight days after treatment (Fig 4 D, G). These differences between low and high density cell cultures can be attributable to a dilution effect of Hoechst due to cell amplification, since low density C2C12 cells proliferated very actively with a doubling time of about 19 hours [24]. Comparison of the differentiation index (% of total nuclei contained within myosine heavy chain-expressing myotubes) between control C2C12 cells and Hoechst treated cells confirmed that Hoechst 33342 staining inhibited C2C12 cell differentiation (Fig. 4H). This effect is even more dramatic when C2C12 cells were cultured in serum-free medium, a culture condition known to increase C2C12 cell fusion (Fig. 4H). Taken together these results demonstrate that Hoechst interferes with C2C12 cell fusion as long as the dye is present in the nucleus. Additional evidence for Hoechst effect on cell differentiation was provided using PC12 cells. These cells have a doubling time of about 92 hours and differentiate in neurite bearing cells

when NGF is added to cell culture at a concentration of 50 ng/ml [22]. Flow cytometry analysis with Hoechst 33342 staining demonstrated PC12 cells included a small SP cells compared to C2C12 cells, and is therefore a suitable experimental model to study the effects of Hoechst retention (Fig. 1B). Hoechst treatment had no effect on neurite outgrowth (data not shown), nor decreased cell proliferation (Fig 2). However, Hoechst staining synergizes with sub-optimal concentration of NGF (10 ng/ml) in inducing neurite outgrowth (Fig 3E and F. As expected from the effects on cell differentiation, Hoechst staining is still evident after 5 days (Fig 4 F and G). The low percentage of SP cells observed both in C2C12 and PC12 cell lines (Fig 1) excludes that the observed effect is ascribable to SP cells only. Taken together these data suggest that Hoechst staining can affect cell differentiation as long as the dye is present in the nucleus. In this regard our data are consistent with a previous report on the effect of Hoechst 33342 on the differentiation of embryonic carcinoma cell lines [21]. In fact, these results are not astonishing if we consider that Hoechst 33342 interaction with DNA alters TATA Box Binding Protein (TBP)/DNA complexes [25], and that this dye is a specific topoisomerase-I poison [26]. Interestingly, alterations in topoisomerase I activity induces embryonal carcinoma, neuroblastoma and leukaemia cell differentiation [27-29]. Likewise, evidence has been presented suggesting that topoisomerase I activity is required for C2C12 myogenic differentiation [24]. However, it should be noted that our results are obtained on C2C12 and PC12 cells. Therefore, possible effects of Hoechst staining on cell differentiation should be systematically studied for other cell lines or primary cell culture. In this regard it is noteworthy that Hoechst sensitivity varies among cell types (30, 31). The finding that Hoechst 33342 can affect differentiation suggests potential complications in the interpretation of data obtained in SP analysis. Hence, Hoechst 33342 effects on cell differentiation will be more critical on MP cells which retain the dye than on SP cells which exclude it. This raises the embarrassing possibility that in some experiments, the differences observed on cell



differentiation between SP and MP cells could be a consequence of the higher exposure of MP cell DNA to this vital fluorescent dye.

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## **Legends to Figures.**

### **Fig. 1. FACS analysis of C2C12 and PC12 cells**

C2C12 (A, C) or PC12 (B, D) cells were incubated with Hoechst 33342. Controls (C, D) were also incubated with verapamil to block the transporter responsible for the Hoechst exclusion. SP cells are indicated.

### **Fig. 2. Growth characteristics of C2C12 and PC12 Hoechst-treated cells versus control cells.**

### **Fig. 3. Effects of Hoechst 33342 on C2C12 and PC12 cell differentiation.**

C2C12 were plated at low cell density (A, B) or high cell density (C, D) as described in Materials and Methods. (A and C) are control cells; (B and D) are Hoechst 33342 treated cells.

PC12 cells (E, F) are incubated with a sub optimal concentration of NGF (10 ng/ml). (E) control cells, (F) Hoechst-treated cells.

### **Fig. 4. Hoechst staining of Hoechst-treated cells and C2C12 differentiation index.**

C2C12 (A, B, C, D) and PC12 (E, F) cells were treated with Hoechst (B, D, F) and fixed 8 days (C2C12) or 4 days (PC12) after treatment. C2C12 were plated at low density (A, B), or high density (C, D). Blue nuclear fluorescence is only observed in Hoechst-treated C2C12

plated at high density (D), and in Hoechst-treated PC12 cells. Nuclei are counterstained with propidium iodide (red). (AU): arbitrary fluorescence unit.

G: Decrease in Hoechst staining detected by FACS analysis.

H: Differentiation index of C2C12 cells. The values reported in the graph are the means  $\pm$  standard deviations (S.D.) from three separate experiments.

Figure 1

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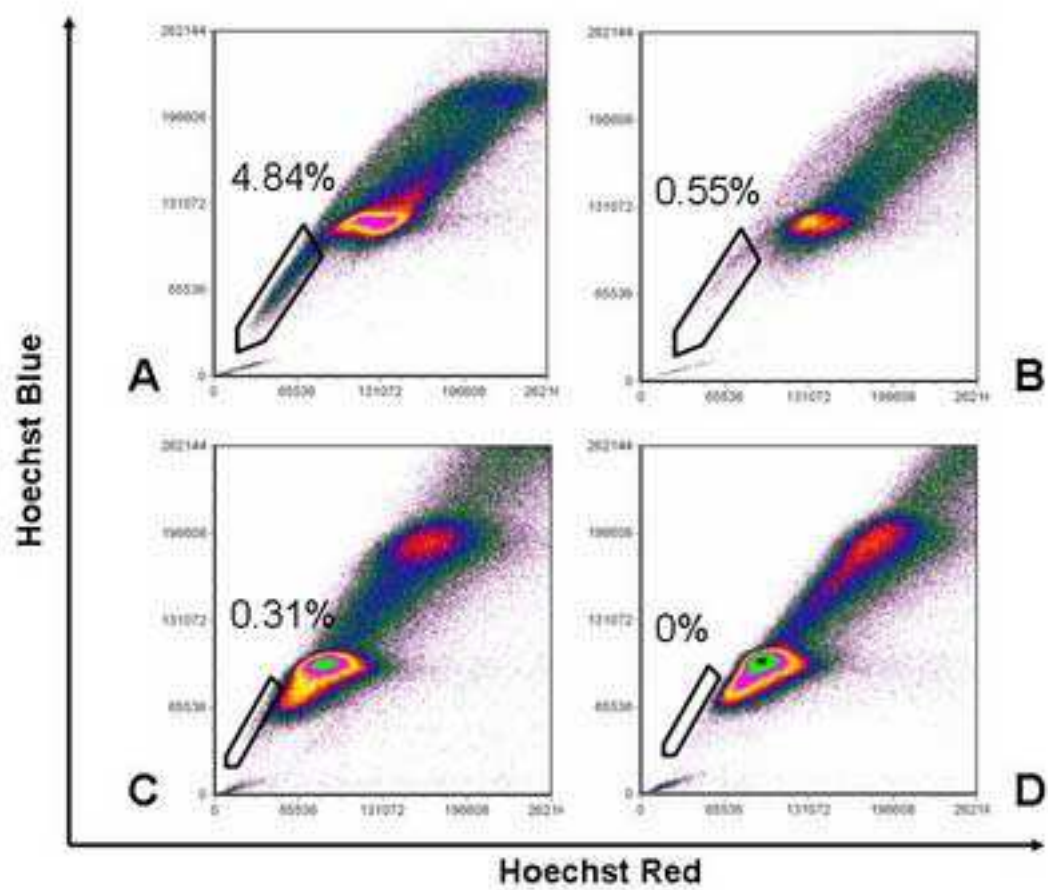


Figure 2

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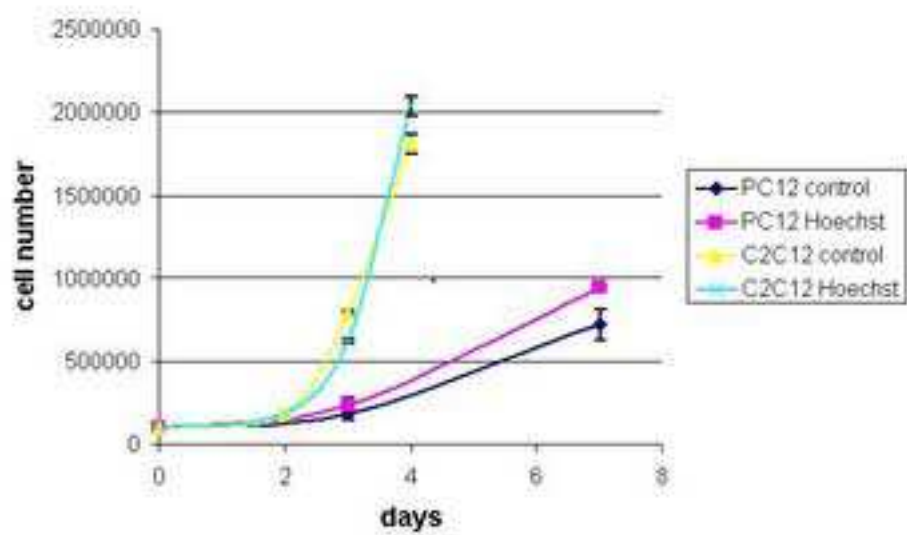




Figure 3

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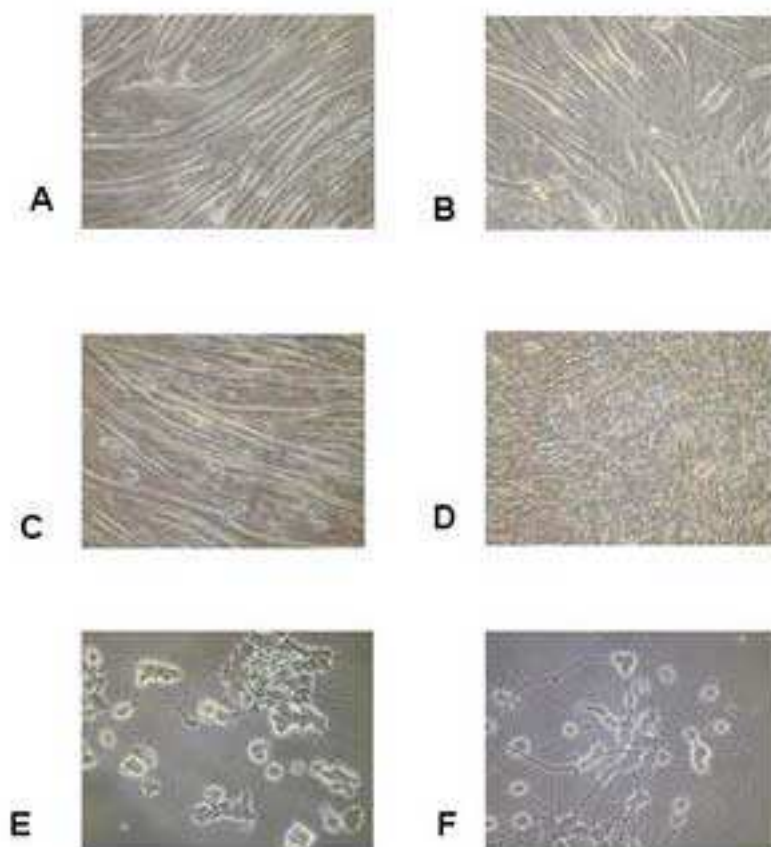


Figure 4

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