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# **Characterization of normal and cancer stem cells: One experimental paradigm for two kinds of stem cells.**

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## **Summary**

The characterization of normal stem cells and cancer stem cells uses the same paradigm. These cells are isolated by a Fluorescent-Activated Cell Sorting step and their stemness is assayed following implantation into animals. However, differences exist between these two kinds of stem cells. Therefore, the translation of the experimental procedures used for normal stem cell isolation into the cancer stem cell research field is a potential source of artefacts. In addition, normal stem cell therapy has for objective the regeneration of a tissue, while cancer stem cell-centred therapy seeks the destruction of the cancer tissue. Taking these differences into account is critical for anticipating problems that might arise in cancer stem cell-centred therapy and for upgrading the cancer stem cell paradigm accordingly.

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### **The cancer stem cell model.**

Accumulating evidence indicates that tumours are organized in a hierarchical pattern like normal tissues, with a small population of stem cells named “cancer stem cells” responsible for both development and maintenance of cancer tissue, and a majority of cancer cells with limited proliferative potential which form the bulk of the tumor (for reviews see references<sup>(1-3)</sup>). In the cancer stem cell experimental paradigm, cancer stem cells are individually defined by their ability to serially regenerate tumours following implantation in animals. This property illustrates their unlimited proliferation potential, their ability to self-renew and their capacity to generate a progeny of differentiated cancer cells with limited proliferative potential. Cancer stem cells share several distinctive properties with normal stem cell i.e. self-renewal and extensive proliferation potentials, and capability to differentiate. However, the term cancer stem cell is not synonymous with “transformed stem cell”, since in many cases the nature of the cell to first acquire fully malignant properties, i.e. stem cell, progenitor or differentiated cell, remains unknown<sup>(1)</sup>. In other words, the term “cancer stem cell” defines a function, and does not refer to the identity of the initial transformed cell that originated the tumor.

The concept of cancer stem cell is of paramount importance. If cancer stem cells represent the only cell population with tumorigenic potential, then one would hypothesize that these cells are not only involved in the maintenance of the primary tumour mass but may also play a critical role in metastasis and relapse<sup>(4-7)</sup>. Regarding metastasis, although the cancer stem cell phenotype is not always sufficient to establish metastases, a subpopulation of migrating cancer stem cells involved in tumour metastasis has already been detected in human breast and pancreatic cancers<sup>(4,5)</sup>. Concerning relapse, the mechanisms involved in the therapeutic resistance of cancer stem cells include for example the preferential activation of the DNA damage checkpoint response, and increased Wnt/ $\beta$ -catenin and Notch signalling<sup>(8)</sup>. Drug

pumps are also preferentially expressed in cancer stem cells<sup>(9)</sup>. Notably, one of these drug transporters involved in chemoresistance, named ABCG2, is the determinant of the Side Population phenotype (SP) which is used for characterizing some normal and cancer stem cells<sup>(10,11)</sup>. Hence, in the cancer stem cell model, a therapy targeting cancer cells but sparing the cancer stem cell population will induce only temporary regression. Therefore, the isolation and the characterization of cancer stem cell are of crucial concern for both basic cancer research and for designing novel efficient therapies.

The purpose of this paper is to discuss the weaknesses of the assays currently used to isolate and characterize the different subpopulations of cancer stem cells that are important for tumour growth and are not efficiently targeted by current therapies. We show that applying the same experimental paradigms for the isolation of normal and cancer stem cell can be misleading because these two kinds of stem cells have fundamental distinct features. Moreover, the goals of cancer stem cell-centred therapy and regenerative therapy are opposite. Cancer stem cell therapy seeks to eliminate a tissue whereas regenerative stem cell therapy looks for tissue growth. Taking into account these fundamental differences is critical for the future of cancer stem cell research.

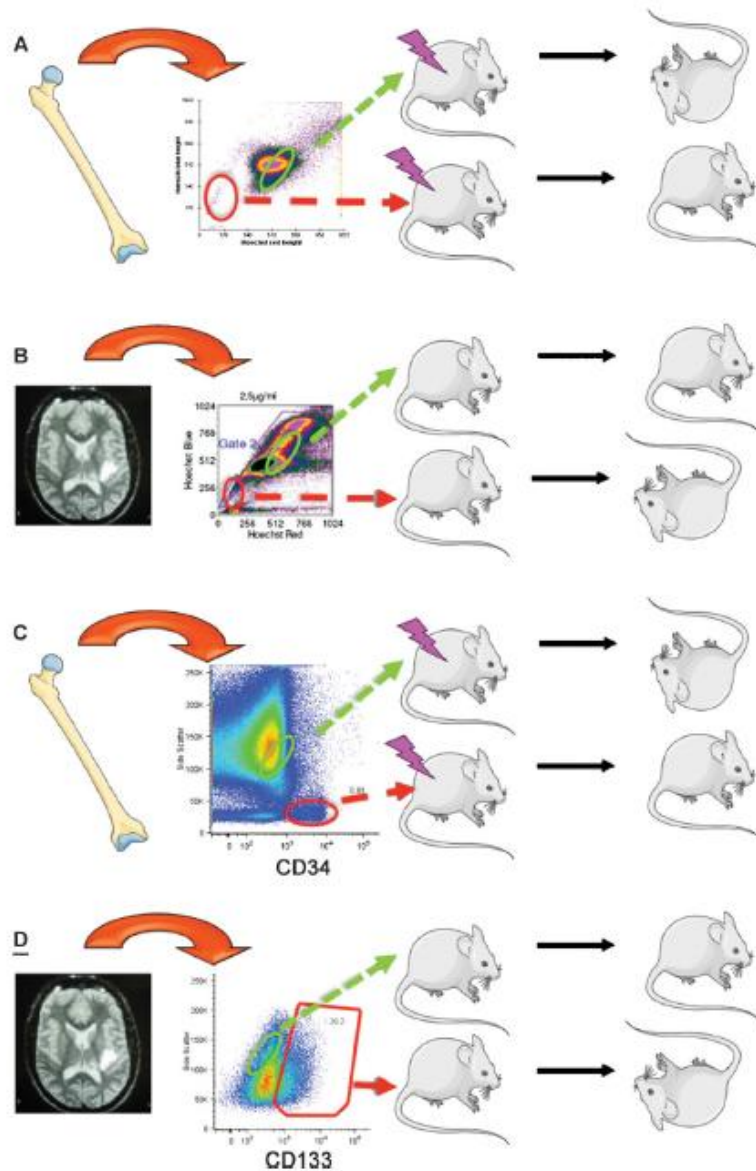
### **Flow cytometry: the gold standard for stem cell isolation.**

Since the introduction of monoclonal antibodies directed against cell surface antigens, flow cytometry is the method of choice for characterizing and sorting normal stem cells. Currently, the multi-parameter analysis based on cell surface antigens expression or on the capability to efflux fluorescent dyes, allows one to assess the phenotype of cells residing at the top of the differentiation pyramid of the haematopoietic system. The conceptual analogy between the hierarchical organization of normal and cancer tissues coincides with experimental analogies in the methods used to isolate normal and cancer stem cells. This similarity is evident when

the experimental methods developed for assaying either haematopoietic or cancer stem cell potentials are compared (Fig 1).

Basically, the isolation of normal or cancer stem cells requires a cell sorting step that is followed by an *in vivo* repopulation/tumorigenicity assay, in which the potential of sorted cells to reconstitute normal or tumoral tissues after implantation in animals is evaluated (Fig. 1). This cell sorting step is based on the detection of cell surface antigens or on the ability of some stem cells to efflux the vital fluorescent dye Hoechst 33342. For example, haematopoietic stem cells (HSC) were first characterized from bone marrow as CD34<sup>+</sup>(12,13). Currently, other cell surface markers such as the CD133 epitope recognized by the mAb AC133 are used to identify human HSC and some cancer stem cells(14,15). On the other hand, a functional assay based on the ability of stem cell to extrude the fluorescent Hoechst 33342 dye (side-population or SP assay) is also widely used to isolate HSC<sup>(10)</sup> or cancer stem cells<sup>(11,16,17)</sup>.

Thus, normal or cancer stem cells characterization requires a Fluorescence Activated Cell Sorter (FACS) analysis step, since even when magnetic-activated cell sorting (MACS) is used, the purity of the sorted cell population needs to be evaluated by FACS. However, even though immunophenotyping and SP assays are the gold standards for identifying normal or cancer stem cells, these two methods are basically different. SP analysis is a functional assay evaluating the capability of cells to extrude a fluorescent dye, while immunophenotyping is a structural assay which detects the expression of cell surface markers.



**Fig. 1**

**Normal stem cell and cancer stem cell assays.**

The experimental steps used to characterize cancer stem cells follow the experimental paradigm of normal stem cell characterization. At first, cells are isolated from tissue and then sorted by FACS either on their capacity to efflux Hoechst 33342 (SP phenotype) (A,B), or on the basis of the expression of CD34 for haematopoietic stem cells and CD133 for brain cancer stem cells (immunophenotyping) (C,D). In a haematopoietic stem cell assay (A,C), normal stem cells prospectively isolated by FACS, are assayed for their capability to rescue lethally irradiated mice from radiation-induced bone marrow aplasia, and for their ability to provide permanent long-term engraftment<sup>(83)</sup>. In a cancer stem cell assay (B,D), cancer stem cells prospectively isolated by FACS are implanted into an orthotopic site of immunocompromised mice and are assayed for their capability to serially induce tumor formation.

## **Sorting cancer stem cells on the basis of a functional test: The SP phenotype**

### ***History.***

Hoechst 33342 is a fluorescent dye designed by the Hoechst firm in 1974 with the aim of providing new chemotherapeutic agents<sup>(18)</sup>. This drug is part of a family of fluorescent vital dyes and has an excitation wavelength at around 350 nm. Hoechst 33342 binds to DNA and has been first used to visualize nuclei, to quantify cell DNA content, and for sorting living cells according to the different phases of the cell cycle<sup>(19)</sup>. Interestingly, this compound emits at two wavelengths of fluorescence, blue and red, when it binds to DNA. This polychromatic fluorescence emission depends on the intracellular concentration of the dye<sup>(20)</sup> and is critical for characterizing the SP phenotype. In 1996 Goodell, demonstrated that mice HSC have the distinct feature to efflux this dye<sup>(10)</sup>; this property is the basis of the SP assay which has been used since in more than one hundred studies to characterize normal or cancer stem cells.

### ***Functional basis.***

The property of some stem cells to efflux Hoechst 33342 defines by FACS analysis a minor population of low labelled cells referred to as “SP” cells<sup>(10,11)</sup>. These low-labelled cells are detected as a distinct population in the left lower quadrant of FACS profiles (Fig 1B and 2A). Bcrp1/ABCG2 expression has been shown to be the determinant of the SP phenotype in bone marrow cells<sup>(21-23)</sup>. However, depending on the tissue, other ABC transporters can contribute to the SP phenotype. For example, both Bcrp1/ABCG2 and Mdr1a/b (Abcb1a/1b) play a part in the SP phenotype in the mammary gland<sup>(24)</sup>. Since a drug transporter such as ABCG2 is the functional basis of Hoechst outflow, a control experiment with verapamil an ABCG2 pump inhibitor, must be included for each experiment. In these control experiments, the presence of

verapamil during the assay leads to the disappearance of SP cell population (Fig. 1B and 2B). An important consequence of the involvement of the Bcrp1/ABCG2 drug-transporter in the determination of the SP phenotype is that SP cells are de facto resistant to several drugs such as mitoxantrone, and methotrexate<sup>(25)</sup>.

### ***Controversies:***

#### *Verapamil-sensitive upper SP cells:*

Although settings for SP analysis place the cell-sorting gate in the left lower quadrant, a more careful examination of FACS profiles obtained in the presence of verapamil demonstrates the existence of other SP cells located in the left upper quadrant of FACS profile<sup>(26,27)</sup>. This upper cell population is evident in cancer cell lines (Fig. 2C). In several cancer stem cell experiments, the addition of verapamil induces a convex-concave transition of FACS profiles (see for example<sup>(11,26)</sup>). The fact that this observation is not readily observable with normal low cycling cells such as HSC suggests that this upper SP cell population could correspond to SP cycling cells or SP polyploid cells<sup>(26,27)</sup>. Unfortunately, this upper SP was not further characterized and goes unnoticed by most investigators working in field of cancer stem cells<sup>(11,26)</sup>, illustrating our tendency to use the same experimental paradigm for studying normal and cancer stem cells regardless the fundamental differences existing between these two cell types.

#### *Hoechst 33342 activities:*

A recurrent question regarding the use of Hoechst 33342 in SP analysis is the consequence of Hoechst staining on cell viability and differentiation since Hoechst 33342 interacts with DNA. Indeed, the biological consequence of Hoechst nuclear staining is a particularly relevant point in cancer research where many DNA-labelling agents have important cancer therapeutic



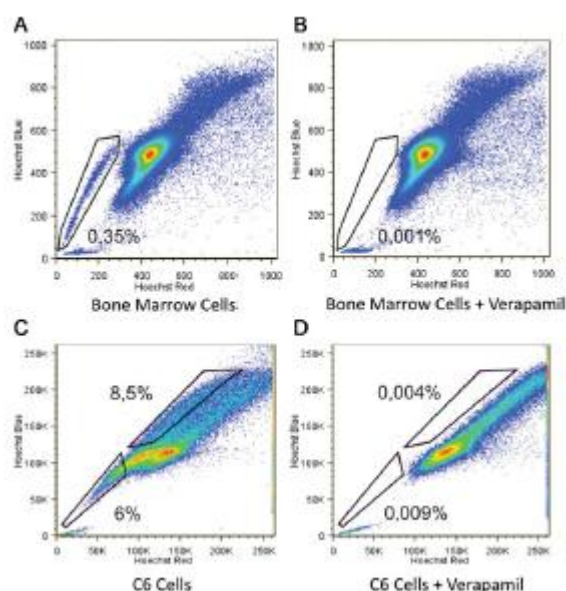
effects. In fact, it has long been known that Hoechst 33342 can be cytotoxic depending on the concentration and the cell type used<sup>(28,29)</sup>. Moreover, it is noteworthy that Hoechst interferes with cancer cell differentiation at concentrations used for SP analysis<sup>(30-32)</sup>. Unfortunately, these data are largely ignored.

Although the mechanism by which Hoechst 33342 influences cell differentiation has not been specifically addressed, several reports demonstrate that Hoechst 33342 poisoned topoisomerase I with a similar mechanism of action as camptothecin<sup>(33)</sup>. This point is of particular concern since topoisomerase I inhibitors are known to induce cancer cell differentiation<sup>(34,35)</sup>. Hence, an evaluation of Hoechst 33342 effects on cancer cell differentiation should be included in any SP analysis demonstrating a difference between SP and non-SP cells. Regrettably, these control experiments, which are much more cumbersome than evaluating Hoechst cytotoxicity, are never performed. The fact that Hoechst 33342 disrupts TATA box-binding protein/DNA complexes<sup>(36)</sup> also raises concerns on the transcriptomics data that compare SP and non-SP cells.

*Functional independence of normal stem cells for SP phenotype.*

Bcrp1/ABCG2, the molecular determinant of the SP phenotype, is expressed in a wide variety of stem cells<sup>(23)</sup>. However, although a large reduction in the number of SP cells is detected in *bcrp1* null mouse, the HSC compartment is not affected in these null mice<sup>(22)</sup>. Moreover, the SP phenotype could be dispensable for stem cell function since these mice show no obvious stem cell pathology. This could explain some conflicting results obtained with cancer stem cells when SP phenotype fluctuates independently of stemness<sup>(26)</sup>. An important point is that, concerning solid tumors, SP analyses are usually done on cancer cell lines or on primary cancer cells which have been amplified in culture. Therefore, the extrapolation of the data to the behaviour of the cells in vivo can be debatable. The situation is different for leukemic cells and for normal hematopoietic cells which are usually analysed

immediately after isolation<sup>(10,37,38)</sup>. Notwithstanding these limitations since Bcrp1/ABCG2, which is the functional determinant of the SP phenotype is an ABC transporter involved in drug resistance, the SP phenotype provides a selective advantage when cells are challenged with anticancer drugs such as the topoisomerase I inhibitor topotecan or the antifolate agent methotrexate<sup>(39)</sup>. Hence, the SP phenotype defines a population of chemo-resistant cancer cells, and this approach is pertinent from a therapeutic standpoint. The recent finding suggesting that therapeutic blockade of either PI3K or Akt might reduce ABCG2 function in cancer stem cell in vivo is, in this context, highly relevant<sup>(40)</sup>.



**Fig.2**

**The upper SP: how the blind adherence to the academic SP cell gating procedures can hinder a relevant cancer SP cell population.**

In a typical SP analysis, cells are labelled with Hoechst 33342 in the presence or absence of an ABCG2 inhibitor such as verapamil. The SP cell population assimilated to cancer stem cells is defined as the population of cells that actively outflow Hoechst by a verapamil sensitive process. Therefore, in FACS profiles, the SP region which corresponds to low labelled cells, is indicated by a trapezoid on the left lower quadrant of FACS profile (A). In the presence of verapamil, cells which are localized in the SP region disappears (B). However, when SP analyses are performed on cancer cell lines, another verapamil sensitive population is readily detectable in the left upper quadrant of FACS profile (C,D). Unfortunately this cell population remains unnoticed in almost all published analyses. In the example shown, which is performed on C6 glioma cells, roughly half of the verapamil-sensitive cells are found in this upper side population. Although these cells have not been fully characterized they probably

correspond to cycling cells. Indeed this upper SP is not detectable when SP analysis is performed on low-cycling diploid cells such as bone marrow cells (A,B). This example illustrates the limitations of transferring a technology validated in normal stem cell studies to cancer stem cell studies without considering the special features of cancer cells.

### **Sorting cancer stem cells on the basis of a structural test. Immunophenotyping cancer stem cells on the basis of cell surface markers.**

#### ***History.***

Cancer stem cells immunophenotyping originates directly from methods used more than 30 years ago to identify the heterogeneous populations of cells within bone marrow<sup>(12)</sup>. Nowadays, prospective cancer stem cells are isolated by flow cytometry on the basis of cell surface markers expression, just like haematopoietic stem cells are<sup>(41-46)</sup>. For example, the cancer stem cell population has been defined as  $CD34^+CD38^-$  in acute myeloid leukaemia<sup>(47)</sup>  $CD133^+$  in brain<sup>(45)</sup>, melanoma<sup>(48)</sup> and lung tumors<sup>(49)</sup>,  $CD133^{+(43,44)}$  or  $EpCAM^{high}/CD44^+/CD166^+$  in colon cancer<sup>(42)</sup>,  $CD44^+/CD24^{-/low}/Lineage^-$  in breast tumors<sup>(41)</sup>,  $CD44^+/CD117^+$  in ovarian tumors<sup>(46)</sup>, and  $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$  in prostate<sup>(50)</sup>. In all cases, the prospective cancer stem cell populations sorted on the basis of cell surface markers are then characterized by their ability to regrow a tumor in xenotransplantation assays. This xenograft assay can be viewed as the parallel of the repopulation assay used in haematopoietic stem cell research. Importantly none of the antigens used for identifying cancer stem cells is exclusively expressed on cancer cells since, for example  $CD34$  and  $CD133$  cell surface antigens are also used to immunophenotype normal stem and progenitor cells in several tissues<sup>(51,52)</sup>.

#### ***Functional basis.***

Contrary to the SP phenotype, which relies on the functionality of a drug transporter involved in multidrug resistance and therefore in cancer chemoresistance, the immunophenotyping assay can target membrane proteins not necessarily involved in the “cancer stem cell” function. For example, AC133 one of the most widely antigens used for cancer stem cell immunophenotyping corresponds to CD133, a protein whose function remains elusive. However, even if the role of CD133 in cancer is not elucidated, CD133<sup>+</sup> cells have nevertheless been reported to have important features such as an increased radioresistance<sup>(53,54)</sup> and increased expression of VEGF<sup>(55)</sup>. On the other hand, other antigens used for immunophenotyping are directly implicated in cancer process. For example, the cell surface marker EpCAM is used for immunophenotyping and is known to up-regulate c-myc and cyclin A/E, and to induce cell proliferation<sup>(56)</sup>.

### ***Controversies.***

*-Effect of cell sorter optical configuration, inter-laboratory variability, and the need for a standard of measurement.*

A large proportion of the cell sorters commercialised during the end of the 1990s and the beginning of the 2000s such as the FACS Vantage SE from BD Bioscience have an optical configuration that does not allow the optimal collection of the fluorescences emitted by stained cells<sup>(57)</sup>. These devices are still used nowadays in numerous flow cytometry core laboratories. Since the optics of the current machines such as the LSR II from BD Bioscience with a quartz device are more efficient at capturing the emitted light than the stream-in-air system of the old sorters, the percentages of positive cells acquired in these two cases widely differ. This point becomes critical when negative and positive cell populations form a continuum. In this regard it is noteworthy that FACS analyses cluster cells on the basis of positive, negative, low, or high fluorescent intensity. In a sense, FACS is a highly efficient

technology of measurement that has forgotten to define an objective standardised unit of measurement relevant to the parameter studied. The judicious selection of a standard for FACS analysis, which could for example consider the number of epitopes by cell, is therefore critical for ensuring the inter-laboratory reproducibility of experimental results<sup>(58,59)</sup>.

*-Immunophenotyping and SP analyses: similarity, overlap and discrepancy.*

The basic tenet of cancer stem cell research is that cancer stem cells represent a unique population of cancer cells responsible for tumour maintenance and recurrence. Hence, it is critical to characterise these cells as they are priority therapeutic targets. In this context, it could have been expected that cancer stem cell populations characterized by immunophenotyping and SP analysis coincide, and that immunophenotyping using different cancer stem cell markers identifies, for a given neoplasm, a unique population of cancer stem cells. However, several studies report a limited overlap between cancer stem cell populations obtained using different cell sorting procedures. For example, in some glioma cell lines, CD133<sup>+</sup> cells are not SP<sup>(60)</sup>. Likewise in DAOY medulloblastoma cells CD133 and SP subsets can be two independent compartments<sup>(61)</sup>.

Although, these data have been obtained with cultured cell lines and might not be representative of the situation found in vivo, other results obtained by immunophenotyping human colorectal xenograft cells which have never been maintained in culture also show some inconsistencies. Thus, the expression of CD133, which has been described as a colorectal cancer stem cell marker<sup>(43,44)</sup>, is variable in the cancer stem cell population characterized on the basis of CD44 expression<sup>(42)</sup> or in colon metastatic cancer stem cell<sup>(51)</sup>. Likewise, although brain cancer stem cells were first identified as CD133<sup>+</sup><sup>(45)</sup>, several recent reports demonstrate the existence of CD133<sup>-</sup> cancer stem cells in brain tumors<sup>(62-65)</sup>. Hence, the use of a single marker such as CD133 for immunophenotyping cancer stem cells could

underscore the heterogeneity of the cancer stem cell phenotype as well as the complexity of cancer disease. This emphasizes the importance of phenotyping cancer stem cells on the basis of cell surface marker repertoires.

-Cancer stem cell marker constancy.

Constancy of antigens expression is critical for the relevance of cancer stem cell immunophenotyping. However, as mentioned earlier, a potential drawback of immunophenotyping is that most of the antigens used do not seem to be directly involved in the maintenance of the cancer stem cell function. This situation contrasts with the principle of SP analysis which targets the functionality of ABCG2/Bcrp1, a protein involved in drug resistance<sup>(25)</sup>. Hence, inconsistencies may occur in the immunophenotyping of cancer stem cells if the markers used are not under selective pressure. Contrary to normal stem cells which have developed several genomic safeguard mechanisms and reside in a unique well-defined microenvironment (the stem cell niche), cancer stem cells are genetically and epigenetically unstable cells which are challenged by changing microenvironments not only in the primary tumor site, but also during metastatic process, and in cell culture. Hence, as cancer is an evolutionary system<sup>(25,66,67)</sup>, the stability of expression over time of a cancer stem cell-associated antigen may depend on the role of this antigen in the maintenance of the cancer stem cell function. This could explain the inconsistency reported above in the use of CD133 to characterise cancer stem cells in brain and colon cancers since the functional contribution of this antigen to cancer stem cell function remains obscure. Thus, both CD133<sup>+</sup> and CD133<sup>-</sup> cells with cancer stem cell functions can appear in a tumour during tumour progression and metastasis.

In this regard it is note-worthy that environmental conditions, such as low oxygen tension has been described to up regulate CD133 expression<sup>(54,68,69)</sup>. This finding suggests that

CD133<sup>+</sup> cells might be cancer cells challenged to a hypoxic microenvironment. Hence the existence of multiple subsets of “cancer stem cells” with different antigenic profiles, even for tumours of a given tissue, must now be integrated into the cancer stem cell paradigm. The characterization of the functional role of the cell surface markers used in cancer stem cell immunophenotyping is therefore critical. In a sense, sorting cancer stem cells on the basis of a functional test such as the SP assay provides both information and understanding since Bcrp1/ABCG2 function is involved in the drug resistance of tumour stem cells. However, immunophenotyping cancer stem cells on the basis of epitopes whose function is not yet defined provides only information.

**FACS in normal stem cell and cancer stem cell medicine: One technical approach for two different therapeutic goals.**

FACS analysis of cell surface molecules, by its ability to isolate rare cell types to high purity, had a critical impact first on haematopoietic stem cell research, and then on leukaemia research and diagnosis<sup>(70,71)</sup>. However, transferring the concepts and methods developed in the context of HSC research to cancer stem cell entities in solid tumors requires a cautious consideration of the clinical difference existing between the therapeutic purpose of a regenerative therapy and that of a *stemicide* therapy. For example, regarding normal stem cell regenerative therapy, although LT-HSC have been first described as CD34<sup>+</sup> or SP, very rare CD34<sup>-</sup> and non-SP subsets of LT-HSCs also exist<sup>(72,73)</sup>. Therefore, sorting HSC on the basis of CD34 expression or SP phenotype eliminates a functionally minor but relevant stem cell population. Omitting these CD34<sup>-</sup> or non-SP LT-HSC, however does not seem to have clinical consequences in the repopulation assay since successful haematopoietic reconstitution can be therapeutically achieved with either CD34<sup>+</sup> or SP LT-HSC cells only. Therefore, in normal stem cell regenerative medicine, the therapeutic achievement can be obtained with only one

the stem cell subpopulation. Likewise, if the cancer stem cell function is supported in the patient by heterogeneous population of cancer stem cells, the implantation of only one of these cancer cell populations is sufficient to regrow the tumour. This outcome fulfils the experimental goal of the xenograft assay model, which is the regeneration of the tumour in the animal. However, in cancer medicine, the therapeutic goal is the eradication of the natural tumour, not its regeneration. If we consider that at the time of diagnosis or recurrence, the primitive cancer stem cell has generated several cancer stem cell sub-populations differing not only genetically or epigenetically, but also according to their surrounding microenvironment and metastatic potential, then a successful *stemicide* therapy requires the characterization and the targeting of all the cancer stem cell populations present in the patient.

Unfortunately, in a xenograft assay, cancer cells implanted in mice are challenged to a microenvironment very different from the cancer mass from which they are issued. Hence, the xenograft assay may fail to detect human cancer stem cell populations unable to grow in immunocompromised animal but nevertheless capable of sustaining tumour growth in patient<sup>(74,75)</sup>. The fundamental difference existing between a regenerative and a *stemicide* stem cell therapy, and the low sensitivity of the xenograft assay, are two possible explanations for a potential disappointing outcome for cancer stem cell-centred therapies.

### **Concluding remarks and perspectives.**

Cancer research is a relatively old field of investigation, and its experimental paradigms have successively evolved throughout the last century according to the most available frames of reference<sup>(74)</sup>. Recently, stem cell biology became a new frame of reference in medical research as evidenced by the increase in the number of journals covering this field (Stem Cells, first issue 1983; Stem Cell and Development, first issue 1999; Stem Cell Research, first issue 2007; Cell Stem Cell, first issue 2007). This could, in part, explain the recent revival of



the cancer stem cell concept whose origin is more than a century old<sup>(76)</sup>. As biology is an experimental science, emergence of new reference frames is often linked to the development of new experimental tools. For example, more than one century ago, the development by Koch and Petri of a solid media enabling the rapid isolation of pure cultures from contaminated specimens was a major breakthrough in the development of medical microbiology<sup>(77,78)</sup>. Interestingly, FACS now achieves the same goal as was achieved by solid media and the Petri dish, that is, cell cloning. However, we must be aware of the current limitations of the FACS experimental procedures used to isolate cancer stem cells (Table 1). The determination of the functional role played by the different cell surface antigens used for cancer stem cell immunophenotyping is critical. Cancer is in development at all times, and cancer cells are engaged in an evolutionary process which occurs in a very short period of time as limited to the timescale of the illness<sup>(66,67)</sup>. This ecological and evolutionary process involves genetically and epigenetically unstable cancer cells confronted with changing physiological and non-physiological microenvironments. In this context the expression of cancer stem cell epitopes not directly involved in critical stemness functions could fluctuate. Therefore, identifying cell surface antigens whose function is mandatory for cancer stemness is of paramount importance.

In the absence of such key markers, FACS analyses need to evolve. More complex analyses, taking into account the intensity of cell labelling (high, medium, low, negative) and involving panels of antigens as recently done for colon and breast cancer stem cells<sup>(41,42)</sup> are required. These analyses could target cell surface proteins involved in critical cancer stem cell functions such as chemokine receptors (CXCR4, CXCR3...)<sup>(79)</sup> adhesion proteins (L1CAM...)<sup>(7)</sup>, or ABC drug transporters<sup>(25)</sup>. Even if these proteins are also expressed on normal cells, a cancer stem cell specific pattern of expression can be expected to emerge. Functional in vitro assays evaluating for example, invasion and angiogenic assays in

Matrigel<sup>(80,81)</sup> could also be included for characterizing cancer stem cell subpopulations. The evolution of cancer stem cell analyses toward a combination of such structural and functional multiparametric assays is necessary if we consider the complexity of the cancer stem cell phenotype.

However, cancer stem cell characterization is not limited to a FACS sorting step. The stemness of cells prospectively isolated by FACS must be functionally assayed in xenograft experiments. In that case, the overall sensitivity of the cancer stem cell isolation procedure is limited by the experimental step with the lowest threshold of detection. One of the weak link of the cancer stem cell experimental paradigm resides in the sensitivity of the xenograft assay which needs to be improved as it can underestimate the frequency of cancer stem cells<sup>(4)</sup>. Notably, it has been recently shown that the use of more highly immunocompromised mice increases the detectable frequency of cancer stem cells<sup>(4)</sup>. Numerous studies have demonstrated that microenvironment can either support or inhibit tumorigenesis<sup>(82,83)</sup>. Cancer stem cell assays are presently performed by implanting cancer cells in normal orthotopic tissue. A tumorigenicity assay based on the injection of cancer cells in pre-tumoral niches could reveal the existence of new class of cancer stem cells not previously detected by the present classical assays.

In conclusion, the parallel between normal and cancer stem cells is both conceptually and experimentally rewarding. However, the striking similarities between normal and cancer stem cells should not understate the fundamental differences existing between these cells (Table 1). Taking into account these distinctions is essential for upgrading the experimental paradigm currently used in cancer stem cell research and for the development of therapies which will be able to target cancer stem cells while sparing normal stem cells.

## **Legends to figures**

### **Fig. 1**

#### **Normal stem cell and cancer stem cell assays.**

The experimental steps used to characterize cancer stem cells follow the experimental paradigm of normal stem cell characterization. At first, cells are isolated from tissue and then sorted by FACS either on their capacity to efflux Hoechst 33342 (SP phenotype) (A,B), or on the basis of the expression of CD34 for haematopoietic stem cells and CD133 for brain cancer stem cells (immunophenotyping) (C,D). In a haematopoietic stem cell assay (A,C), normal stem cells prospectively isolated by FACS, are assayed for their capability to rescue lethally irradiated mice from radiation-induced bone marrow aplasia, and for their ability to provide permanent long-term engraftment<sup>(83)</sup>. In a cancer stem cell assay (B,D), cancer stem cells prospectively isolated by FACS are implanted into an orthotopic site of immunocompromised mice and are assayed for their capability to serially induce tumor formation.

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**The upper SP: how the blind adherence to the academic SP cell gating procedures can hinder a relevant cancer SP cell population.**

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