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► To cite this version:

Jean-François Mayol, Corinne Loeuillet, Francis Hérodin, Didier Wion. Characterization of normal and cancer stem cells: One experimental paradigm for two kinds of stem cells. *BioEssays*, 2009, pp.993-1001. 10.1002/bies.200900041 . inserm-01194520

HAL Id: inserm-01194520

<https://inserm.hal.science/inserm-01194520>

Submitted on 7 Sep 2015

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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⁽¹⁻³⁾). 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-renewed 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⁽¹⁾. 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⁽⁴⁻⁷⁾. 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^(4,5). 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/ β -catenin and Notch signalling⁽⁸⁾. Drug

pumps are also preferentially expressed in cancer stem cells⁽⁹⁾. 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^(10,11). 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⁺^(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⁽¹⁰⁾ or cancer stem cells^(11,16,17).

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.

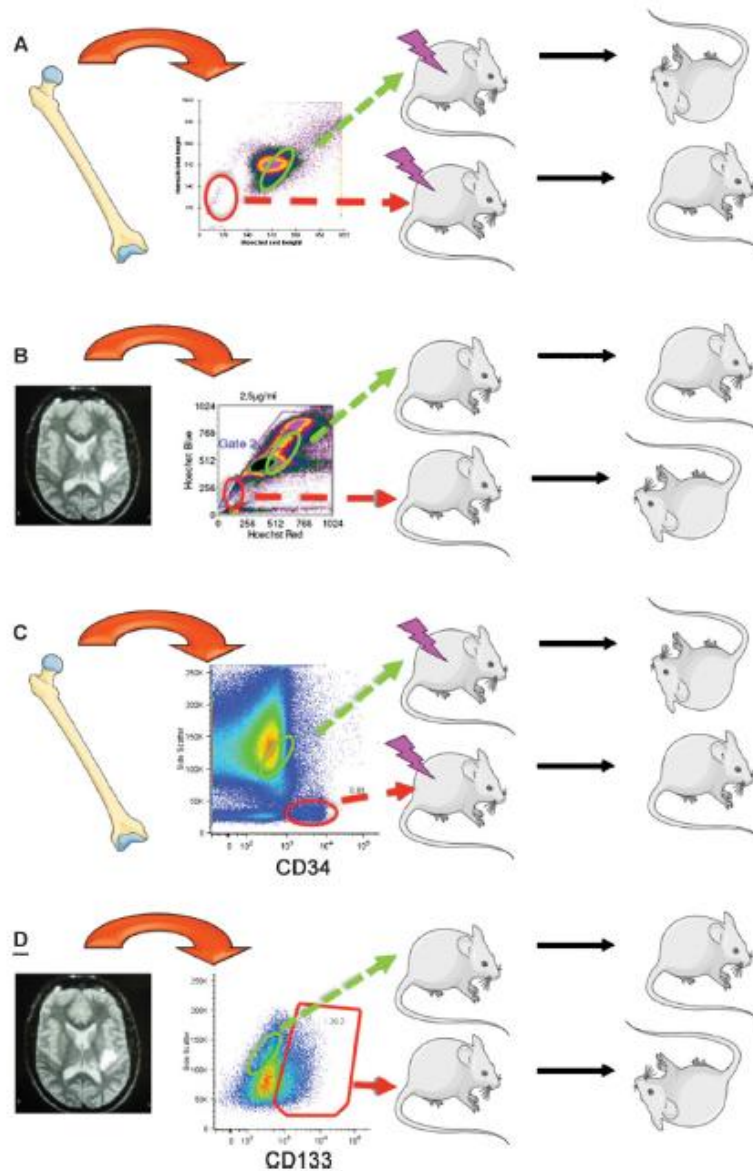


Fig. 1

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⁽⁸³⁾. 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⁽¹⁸⁾. 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⁽¹⁹⁾. 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⁽²⁰⁾ and is critical for characterizing the SP phenotype. In 1996 Goodell, demonstrated that mice HSC have the distinct feature to efflux this dye⁽¹⁰⁾; 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^(10,11). 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⁽²¹⁻²³⁾. 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⁽²⁴⁾. 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⁽²⁵⁾.

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^(26,27). 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^(11,26)). 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^(26,27). Unfortunately, this upper SP was not further characterized and goes unnoticed by most investigators working in field of cancer stem cells^(11,26), 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^(28,29). Moreover, it is noteworthy that Hoechst interferes with cancer cell differentiation at concentrations used for SP analysis⁽³⁰⁻³²⁾. 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⁽³³⁾. This point is of particular concern since topoisomerase I inhibitors are known to induce cancer cell differentiation^(34,35). 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⁽³⁶⁾ 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⁽²³⁾. 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⁽²²⁾. 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⁽²⁶⁾. 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^(10,37,38). 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⁽³⁹⁾. 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⁽⁴⁰⁾.

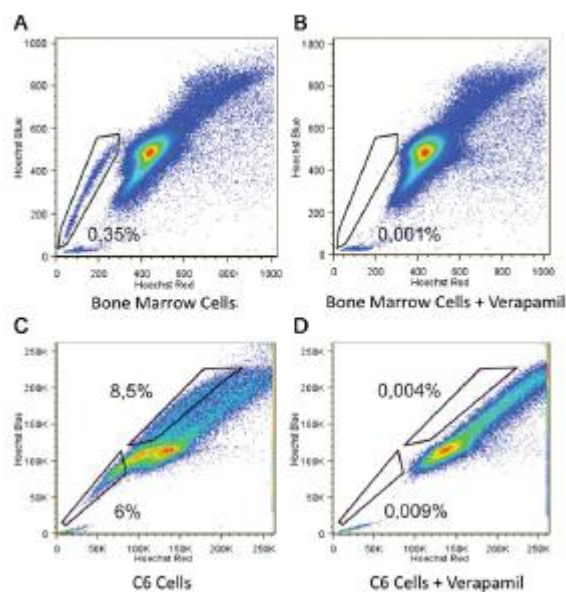


Fig.2

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⁽¹²⁾. Nowadays, prospective cancer stem cells are isolated by flow cytometry on the basis of cell surface markers expression, just like haematopoietic stem cells are⁽⁴¹⁻⁴⁶⁾. For example, the cancer stem cell population has been defined as CD34⁺CD38⁻ in acute myeloid leukaemia⁽⁴⁷⁾ CD133⁺ in brain⁽⁴⁵⁾, melanoma⁽⁴⁸⁾ and lung tumors⁽⁴⁹⁾, CD133⁺^(43,44) or EpCAM^{high}/CD44⁺/CD166⁺ in colon cancer⁽⁴²⁾, CD44⁺/CD24^{~low}/Lineage⁻ in breast tumors⁽⁴¹⁾, CD44⁺/CD117⁺ in ovarian tumors⁽⁴⁶⁾, and CD44⁺/α₂β₁^{hi}/CD133⁺ in prostate⁽⁵⁰⁾. 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^(51,52).

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⁺ cells have nevertheless been reported to have important features such as an increased radioresistance^(53,54) and increased expression of VEGF⁽⁵⁵⁾. 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⁽⁵⁶⁾.

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⁽⁵⁷⁾. 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^(58,59).

-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⁺ cells are not SP⁽⁶⁰⁾. Likewise in DAOY medulloblastoma cells CD133 and SP subsets can be two independent compartments⁽⁶¹⁾.

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^(43,44), is variable in the cancer stem cell population characterized on the basis of CD44 expression⁽⁴²⁾ or in colon metastatic cancer stem cell⁽⁵¹⁾. Likewise, although brain cancer stem cells were first identified as CD133⁺⁽⁴⁵⁾, several recent reports demonstrate the existence of CD133⁻ cancer stem cells in brain tumors⁽⁶²⁻⁶⁵⁾. 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⁽²⁵⁾. 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^(25,66,67), 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⁺ and CD133⁻ 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^(54,68,69). This finding suggests that

CD133⁺ 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^(70,71). 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⁺ or SP, very rare CD34⁻ and non-SP subsets of LT-HSCs also exist^(72,73). Therefore, sorting HSC on the basis of CD34 expression or SP phenotype eliminates a functionally minor but relevant stem cell population. Omitting these CD34⁻ 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⁺ 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^(74,75). 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⁽⁷⁴⁾. 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⁽⁷⁶⁾. 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^(77,78). 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^(66,67). 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^(41,42) are required. These analyses could target cell surface proteins involved in critical cancer stem cell functions such as chemokine receptors (CXCR4, CXCR3...)⁽⁷⁹⁾ adhesion proteins (L1CAM...)⁽⁷⁾, or ABC drug transporters⁽²⁵⁾. 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^(80,81) 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⁽⁴⁾. Notably, it has been recently shown that the use of more highly immunocompromised mice increases the detectable frequency of cancer stem cells⁽⁴⁾. Numerous studies have demonstrated that microenvironment can either support or inhibit tumorigenesis^(82,83). Cancer stem cell assays are presently performed by implanting cancer cells in normal orthotopic tissue. A tumourigenicity 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⁽⁸³⁾. 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.

Fig.2

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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Acknowledgements. We thank Dr Richard Foucault for critical reading of the manuscript, Dr JP Issartel for suggesting the use of the term “*stemicide*”, and Pr F Berger for support. DW is supported by the Ligue contre le Cancer Comité Rhône..

References

1. Clarke MF, Dick JE, Dirks PB et al. 2006. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 66(19):9339-9344.
2. Reya T, Morrison SJ, Clarke MF et al. 2001. Stem cells, cancer, and cancer stem cells. *Nature* 414(6859):105-111.
3. Visvader JE, Lindeman GJ. 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 8(10):755-768.
4. Quintana E, Shackleton M, Sabel MS et al. 2008. Efficient tumour formation by single human melanoma cells. *Nature* 456(7222):593-598.
5. Balic M, Lin H, Young L et al. 2006. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* 12(19):5615-21.
6. Li F, Tiede B, Massague J et al. 2007. Beyond tumorigenesis: cancer stem cells in metastasis. *Cell Res* 17(1):3-14.
7. Brabletz T, Jung A, Spaderna S et al. 2005. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* 5(9):744-9.
8. Hermann PC, Huber SL, Herrier T et al. 2007. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1: 313-323.
9. Eyler CE, Rich JN. 2008. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol* 26(17):2839-45.
10. Goodell MA, Brose K, Paradis G et al. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 183(4):1797-1806.
11. 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* 101(3):781-786.
12. Berenson RJ, Andrews RG, Bensinger WI et al. 1988. Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J Clin Invest* 81(3):951-955.
13. Osawa M, Hanada K, Hamada H et al. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 273(5272):242-245.
14. Miraglia S, Godfrey W, Yin AH et al. 1997. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 90(12):5013-5021.
15. Wu A, Oh S, Wiesner SM et al. 2008. Persistence of CD133+ cells in human and mouse glioma cell lines: detailed characterization of GL261 glioma cells with cancer stem cell-like properties. *Stem Cells Dev* 17(1):173-184.
16. Hirschmann-Jax C, Foster AE, Wulf GG et al. 2004. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A* 101(39):14228-14233.

17. Patrawala L, Calhoun T, Schneider-Broussard R et al. 2005. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. *Cancer Res* 65(14):6207-6219.
18. Loewe H, Urbanietz J. 1974. [Basic-substituted 2,6-bisbenzimidazole derivates, a novel class of substances with chemotherapeutic activity]. *Arzneimittelforschung* 24(12):1927-1933.
19. Arndt-Jovin DJ, Jovin TM. 1977. Analysis and sorting of living cells according to deoxyribonucleic acid content. *J Histochem Cytochem* 25(7):585-589.
20. Petersen TW, Ibrahim SF, Diercks AH et al. 2004. Chromatic shifts in the fluorescence emitted by murine thymocytes stained with Hoechst 33342. *Cytometry Part A* 60A(2):173-181.
21. Scharenberg CW, Harkey MA, Torok-Storb B. 2002. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99(2):507-512.
22. Zhou S, Morris JJ, Barnes Y et al. 2002. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. *Proc Natl Acad Sci U S A* 99(19):12339-12344.
23. Zhou S, Schuetz JD, Bunting KD et al. 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* 7(9):1028-1034.
24. Jonker JW, Freeman J, Bolscher E et al. 2005. Contribution of the ABC transporters Bcrp1 and Mdr1a/1b to the side population phenotype in mammary gland and bone marrow of mice. *Stem Cells* 23(8):1059-1065.
25. Dean M, Fojo T, Bates S. 2005. Tumour stem cells and drug resistance. *Nat Rev Cancer* 5(4):275-284.
26. Platet N, Mayol JF, Berger F et al. 2007. Fluctuation of the SP/non-SP phenotype in the C6 glioma cell line. *FEBS Lett* 581(7):1435-1440.
27. Smalley MJ, Clarke RB. 2005. The mammary gland "side population": a putative stem/progenitor cell marker? *J Mammary Gland Biol Neoplasia* 10(1):37-47.
28. Durand RE, Olive PL. 1982. Cytotoxicity, Mutagenicity and DNA damage by Hoechst 33342. *J Histochem Cytochem* 30(2):111-116.
29. Fried J, Doblin J, Takamoto S et al. 1982. Effects of Hoechst 33342 on survival and growth of two tumor cell lines and on hematopoietically normal bone marrow cells. *Cytometry* 3(1):42-47.
30. Adamski D, Mayol JF, Platet N et al. 2007. Effects of Hoechst 33342 on C2C12 and PC12 cell differentiation. *FEBS Lett* 581(16):3076-3080.
31. Steuer B, Breuer B, Alonso A. 1990. Differentiation of EC cells in vitro by the fluorescent dye Hoechst 33342. *Exp Cell Res* 186(1):149-157.
32. Zhong Y, Zhou C, Ma W et al. 2007. Most MCF7 and SK-OV3 cells were deprived of their stem nature by Hoechst 33342. *Biochem Biophys Res Commun* 364(2):338-43.
33. Chen AY, Yu C, Bodley A et al. 1993. A new mammalian DNA topoisomerase I poison Hoechst 33342: cytotoxicity and drug resistance in human cell cultures. *Cancer Res* 53(6):1332-1337.
34. Kondo K, Tsuneizumi K, Watanabe T et al. 1991. Induction of in vitro differentiation of mouse embryonal carcinoma (F9) cells by inhibitors of topoisomerases. *Cancer Res* 51(19):5398-5404.

35. Larsen AK. 1994. Involvement of DNA topoisomerases and DNA topoisomerase inhibitors in the induction of leukemia cell differentiation. *Ann Oncol* 5(8):679-688.
36. Zhang X, Kiechle FL. 1998. Hoechst 33342 induces apoptosis and alters tata box binding protein/DNA complexes in nuclei from BC3H-1 myocytes. *Biochem Biophys Res Commun* 248(1):18-21.
37. Moshaver B, van Rhenen A, Kelder A et al. 2008. Identification of a small subpopulation of candidate leukemia-initiating cells in the side population of patients with acute myeloid leukemia. *Stem Cells* 26(12):3059-67.
38. Wulf GG, Wang RY, Kuehnle I et al. 2001. A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. *Blood* 98(4):1166-73.
39. Noguchi K, Katayama K, Mitsuhashi J et al. 2009. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. *Adv Drug Deliv Rev* 61(1):26-33.
40. Bleau AM, Hambardzumyan D, Ozawa T et al. 2009. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell* 4(3):226-35.
41. Al-Hajj M, Wicha MS, Benito-Hernandez A et al. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 100(7):3983-3988.
42. Dalerba P, Dylla SJ, Park IK et al. 2007. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A* 104(24):10158-10163.
43. O'Brien CA, Pollett A, Gallinger S et al. 2007. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445(7123):106-110.
44. Ricci-Vitiani L, Lombardi DG, Pilozzi E et al. 2007. Identification and expansion of human colon-cancer-initiating cells. *Nature* 445(7123):111-115.
45. Singh SK, Hawkins C, Clarke ID et al. 2004. Identification of human brain tumour initiating cells. *Nature* 432(7015):396-401.
46. Zhang S, Balch C, Chan MW et al. 2008. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 68(11):4311-4320.
47. Lapidot T, Sirard C, Vormoor J et al. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367(6464):645-648.
48. Monzani E, Facchetti F, Galmozzi E et al. 2007. Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. *Eur J Cancer* 43(5):935-946.
49. Eramo A, Lotti F, Sette G et al. 2008. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 15(3):504-514.
50. Collins AT, Berry PA, Hyde C et al. 2005. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 65(23):10946-10951.
51. Shmelkov SV, Butler JM, Hooper AT et al. 2008. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. *J Clin Invest* 118(6):2111-2120.
52. Krause DS, Fackler MJ, Civin CI et al. 1996. CD34: structure, biology, and clinical utility. *Blood* 87(1):1-13.
53. Bao S, Wu Q, McLendon RE et al. 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444(7120):756-760.
54. Blazek ER, Foutch JL, Maki G. 2007. Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia. *Int J Radiat Oncol Biol Phys* 67(1):1-5.

55. Yao XH, Ping YF, Chen JH et al. 2008. Glioblastoma stem cells produce vascular endothelial growth factor by activation of a G-protein coupled formylpeptide receptor FPR. *J Pathol* 215(4):369-376.
56. Munz M, Kieu C, Mack B et al. 2004. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. *Oncogene* 23(34):5748-5758.
57. Shapiro HM. 2003. *Practical Flow Cytometry*, 4th Edition New-York: Wiley-Liss.
58. Lee JA, Spidlen J, Boyce K et al. 2008. MIFlowCyt: the minimum information about a Flow Cytometry Experiment. *Cytometry A* 73(10):926-930.
59. Schwartz A, Fernandez-Repollet E. 2001. Quantitative flow cytometry. *Clin Lab Med* 21(4):743-761.
60. Wu C, Alman BA. 2008. Side population cells in human cancers. *Cancer Lett* 268(1):1-9.
61. Srivastava VK, Nalbantoglu J. 2008. Flow cytometric characterization of the DAOY medulloblastoma cell line for the cancer stem-like phenotype. *Cytometry A* 73(10):940-948.
62. Beier D, Hau P, Proescholdt M et al. 2007. CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res* 67(9):4010-4015.
63. Joo KM, Kim SY, Jin X et al. 2008. Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. *Lab Invest* 88(8):808-815.
64. Ogden AT, Waziri AE, Lochhead RA et al. 2008. Identification of A2B5+CD133-tumor-initiating cells in adult human gliomas. *Neurosurgery* 62(2):505-514; discussion 514-515.
65. Wang J, Sakariassen PO, Tsinkalovsky O et al. 2008. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *Int J Cancer* 122(4):761-768.
66. Merlo LM, Pepper JW, Reid BJ et al. 2006. Cancer as an evolutionary and ecological process. *Nat Rev Cancer* 6(12):924-935.
67. Nowell PC. 1976. The clonal evolution of tumor cell populations. *Science* 194(4260):23-28.
68. Griguer CE, Oliva CR, Gobin E et al. 2008. CD133 is a marker of bioenergetic stress in human glioma. *PLoS ONE* 3(11):e3655.
69. Platet N, Liu SY, Atifi ME et al. 2007. Influence of oxygen tension on CD133 phenotype in human glioma cell cultures. *Cancer Lett* 258(2):286-290.
70. Bene MC. 2005. Immunophenotyping of acute leukaemias. *Immunol Lett* 98(1):9-21.
71. Di Noto R, Mirabelli P, Del Vecchio L. 2007. Flow cytometry analysis of acute promyelocytic leukemia: the power of 'surface hematology'. *Leukemia* 21(1):4-8.
72. Engelhardt M, Lubbert M, Guo Y. 2002. CD34(+) or CD34(-): which is the more primitive? *Leukemia* 16(9):1603-1608.
73. Morita Y, Ema H, Yamazaki S et al. 2006. Non-side-population hematopoietic stem cells in mouse bone marrow. *Blood* 108(8):2850-2856.
74. Garcion E, Naveilhan P, Berger F et al. 2008. Cancer stem cells: Beyond Koch's postulates. *Cancer Lett in press*. Doi:10.1016/j.canlet.2008.09.006.
75. Hill RP. 2006. Identifying cancer stem cells in solid tumors: case not proven. *Cancer Res* 66(4):1891-1895; discussion 1890.
76. Sell S. 2004. Stem cell origin of cancer and differentiation therapy. *Crit Rev Oncol Hematol* 51(1):1-28.

77. Koch R. 1881. Zur Untersuchung von pathogen Organismen. Mitt. Kaiserlichen Gesundheitsamte 1:25-26.
78. Petri RJ. 1887. Eine kleine Modification des Koch'shen Platten-verfahrens. Zentralbl. Bakteriol. Parasitenkd. 1:279-280.
79. Vandercappellen J, Van Damme J, Struyf S. 2008. The role of CXC chemokines and their receptors in cancer. *Cancer Lett* 267(2):226-44.
80. Benelli R, Albini A. 1999. In vitro models of angiogenesis: the use of Matrigel. *Int J Biol Markers* 14(4):243-6.
81. Albini A, Benelli R, Noonan DM et al. 2004. The "chemoinvasion assay": a tool to study tumor and endothelial cell invasion of basement membranes. *Int J Dev Biol* 48(5-6):563-71.
82. Hu M, Polyak K. 2008. Microenvironmental regulation of cancer development. *Curr Opin Genet Dev* 18(1):27-34.
83. Bissell MJ, Labarge MA. 2005. Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? *Cancer Cell* 7(1):17-23.
84. van Os R, Kamminga LM, de Haan G. 2004. Stem cell assays: something old, something new, something borrowed. *Stem Cells* 22(7):1181-1190.