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From where do Cancer-Initiating Cells Originate?

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1. Introduction

Cancer development is generally depicted as successive waves of Darwinian selection of cells harbouring genetic and epigenetic abnormalities, providing them with proliferative, survival and adaptive advantages. As genetic alterations preferentially operate on naked DNA, original targeted cells are presumably either proliferating or engaged in a reprogramming process, both cellular mechanisms being associated with chromatin decondensation. Taking this point in consideration, appropriate candidates include a large set of embryonic cells (or embryonic stem-cells) as well as adult stem/progenitor cells when engaged in a repopulation process, a mechanism either permanent as in regenerative tissues such as the intestine, the colon or the skin, or sporadically induced in response to insults, such as wound healings. Studies of hematopoietic cancers point out that the malignancy might originate from the alteration of a single cell displaying both self-renewal and differentiation potentials. By similarity with normal stem-cells, that are able to reconstitute a complete tissue, this observation led to the development of the "cancer stem-cell" (CSC) concept. Indeed, in chronic myeloid leukaemia (CML), several type of blood cells including their most primitive precursors display a similar chromosomal recombination (named the Philadelphia chromosome) leading to the production of the aberrant BCR-ABLp120 fusion protein. This genetic alteration was therefore likely to drive transformation of precursor cells or stem-cells, deregulating the production of mature cells without affecting their ability to execute their normal differentiation (Bonnet and Dick, 1997). Accordingly, the restricted expression of the aberrant BCR-ABLp120 fusion protein in $Sca1^+$ stem-cells was shown, in transgenic mice, to mimic human CML, characterized by a progression from chronic towards an acute phase (Perez-Caro et al., 2009). While the inhibition of the activity of the kinase by the ST1571 chemical compound, according to the resistance of the human leukaemia stem cells to the chemical (Graham et al., 2002; Hu et al., 2006; Primo et al., 2006; Jiang et al., 2007), did not modify the survival of the transgenic mice, CSC ablation eradicated tumours, demonstrating undoubtedly their role in AML development and the therapeutic interest of eradicating them (Perez-Caro et al., 2009). Since then, a large number of laboratories attempt to extend the CSC theory to solid tumours. The observation that

metastases and their original primary tumour share a similar heterogeneity indeed argue in favour of the presence of a subset of CSCs displaying both self-renewing and differentiation capabilities. In such a scenario, CSCs are expected to represent a minor population of the tumour, giving rise to differentiated cells that, per definition, would have lost their self-renewal capabilities and thereby their tumour driving potential. In the last decade, based on phenotypic and/or functional similarities with their normal counterparts, CSCs have been successfully isolated form numerous cancer types, including breast tumours, gliomas and melanomas and described as displaying self-renewal and differentiation properties. Validating the concept that a limited number of cells resulting from the transformation of normal stem-cells continuously fuel the tumour has constituted a real breakthrough in the cancer field and has had major repercussions in the design of novel therapeutic approaches. Nonetheless, as discussed below, several of the experimental assays commonly used to evaluate stem-like properties are individually questionable. These doubts raise some concerns on the real biological properties of the isolated CSC subpopulations and impact on the current debate concerning their potential origin. Noticeably, even the term of "cancer stem-cells" is probably not appropriated referring to their normal counterparts. Although some adult normal stem-cells were found to be highly proliferative (Barker et al., 2009), they generally are depicted as poorly proliferating cells, able to concomitantly maintain their pool and generate their progeny through asymmetric divisions. As far as we know, if the proportion of CSC is maintained during tumour growth, this is far away of demonstrating that they actually share this same property. The potential filiation between normal stem-cells and CSCs thus remains a matter of discussion, leading to the emergence of the alternative "tumour-initiating cells" terminology.

The questionable characterisation of CSC

In this first section, we will attempt to demonstrate the limit of the techniques currently used for isolating CSCs and the conflicting results they provide. These techniques consist in identifying CSCs by exploiting expected similarities with their normal counterparts, including some phenotypic features, their ability to efflux drugs and to grow as colonospheres, when cultured in low adherent conditions. Sorting CSC from tumours or tumour cell lines, taking advantage of specific stem-cell markers, is a commonly used approach but *in fine* turned out to be more difficult as previously thought. A major reason is that this notion of "specificity" is often biased by the quality of the available antibodies used and by our current limited knowledge on normal stem cell features. A significant example is provided by the contradictory results generated by using the transmembrane protein CD133 as a stem-cell marker. In numerous studies, monoclonal antibodies to CD133 were defined as appropriate tools to isolate CSC from various tumour types (Barker et al., 2009; Yin et al., 1997; Uchida et al., 2000; Lee et al., 2005; Sagrinati et al., 2006; Richardson et al., 2004; Kordes et al., 2007; Oshima et al., 2007; Sugiyama et al., 2007; Ito et al., 2007). Nonetheless, by generating transgenic mice expressing the LacZ reporter gene under the control of the CD133 promoting sequences, the transmembrane protein was found expressed by mature luminal ductal epithelial cells in adult organs, suggesting that it is not a specific marker of stem-cells (Shmelkov et al., 2008). The interest in using CD133 was further challenged, as these authors next demonstrated, taking advantage of IL10 knock-out mice, that cancer cells in primary colon carcinomas uniformly express CD133. Evenmore, $CD133^+$ and $CD133^-$ cells

isolated from secondary tumours display similar tumorigenic potential, as assessed by serial transplantations into immuno-compromised mice, and were both capable of forming colonospheres *in vitro* at a similar rate (Shmelkov et al., 2008).

The ability of stem cells to efflux drugs, due to a high expression level of transporters, was also exploited for isolating CSCs. This approach led to the detection by flow cytometry of a population of cells named side population (SP), able to efflux the DNA binding Hoechst 3342 dye. Unfortunately SP and CSC populations do not always match. In mice bone marrows, SP subpopulation was originally found to be enriched in hematopoietic stem cells (Goodell et al., 1996). Consistently, progenitor cells were restricted to the SP fraction of mammospheres (Dontu et al., 2003) and SP purified from several cancer cell lines show enhanced tumorigenicity *in vivo* relative to their non-SP cohorts (Ho et al., 2007; Patrawala et al., 2005). Nonetheless, in some tumor types, SP populations are not enriched in SSC (Mitsutake et al., 2007; Stingl et al., 2006; Burger et al., 2004) and purified mouse mammary SP cells do not efficiently repopulate the mammary gland in a reconstitution assay (Alvi et al., 2003). This discrepancy is likely to reflect the existence of various cell populations that actually share with stem-cells a set of common properties.

Enrichment in stem-cells in low adherent culture conditions is an additional commonly used approach to isolate CSC. This technology was originally performed to evaluate the self-renewal capacity of neural cells (Reynolds and Weiss, 1996), next adopted for human breast epithelial cells to form mammospheres (Dontu et al., 2003) and finally extended to various cancer types. Individual cells able to grow in low adherent conditions for up to five consecutive passages indeed display a gene expression profile consistent with progenitor properties, validating the experimental approach. These conditions might however simply select for cells displaying resistance to anoïkis. One could easily envisage that the stress conditions provided by the low adherence actually enforce cells to adapt through a genomic reprogramming, potentially a partial dedifferentiation, leading to the expression of some stem cell-associated genes. Evenmore, the function of normal stem cells is highly regulated by their niche through direct and paracrine interactions with supporting cells and the extracellular matrix. One could then wonder why in sphere cultures, in absence of this niche, cells might display stem-cell properties.

A more recent assay has consisted in purifying CSC based on the detoxifying aldehyde dehydrogenase 1 (ALDH1) enzymatic activity, previously detected in a set of normal stem-cells (Armstrong et al., 2004; Matsui et al., 2004; Hess et al., 2004). Nonetheless, attempts to isolate breast CSCs according to their antigenic phenotype or to their ALDH1 activity led again to the isolation of different cell subpopulations that at the most partially overlap, suggesting that actually any of these markers are strictly allotted to stem-cells (Al-Hajj et al., 2003; Fillmore and Kuperwasser, 2008; Ginestier et al., 2007).

The stem cell potentiality of the presumed isolated CSC subpopulations is next evaluated through various functional assays. As theoretically, a single CSC should be able to reconstitute a complete tumour, a commonly used assay consists in evaluating their tumorigenic potential when xenografted at limit dilutions in immunosuppressive mice. This assay turns out being also questionable. Considering that cells have to evade from the immune system (even in immuno-compromised hosts), their antigenic phenotype and their immunosuppressive properties might impinge on their tumorigenic potential. Moreover, their ability to interfere with the host environment is undoubtedly a limiting factor. Taking

this information in consideration, optimisation of the experimental conditions, including selection of more highly immuno-compromised or humanised mice, dramatically increased the detectable frequency of tumorigenic cells (Quintana et al., 2008). One fourth of melanoma cells were thus found to display a tumorigenic potential, independently of their CD133 antigenic phenotype (Quintana et al., 2008). Consistently, a large proportion of cells isolated from primary Eμ-Myc pre-B/B lymphoma, Eμ-N-Ras thymic lymphomas and PU.1$^{-/-}$ acute myeloid leukaemia sustain tumour growth when transplanted in NOD/SCID immuno-deficient mice, challenging the concept that tumours arise from rare CSCs, at least for malignancies with substantial homogeneity (Kelly et al., 2007). Recently, the Herlyn laboratory actually demonstrated that CSCs did not contribute to tumour initiation but were rather found as essential for long term maintenance, as judged by serial transplantations in *nude* mice (Roesch et al., 2010). Finally, transplantations in mice are generally performed with individualised cells, although maintaining them in a niche has recently been shown as determinant for their tumorigenic potential (Liu et al., 2009). Conclusions based on xenograft experiments should therefore be considered with caution.

If CSCs are able to reconstitute the heterogeneous populations of a primary tumour, they are additionally suggested to display a differential potential (Dirks, 2008). As previously mentioned, CSCs are often sorted out of primary tumours/cell lines based on the expression of specific antigens. By definition, the non cancer stem-cell subpopulation that presumably represents the large pool of differentiated cells constituting the bulk of the tumour is represented by the cellular fraction lacking this specific marker. The differentiation potential of the presumed isolated CSCs often relies on their ability to evolve into their differentiated counterparts. While this shift is likely to reflect some reprogramming, these data are far away from demonstrating pluri-potentiality, with a potential to commit into various differentiation programs. At the most, transplantation of these cells in mice gives rise to tumours that display a similar heterogeneity as the primary tumours they originate from. Whether this heterogeneity reflects an adaptive partial reprogramming rather than a dedifferentiation-differentiation process is plausible.

In conclusion, various recent observations reveal the intrinsic limits of each of these experimental approaches. While combining them is probably helpful in interpreting the results, it is obviously not sufficient, implying the development of additional tools. The establishment of novel transgenic mouse models is undoubtedly a promising alternative in further exploring tumour initiation. As a first example, the activation of the Wnt pathway in LG5^{+}/CD133^{+} or Bmi1^{+} intestine stem cells was recently found to promote adenomas while it fails to do so when induced in short-lived transit amplifying cells (Barker et al., 2009; Zhu et al., 2009). These studies provide first evidences that a window of time exists for mutations in intestinal epithelial cells to initiate tumour formation. More sophisticated engineered transgenic mouse models, recapitulating the sequential accumulation of genetic alterations will probably be of further help in understanding the tumour progression process in the next future.

Origins of CSCs

While some studies suggest that CSC may arise from the transformation of their normal counterparts, recent observations rather suggest that they originate from fully differentiated cells through an adaptive transdifferentiation program (Figure 1). This hypothesis originally

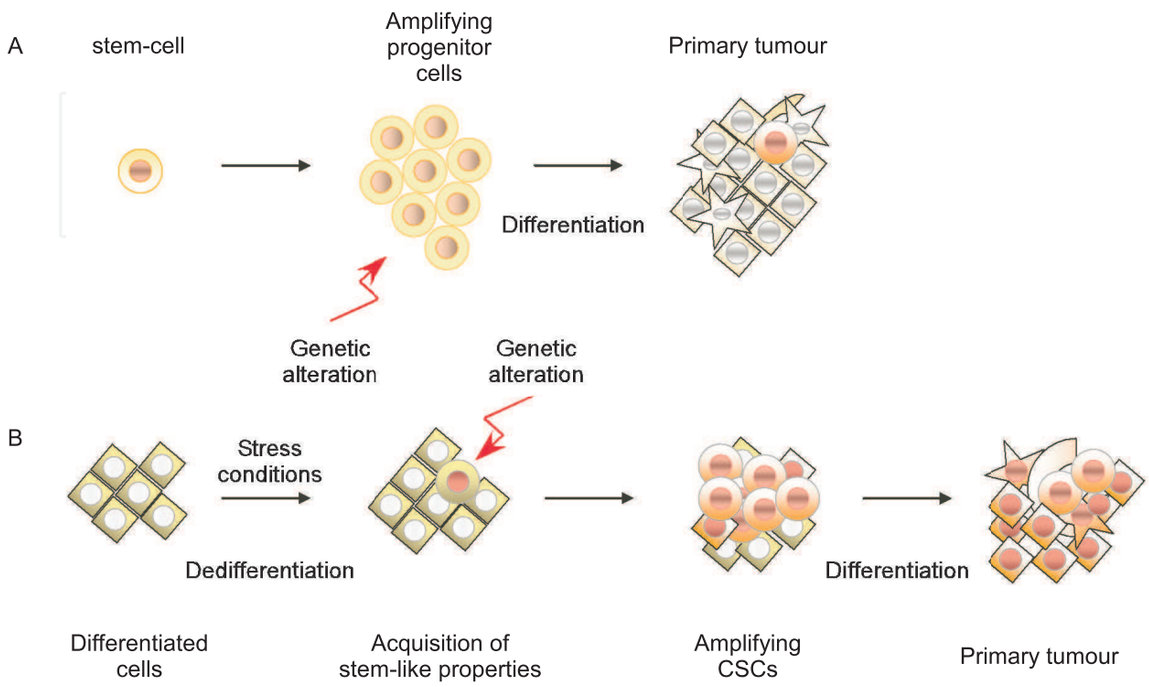

Fig. 1. The "cancer stem-cell theory" (panel A) is based on the assumption that during tissue regeneration, the amplification of progenitor cells opens a window of time suitable for accumulating genetic alterations, leading to the emergence of cancer cell-stems (CSCs). CSCs would thus initiate and sustain tumour growth.
Alternatively, under stress conditions, fully differentiated cells reacquire stem-like properties, including self-renewal properties (panel B). This gain of function is influenced by cellular intrinsic properties as well as micro-environmental conditions. These cells could potentially be prone to transformation and give rise to CSCs.
Both models are not exclusive. CSCs and cell dedifferentiation would thus constitute the initial and secondary tumour drivers, respectively.

emerges from *in vitro* cell transformation assays. Transformation of human mammary epithelial cells (HMECs) consisted in sequentially infecting cells with the catalytic sub-unit of the telomerase (immortalisation step), the SV40 T/t antigens (these viral proteins have pleiotropic effects including the neutralisation of both Rb- and p53-dependent-oncosuppressive pathways) and an activated version of the mitogenic protein Ras (H-Ras^{G12V}) (Elenbaas et al., 2001). Cell transformation was found to be invariably associated with cellular morphological changes associated with an epithelial-mesenchymal transition (EMT) (Morel et al., 2008; Mani et al., 2008). EMT is a trans-differentiation process that

consists in turning polarized and adjacent epithelial cells into individual and motile mesenchymal ones. Originally identified as a biological process essential for the morphogenetic movements during the embryonic development, its aberrant reactivation in cancers is currently considered as one of the main driving cancer cell dissemination (Thiery et al., 2009). Studying the contribution of EMT in cell transformation led to the demonstration that it actually constitutes a dedifferentiation process, providing cells with some stem-like properties (Morel et al., 2008; Mani et al., 2008; Vesuna et al., 2009). Cells that have undergone an EMT were thus found to form mammospheres in low adherent conditions and to be highly tumorigenic when orthotopically xenografted at limit dilution in *nude* mice. They additionally display a $CD44^{high}$ $CD24^{low}$ antigenic phenotype that was previously allotted to mammary CSCs (Al-Hajj et al., 2003). EMT being by definition a reversible process, these cells continuously generate $CD44^{low}$ $CD24^{high}$ epithelial cells that interestingly lack a tumorigenic potential (Morel et al., 2008; Mani et al., 2008; Vesuna et al., 2009). In regards to the EMT-associated properties, the transdifferentiation process was thus considered as a biological process able to convert differentiated epithelial cells into CSCs. EMT being strongly impacted by micro-environmental conditions, the balance between differentiated cells and CSCs was then proposed to be a highly dynamic process with important repercussions on therapeutic approaches, eradication of the entire primary tumour, including differentiated cells, being henceforth a requisite to prevent recurrence (Gupta et al., 2009).

Despite the obvious interest of these works, we still can emit some reserve about their meaning. Obviously, EMT is a reversible transdifferentiation process associated with a profound genetic reprogramming and major consequent phenotypic changes. Considering that mesenchymal cells display a pluripotency based on their ability to turn into epithelial ones, is probably a miss-interpretation, rather reflecting the equilibrium between the two cell fates of this transdifferentiation process. Recently, in appropriate culture conditions, HMEC-transformed mesenchymal derivatives were found to initiate chondrocytic, adipocytic or osteoblastic differentiation programs, highlighting their pluripotency (Battula et al., 2010). Nonetheless, as previously mentioned, these cells harbour a set of genetic alterations, including the expression of viral proteins which are known to impact on multiple cellular functions. Whether similar results would be obtained in more "physiological" conditions, by combining EMT-permissive conditions with a restricted number of genetic events, is warranted to further evaluate the relevance of these observations. The CSC features of these HMEC derivatives were next supported by their tumorigenic potentials at limiting conditions. If CSCs are rather important for tumour maintenance than for tumour initiation (Roesch et al., 2010), this result would more highlight a direct role of EMT in facilitating cell transformation and tumour initiation. Finally, these cells were described as displaying a similar antigenic phenotype as the one originally attributed to mammary CSCs (Al-Hajj et al., 2003) Nonetheless, likewise the $CD133^{+}$ population, $CD44^{high}CD24^{low}$ cells might actually include much more than the CSCs, which antigenic phenotype has been restricted to $CD44^{high}CD24^{low}ESA^{+}$ or $CD44^{high}CD24^{low}ALDH1^{+}$ cells (Fillmore and Kuperwasser, 2008; Ginestier et al., 2007). Rather than providing cells with real stem-like properties, EMT might actually provide cells with some plasticity, facilitating potentially the transformation process and helping them to

adapt to microenvironmental changes. In other terms, this plasticity and adaptation to microenvironmental changes implies that $CD44^{high}CD24^{low}$ mesenchymal cells constitute a pool of tumour-driving cells whereas the $CD44^{low}CD24^{high}$ epithelial counterparts behave as a latent reserve of cancer cells reactivated in hostile conditions. In line with such a model, when exposed to EGFR tyrosine kinase inhibitor (TKI), a minor subpopulation of non small cell lung cancer derived cells that express some stem-cell-associated antigens (such as CD133) adopt a quiescent phenotype and resistance. Emergence of these resistant clones is abrogated in presence of trichostatin, an inhibitor of histone deacetylases, suggesting that it reflects a transient reprogramming, involving epigenetic changes, rather than an enrichment of a pre-existing cell subpopulation. When maintained in presence of TKI, a proportion of these cells restarts proliferating, giving rise to resistant cell lines that revert to a sensitive stage when released from the drug (Sharma et al., 2010). Cell reprogramming thus provides a route for cells to adapt to hostile conditions, a mechanism that the authors interestingly compare to the antibiotic-tolerant bacterial subpopulations termed "persisters" (Sharma et al., 2010). By similarity, EMT might be an escape from hypoxic conditions and mechanical constrains and the stem-like features associated with, just be a mirror of this adaptative process. Whether these cells are particularly prone to transformation, in light of their proliferation capabilities, remains to be determined. Some genetic events might similarly favour cell dedifferentiation into CSCs. Indeed, murine fibroblasts lacking the RB proteins were found to generate colonospheres at confluency and to reconstitute monolayers when plated at lower density. Interestingly, these colonospheres were found to be tumorigenic when xenografted in mice at limit dilutions, to include a SP, to express stem-cell markers and to additionally display differentiation properties (Liu et al., 2009). In conclusion, this plasticity might provide cells with survival advantages, when placed in hostile conditions. Overall, these recent observations demonstrate that the stem-like properties harboured by numerous cancer cells do not rely on any particular relationship to normal stem-cells but rather reflect the Darwinian selection that operates within a tumour.

Evolution of the concepts and therapeutic consequences

According to the CSC theory, eradicating the rare CSCs would be sufficient to clear tumours. A selection step implying a gain in plasticity and adaptation potential rather suggests that the eradication of all cancer cells, including the differentiated ones, is actually a requisite to eliminate all risks of recurrence. Beyond the cognitive interest, the origin of CSCs might impact on the design of future therapies. If CSCs display a low proliferation potential, they are supposed to be resistant to standard radio- or chemotherapies. Evenmore, these treatments could have the noxious effect to enforce differentiated cancer cells to evolve into tumour-driving ones. Numerous studies are currently engaged to determine the relative importance of various signalling pathways in these cells. The design of additional drugs that might additionally annihilate the dedifferentiation potential of the differentiated cancer cells should also be considered. Obviously, drugs preventing transient epigenetic changes, such as the histone deacetylase (HDAC) inhibitor trichostatin (TSA) might be appropriate (Sharma et al., 2010). Recently, numerous histone deacetylase inhibitors have been identified and some were recently found as efficient in clinical trials for cancer treating (for recent reviews see Lane and Chabner, 2009; Sebova and Fridrichova, 2010). Alternatively, one could also envisage that the plasticity is maintained to some extent and

engaging cells further in a differentiation program might avoid them to rescue from insults, potentially explaining the synergistic effect of some differentiation agents and radiation in eradicating xenografted tumours (Kawamata et al., 2006).

2. Conclusions

The relevance of the cancer-stem cell theory and the origin of CSCs remains currently a matter of discussion. The interpretation of the data obtained in this field is complicated by the fact that selection pressures enforce cancer cells to constantly evolve and gain in plasticity. Adaptation to hostile environment is likely driven by transient dedifferentiation processes, likely associated with the acquisition of some stem-like properties. The co-existence of various cancer cell populations within a primary tumour makes the interpretation of the results somehow difficult. Further investigations with help from novel techniques, including sophisticated transgenic mouse models, will probably clarify the current debate. Undoubtedly, these fields of research will shed light on impenetrable aspects of the tumorigenesis and open up new horizons for eradicating cancers.

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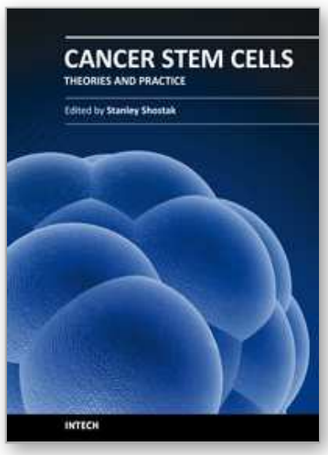

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Cancer Stem Cells Theories and Practice does not 'boldly go where no one has gone before!' Rather, Cancer Stem Cells Theories and Practice boldly goes where the cutting edge of research theory meets the concrete challenges of clinical practice. Cancer Stem Cells Theories and Practice is firmly grounded in the latest results on cancer stem cells (CSCs) from world-class cancer research laboratories, but its twenty-two chapters also tease apart cancer's vulnerabilities and identify opportunities for early detection, targeted therapy, and reducing remission and resistance.

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