Limitations of the cancer stem cell theory

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Abstract Cancer stem cells (CSCs) can be operationally defined as a subset of neoplastic cells which are responsible for the growth and re-growth of primary and metastatic tumors. Although the existence of perpetually dividing cells is a logical necessity to explain the malignant properties of human tumors, experimental data supporting their existence have only recently been obtained. New knowledge in basic stem cell biology and the availability of several cell surface markers for the definition and isolation of small subsets of immature cells coupled to the use of the classical model of xenotransplantation in immune deficient mice has identified putative CSCs in several solid tumors such as mammary, colon, brain, pancreas, prostate, melanoma and others. However, the theory must be considered as still in its infancy, since tumors grown in mice only partially recapitulate the biology of human cells. In addition, whether the “transformed” cell is the neoplastic counterpart of a normal stem cell or whether complete malignant behaviour can occur in a more differentiated cell has still to be demonstrated. In spite of these difficulties, the CSC hypothesis could be of clinical relevance, especially in the definition of new ways to assay drug sensitivity of primary human tumors.

Keywords Cancer stem cell · Tumor xenotransplantation · Differentiation · Tumor heterogeneity

Background

Defining “stem cells” is not so easy. Most researchers agree that the only true totipotent stem cell is the zygote and, possibly, the earliest blastomere (Jaenisch and Young 2008). Pluripotent stem cells are those of the blastocyst inner cells mass which in mouse give rise to the so called “embryonal stem cells” (ESC), which can give rise to all cells but not the trophoectodermal ones. Multipotent cells are usually defined as those able to produce cells pertaining to multiple cell types of the same lineage: haematological stem cells (HSC) are the best defined example of this category. Unipotent stem cells form only one cell type, as the spermatogonial stem cells do.

A stem cell is one which is able to self-renew while generating other cells which commit to differentiation. Recent studies have elucidated a further characteristic of stem cells which seems of great physiological and pathological relevance: the ability to divide asymmetrically, giving rise to an identical cell remaining in situ and to a daughter cell which starts the differentiation process, being “committed” to a particular lineage (Knoblich 2008). This feature is more a logical necessity than an experimental

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demonstration, since it is needed to explain the continuous renewal of tissues such as the skin or the intestine epithelium. However, the alternative possibility that a finite, albeit large, number of stem cells exist after birth and provide differentiated cells for all the life of the organism by stochastic activation and commitment of both daughter cells to differentiation, has not yet been ruled out. Indeed, at least for the female germline cells, whose stem cell pool becomes depleted in the fourth-fifth decade of human female life, this alternative could hold true.

Recently, the concept of stem cells has also entered the cancer field (Lobo et al. 2007). Several seminal papers have suggested the existence of a small percentage of cells which are responsible for the initial growth, maintenance and, more importantly, re-growth of all the tumor cells after therapy (Al-Hajj et al. 2003; Bao et al. 2006; Chiba et al. 2006; Dalerba et al. 2007; Dontu et al. 2007; Fang et al. 2005; Galli et al. 2004; Ho et al. 2007; Lapidot et al. 1994; Li et al. 2007; Patrawala et al. 2006; Piccirillo et al. 2006; Ponti et al. 2005; Prince 2007; Ricci-Vitiani et al. 2007; Schatton et al. 2008; Singh et al. 2003, 2004; Szotek et al. 2006; Wang et al. 2007). In addition to being biologically relevant, this concept might be of great clinical importance, since it could explain the clinical observation that in spite of the fact that more than 99% of tumor cells can often

be destroyed by chemotherapy, many tumors are eventually able to grow again.

The experimental plan

The studies performed follow a general scheme (Fig. 1). Cells from primary tumors or cell lines are separated by FACS or other classical means in several fractions defined by surface markers or other properties, some of which are known to be shared by normal stem cells. Table 1 shows several markers used in different tumors: some are common to many histological types while others are, so far, more restricted. These fractions are then individually inoculated in immunocompromised mice and their ability to form tumors is scored. In many reports it has been shown that the ability to form tumors differs from fraction to fraction and that the populations bearing markers associated to “stemness” give rise to tumors with a frequency that is statistically different from that produced by other fractions. Some of these data are summarised in Table 2. Additional common features of the experiments are the ability of the transplanted CSCs to recapitulate the original heterogeneity of the tumors and the maintenance of their properties even after serial transplantation since CSCs from both the original and transplanted tumors

Fig. 1 Schematic representation of the xenotransplantation approach to the study of cancer stem cells. The mammary cancer is taken as example. Tumor cells are dissociated from a primary breast cancer and sorted according to the expression of surface cell markers. The various fractions are separately

injected into the immunocompromised mouse and tumor formation is followed. In secondary transplants, the cells from the transplanted tumor are again dissociated, sorted and re-injected into another immune compromised animal

Table 1 Cancer stem cell markers of histologically different human tumors^a

Tumor	Markers	Frequency (%)	References
Leukemia (AML)	CD34 ⁺ , CD38 ⁻ , Lin ⁻	0.0004 ^b	Bonnet and Dick (1997)
Breast cancer	ESA ⁺ , CD44 ⁺ , CD24 ^{-/low}	11–35	Al-Hajj et al. (2003)
	CD44 ⁺ , CD24 ⁻ , Oct-4 ⁺	10–20	Ponti et al. (2005)
Brain tumors	CD133 ⁺	5–30	Galli et al. (2004)
Colorectal cancer	CD133 ⁺	1.8–24.5	Ricci-Vitiani et al. (2007)
	CD44 ⁺ , CD166 ⁺ , ESA ⁺	1.8–9.2	Dalerba et al. (2007)
	CD133 ⁺	0.057	O'Brien et al. (2007)
Melanoma	ABCB5 ⁺	1.6–20.4	Schatton et al. (2008)
Pancreatic cancer	ESA ⁺ , CD44 ⁺ , CD24 ⁺	0.2–0.8	Li et al. (2007)
Prostate cancer	CD44 ⁺ , CD133 ⁺ (Hoechst excluding cells)	0.04–0.2	Patrawala et al. (2007)
Ovarian cancer	Side population (Hoechst excluding cells CD44 ⁺)	N.D.	Szotek et al. (2006)
Head and neck cancer	CD44 ⁺ , CK5/14 ⁺ , BMI-1 ⁺	<10	Prince (2007)

^a None of these markers are expressed exclusively by stem cells^b Frequency of the leukemia-initiating cell in peripheral blood of AML patients is 1 engraftment unit/250.000 cells

N.D., Not determined

Table 2 Tumor cell initiating potential according to selected markers

Hystological type	Cell source	Cell dose	Total number of mice with tumors (%)	
Colorectal cancer (Ricci-Vitiani et al. 2007)	Bulk	1×10^6	19/64 (29.69)	
		3×10^3	15/26 (57.69)	
	CD133 ⁺	1×10^4	19/32 (59.38)	
		CD133 [−]	5×10^3	0/10 (0)
			1×10^4	0/20 (0)
Colorectal cancer (O'Brien et al. 2007)	Bulk	1×10^4	0/8 (0)	
		5×10^4	4/10 (40)	
		1×10^5	10/10 (100)	
	CD133 ⁺	1×10^2	1/4 (25)	
		5×10^2	5/6 (83.33)	
		1×10^3	7/7 (100)	
	CD133 [−]	1×10^5	0/8 (0)	
		2.5×10^5	1/9 (11.1)	
		Colorectal cancer (Dalerba et al. 2007)	CD44 ⁺ , ESA ^{high}	2×10^2
4×10^2	16/20 (80)			
2×10^3	17/19 (89,47)			
5×10^3	4/5 (80)			
CD44 [−] , ESA ^{low}	4×10^2		0/10 (0)	
	2×10^3		1/30 (3,33)	
	8×10^3		0/5 (0)	

can give rise to tumors similar to those from which they originated.

The significance of all these findings are not completely clear. Strictly speaking, they only

demonstrate that the xenotransplantability in an immunocompromised host of human neoplasias is the property of a small number of cells, which can reproduce the heterogeneity of the tumor, while the

remaining >95% of the cells are not capable of malignant growth in a heterologous host. This property is taken as evidence that also *in vivo* and *in homine* these cells are the only ones whose replication is responsible for the growth of the tumor and, more importantly, that they are the ones which are not killed by chemotherapy, thus being responsible for the recurrence of the neoplasia in humans. This seems reasonable but has not been formally proven and there is the possibility that the xenotransplantation model underestimates the frequency of cells able to reproduce tumors (Kelly et al. 2007; Adams et al. 2007).

Reasons for which this conclusion could be too extreme include (Wicha et al. 2006) the following arguments:

- (a) Disruption of the primary tumor structure: recent studies have emphasized the fact that in primary tumors different populations interact with each other and with normal ones. The disruption of these interactions could alter the behaviour of neoplastic cells which could lose some of their malignant features.
- (b) Cell damage during the isolation of cell populations: the procedure for fraction isolation is stressful to the cell and activate intracellular mechanisms (for example: p53 activation) impairing the growth potential of the cell; in addition, the procedure often involves the use of proteolytic enzymes which could remove important molecules from the cell membrane.
- (c) The transplantation into tissue environments which are often different from the normal one could make the growth of the cells difficult.

CSC theory *sensu stricto* and *sensu lato*

A major limitation of the CSC hypothesis is however the definition of CSC. The term “cancer stem cell” has acquired broad usage in the last few years, following the appearance in the scientific and social arena of the embryonal and adult stem cells, which are of great importance for their possible role in regenerative medicine. This has lead to some confusion, since the CSC term seems now to be used with two different meanings, which have different consequences for the hypothesis itself.

According to one interpretation, which we could dub the CSC theory “*sensu stricto*” (in the strictest sense), the existence of CSCs would mean that the cells responsible for tumor growth are the neoplastic counterpart of a normal stem cell of the tissue from where the tumor originated (Fig. 2, panel A). This interpretation seems suggested by the technical approach used to identify CSCs, since most studies use markers known to be present in normal stem cells in order to isolate CSCs. This would imply that the original event(s) leading to neoplastic transformation occurred in a normal tissue-specific adult stem cells, whose deregulation is responsible for the excessive auto-reproduction and the production of daughter cells maintaining, to some extent, the ability to differentiate, explaining the heterogeneity of the tumor. The best example that this could really occur is the human chronic myeloid leukaemia (CML), in which all the hematological lineages are involved and bear the same Philadelphia chromosome marker, which is the hallmark of the disease. The original mutation(s) allow for maintenance of a complete differentiation capacity, although subsequent events could change this condition, limiting this ability to differentiate, as occurs in the so-called “blastic crisis”. Many other human leukemias could be explained by a mutation in a “*bona fide*” stem cell with loss of the ability to complete all the differentiation steps. The theory of leukemias as expansion of clones frozen at some specific stage of the maturation (Greaves and Janossy 1978) is compatible with the CSC theory “*sensu stricto*”, assuming that the mutation occurred at the level of an HSC cell in genes which are involved in “committing” cells to different lineages, leading to an imbalance in normal differentiation of the various lineages.

The “*sensu stricto*” CSC theory is obviously untrue in several human lymphoid neoplasias. Both T and B lymphoid cells rearrange their genes during their maturation, well after the formation of the committed lymphoid progenitor. In the T-cell lineage, TCR rearrangement occurs in the thymus and is known to some extent. Every rearrangement is unique, and had the mutation occurred in the HSC, cells from pre-T and T lymphoid leukemias would bear polyclonal rearrangements, since every daughter cell would rearrange its TCR in a unique way. This is not the case, since these leukemias have clonal TCR rearrangements. The same is true for B-cell neoplasias.

Fig. 2 Schematic representation of the cell targeted by transforming mutation(s) and of the possible consequences on differentiation and neoplastic cell growth. (Panel A) The mutation occurred in a true “stem cell”. This mutation does not alter the differentiation potential of the lineage(s) and the tumor is composed of cells at various differentiation stages including the terminal one. Alternatively, the mutation in the stem cell does not allow for complete differentiation and a block at various levels occurs. All the mentioned cases are compatible with the CSC theory “*sensu stricto*”. (Panel B) The mutation does not occur in a true immature stem cell.

Therefore normal differentiation can take place (at least before the neoplastic cells invades all the niches of the normal stem cells), as shown in the left lane. The mutation occurs in a more differentiated cell which maintains the ability to differentiate (central lane). Finally, the mutation in a differentiated cell does not allow for terminal differentiation, giving rise to an exaggerated growth of cells frozen at a specific differentiative stage. These are examples of the CSC “*sensu lato*” theory. These examples do not cover all the possibilities of cancer development

The most evident case are B-cell CLL and myeloma, in which monoclonal immunoglobulin are produced.

Irreversible modifications of the genome occurring at specific advanced stages of maturation are so far known only for lymphoid cells, therefore it is not possible to investigate neoplasias arising from other tissue with this approach. However, some human neoplasias, such as thyroid or prostate cancers, are of such a low-grade malignancy, which are hardly compatible with a theory of CSC *sensu stricto*. In addition, experiments in transgenic mice also suggest that mutations arising in more differentiated cells can give rise to tumors. In these models, oncogene activity can be directed to specific differentiation stages by putting them under the control of specific promoter/enhancers which are active only on differentiated cells (Hanahan 1989): tumors arising in this way clearly show that the occurrence of the transforming event in a normal stem cell is not an absolute

requirement, but that also more differentiated cells can sustain the growth of fully malignant tumors.

“*Sensu lato*” (in the broad sense) CSC theory could be defined as suggesting that the malignant growth properties of a tumor are found in only a small fraction of cells which are responsible for the growth, maintenance and recurrence of the tumor. Although these cells show some features of immature cells and are able to produce all the other (more mature) cells, being responsible for the heterogeneity of the neoplasia, their gene expression pattern (and the chromatin conformation on which it depends) do not match the one of stem cells. In this case, it might be concluded that the initiating event(s) occurred in a cell which has already completed one or more stage in the normal differentiation pathway (Fig. 2, panel B). This could elucidate the well known correlation between the degree of differentiation and the aggressiveness of the tumor, which cannot be easily

explained by mutations always occurring in the same stem cell.

This second interpretation of the CSC theory clearly is compatible for all the difficulties cited above which do not fit with the strict interpretation of the CSC theory. Indeed, a recent workshop seems to endorse this interpretation of the theory, also suggesting that more differentiated cells can eventually evolve to become a CSC (Clarke et al. 2006). However, the real innovative potential of this “*sensu lato*” theory is limited. Indeed, the existence of a more aggressive fraction in human tumors has always been recognized on experimental and clinical grounds, not least from the ability to recur when 99.99% of the tumor has disappeared. With this interpretation, the marker analysis and the inoculation in immunodepressed mice is only an additional approach to the study of the self-renewing fraction of the tumor. However, these studies could lead to a new approach to isolate and investigate these cells and test them for sensitivity to therapy, with the hope that the results obtained on these purified fractions in vitro could match the sensibility in vivo of the whole tumor. Therefore, from a practical point of view, even in this case the theory could be clinically relevant, but the conceptual novelty will be low.

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