Melanoma initiating cells: where do we stand?

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A growing body of evidence has shown over the last 15 years that tumor progression is driven by cancer cells which display stem-like properties [1,2]. According to the cancer stem cell theory, cancer stem cells (CSCs), or cancer initiating cells, are a subpopulation of cancer cells which, like physiologic stem cells, have the ability to self-renew and to divide asymmetrically, generating progenies of both differentiated cancer cells and CSCs (Figure 1). In addition, they are chemo- and radio-resistant, and play a major role in disease recurrence and metastatic spread, the two major causes of patients' mortality and morbidity. Therefore to effectively 'cure' a patient from a malignancy, CSCs need to be eradicated along with differentiated tumor cells, as the latter have been shown to de-differentiate into CSCs [3,4]. These findings have stimulated interest in the identification and characterization of CSCs in several types of malignancies and in the development of strategies to eradicate them.

Many cell surface markers, cellular activities, expression of genes and functional assays have been and are utilized to identify CSCs. Some of the most commonly-adopted

cell surface markers include CD24, CD44 and CD117, and CD133 as well as an adenosine triphosphate-binding cassette (ABC). Aldehyde dehydrogenase (ALDH) activity is the most commonly used cellular activity [5]. Stemness genes used to identify CSCs include *SOX2*, *NANOG*, *OCT4* and *KLF4*. Lastly, the *in vitro* and *in vivo* functional tests developed to identify CSC populations include colony formation, sphere formation and serial xenotransplantation in immunodeficient mice. The interpretation of the results generated by *in vitro* and *in vivo* assays suffers from the different experimental conditions used in various laboratories. They include: different antibodies which are reported to recognize the same marker, but have not been compared in their reactivity patterns with the same substrates; different cell culture conditions in terms of fetal calf serum concentration, time of cell starvation, hypoxic conditions and seeded cell density; and extent of immunodeficiency of the mice grafted with cancer cells. Nevertheless, in some of the malignancies investigated, there is an agreement on the markers used to identify CSC, such as high activity of ALDH in

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Figure 1. Bidirectional relationship between cancer stem cells and differentiated cancer cells.

Cancer stem cells can undergo asymmetric division: they can self-renew and differentiate into non-stem cancer cells (either by asymmetric division or by differentiation). Differentiated cancer cells undergo symmetric division; however, under some circumstances, they can mutate and acquire stem-like properties.

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breast cancer and in pancreatic adenocarcinoma. In contrast, in melanoma not only there is no general agreement about the markers used to identify CSCs, but there is also conflicting information about the percentage of cells that have stem-like properties in melanoma biopsies and in xenografts. In this regard, Morrison *et al.* have reported that up to 25% of nonfractionated cells obtained directly from melanoma patients have the capacity to form tumors when transplanted in Matrigel in nonobese diabetic (NOD)-scid IL2 γ^{null} (NSG) mice; this subpopulation cannot be identified with any of the stem cell markers that have been commonly used by the scientific community. Whether these conclusions reflect the use of techniques which may influence the experimental outcomes, such as severely immunodeficient mice, Matrigel as a vehicle for cell transplantation and/or cell dissociation with trypsin, remains to be determined. Enrichment of cells for CD133 and CD166 did not increase the tumorigenic potential [6]. A later study by the same group showed that cell subpopulations selected using CD271 and ABCB5 as biomarkers did not differ from subpopulations lacking these markers in their tumorigenic potential in an NSG mouse model [7].

MICs: putative cellular markers & enzymatic activities

Table 1 lists the many markers proposed for the identification of melanoma initiating cells (MICs). The first marker reported to identify MICs was CD20, a marker of mature B cells, which Fang *et al.* found to be expressed in a particularly aggressive subpopulation of melanoma cells directly harvested from patients [8]. Cells expressing CD20 had an increased clonogenic capacity *in vitro* and an increased tumorigenicity in immunodeficient mice. These results prompted Fang *et al.* to suggest targeting MICs with anti-CD20 antibodies to eradicate melanoma. This possibility was tested in a Phase I trial, in which stage IV melanoma patients, without evidence of disease after surgical treatment, were treated with the CD20-specific monoclonal antibody (mAb) rituximab. Five out of the nine enrolled patients were recurrence-free after a median follow-up of 42 months [9]. Although encouraging, these results failed to identify CD20 as a biomarker of response to the treatment, since CD20 expression by melanoma cells did not correlate with disease-free recurrence. In addition, the analysis of the role of CD20 in a xenogeneic model generated questionable results [10]. Another putative marker

Table 1. List of selected studies investigating putative melanoma initiating cell markers.

Study (year)	MIC putative marker	Positive population in primary tumor	Enzymatic digestion	Matrigel	Transplant site	Animal model	Serial xenotransplantation (number of passages)	Ref.
Fang <i>et al.</i> (2005)	CD20	1.0%	Collagenase	No	sc.	SCID	No	[8]
Monzani <i>et al.</i> (2007)	CD133 ⁺	0.2–0.8%	Collagenase	No	sc.	NOD/SCID	No	[12]
Boiko <i>et al.</i> (2010)	CD271 ⁺	2.5–41.2%	Collagenase (Blendzyme)	Yes	Intradermal	Rag ^{+/+} γC ^{-/-} , NSG	Yes (n = 1)	[14]
Civenni <i>et al.</i> (2011)	CD271 ⁺	15.0 ± 2.0%, 8.0 ± 4.0% [†]	Collagenase + dispase	Yes	sc.	Nude, NOD/SCID, NSG	Yes (n = 4)	[15]
Quintana <i>et al.</i> (2010)	CD271 ⁺	2.0–12.0%	Collagenase + trypsin	Yes/no	sc.	NSG	Yes (n = 2)	[7]
Schatton <i>et al.</i> (2008)	ABCB5	10.1 ± 2.9%	Collagenase	No	sc.	NOD/SCID	Yes (n = 1)	[16]
Quintana <i>et al.</i> (2010)	ABCB5	0.0–5.3%	Collagenase + trypsin	Yes	sc.	NSG	Yes (n = 2)	[7]
Boonyaratankornkit <i>et al.</i> (2010)	ALDH	2.0%	Collagenase + hyaluronidase	No	sc.	NOD/SCID, NSG	Yes (n = 5)	[17]
Luo <i>et al.</i> (2012)	ALDH	0.0–1.2%	Collagenase + hyaluronidase	No	Intradermal	NOD/SCID, NSG	Yes (n = 2)	[18]
Quintana <i>et al.</i> (2008)	No marker		Collagenase+trypsin	Yes	sc.	NOD/SCID, NSG	Yes (n = 2)	[6]

[†]Data for only two patients out of 19 reported.

NOD: Nonobese diabetic; NSG: NOD-scid IL2γ^{null}; sc.: Subcutaneous; SCID: Severe combined immunodeficiency.

used to identify MICs has been CD133, a hematopoietic stem cell marker that is expressed on both melanoma cell lines and melanoma tumor biopsies [11]. It has been shown that CD133⁺ cells have an enhanced capability of initiating primary tumors in NOD/SCID mice as compared with CD133⁻ cells [12]. In addition, CD133⁺ cells have been reported to be resistant to chemotherapeutic agents such as taxol [13]. Another putative biomarker of MICs is CD271⁺, also known as nerve growth factor receptor. Boiko *et al.* have demonstrated that CD271⁺ cells are more tumorigenic than CD271⁻ melanoma cells when injected in a human skin graft placed on Rag^{+/+}γC^{-/-} mice [14]. Furthermore Civenni *et al.* have demonstrated that CD271⁺ cells but not CD271⁻ cells display stemness-defining properties such as self-renewal and differentiation [15].

Other studies to identify MICs have relied on the expression of ABCB5, a detoxifying transmembrane drug efflux transporter which is a member of the ABC family. ABCB5 has been implicated in the resistance of several types of cancer cells to chemotherapeutic agents and, not surprisingly, its expression has been associated with tumor progression in melanoma. In a NOD/SCID xenograft model, ABCB5⁺ cells showed stem-like properties, such as tumorigenicity,

self-renewal and differentiation [16]. In addition, ABCB5⁺ cells were shown to have a downregulation of HLA class I antigens and melanoma antigens, and to display immunosuppressive properties [19]. These authors have also suggested a statistically significant association of CD271 and ABCB5 [20] in melanoma tumor biopsies. However, the latter findings have been questioned by Cheli *et al.*, on the basis of the results obtained with freshly established melanoma cell lines [21]. In addition, Morrison *et al.*, using the ABCB5-specific mAb produced by Frank *et al.*, were not able to isolate MICs from melanoma cell lines and surgically removed tumors [7]. Lastly, our group was not able to confirm the specificity of the ABCB5 mAb produced by Frank *et al.* [SFERRONE, UNPUBLISHED DATA]. An additional marker proposed to identify MICs, is the high activity of ALDH [22]. Human melanoma cells displaying a high ALDH activity, referred as ALDH^{bright} cells, have been shown to have an elevated tumorigenic potential in both NOD/SCID and NSG mice [17,18]. Furthermore, ALDH^{bright} cells have been reported to express NANOG and to display increased levels of SOX2 protein [23,24]. In our experience, neither ABCB5 expression nor ALDH activity alone is sufficient to identify MICs. Given the conflicting information about

the specificity of the ABCB5-specific mAb in the literature, we have produced our own mAbs. To eliminate questions about its specificity we had it tested by leading investigators in the field of ABCB5. By using this reagent we have found that ALDH^{bright} melanoma cells which express ABCB5 display the characteristics of MICs in terms of clonogenicity and tumorigenicity in immunodeficient mice [S FERRONE, MANUSCRIPT IN PREPARATION].

MICs: functional assays

As with their physiologic counterparts, the definition of CSCs is based on operational models which test their tumorigenicity in immunodeficient mice. Although these artificial experimental models provide useful information they have several limitations, caused by the influence of three potential variables on the *in vivo* tumorigenicity of MICs: preparation of cells, site of implant and mouse strain used. The enzymatic digestion used for melanoma cell preparation may affect the expression of markers utilized to identify MICs, resulting in the erroneous classification of tumor cells. A number of investigators have utilized collagenase (with or without dispase) to dissociate tumor tissue, while others, including those who question the usefulness of the commonly used MIC markers, have relied on harsher methods

of tumor dissociation, such as incubation with trypsin (Table 1). Trypsin is known to have a lytic activity on many, if not all, cell surface markers; therefore its use as a reagent in melanoma cell preparation may select subpopulations of cancer cells which do not express the CSC markers, but retain stemness properties ('false-negative' MICs). As a result, methods which rely on the expression of the removed markers could not separate stem- from non-stem cell populations, leading to the conclusion that most of the melanoma cells can initiate and propagate tumors.

The importance of establishing the right niche environment for cell growth has been demonstrated in a number of studies on physiologic and cancer stem cells. The most commonly-used model in MIC investigation is subcutaneous cell transplantation. The subcutaneous tissue, however, is mainly constituted by adipose cells, which can hardly provide the physiologic environment of the dermal-epidermal junction where melanoma tumors arise. The different vascularization and local milieu of the dermis can conceivably influence tumor engraftment and metastatic spread. In addition, a number of studies have also utilized Matrigel to facilitate tumor engraftment; however Matrigel itself provides growth factors that may promote changes in the tumor cell subpopulations. Both site of

Figure 2. Immunologic characteristics of animal model systems commonly used in melanoma research.

NK: Natural killer; NOD: Nonobese diabetic; NSG: NOD-scid IL2ry^{null}; SCID: Severe combined immunodeficiency.

injection and vehicle matrix have to be carefully evaluated when setting up a melanoma xenograft model system.

Finally, the immune environment of the mouse strain utilized to implant the identified MICs has a great relevance in selecting the cell subpopulations which can initiate and propagate the tumor. In the physiologic scenario, a melanoma undergoes a Darwinian process of immune selection, in which the less immunogenic cells have a replicative advantage. In an immunocompetent host the tumor not only changes its own phenotype, but also modulates the host immune response, and finally achieves operative tolerance. The xenograft model is by definition an artificial model in which there is no immunoediting of the host's environment and tolerance of the grafted tumor is achieved by the absence of relevant immune cell populations (Figure 2). In this context, a more immunocompromised mouse will facilitate tumor engraftment, but probably will also provide a favorable environment to non-stem cell subpopulations. It is important to consider that *bona fide* CSCs have an inexhaustible renewal capacity, while lower hierarchy tumor cells may show a limited renewal capacity; the difference between these two populations may be revealed only by serial passages in immunodeficient mice. This information should be taken into account when designing experiments to show the CSC nature of a tumor cell population (Table 1).

Conclusion & future perspective

In conclusion, the interest in MICs has led to the identification of several putative markers. The field would greatly benefit from the analysis of the relationship among the various markers and from the standardization of reagents and methods utilized to identify MICs. Only a few studies have assessed the clinical significance of biomarkers to identify and target MICs, and they have generated negative results. This line of studies is however very important to design therapeutic strategies which might cure patients with melanoma, since according to the CSC theory MICs are responsible for tumor relapse and metastatic spread.

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