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Mini-Review

Testing the cancer stem cell hypothesis in melanoma: the clinics will tell

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Abstract

Whether tumorigenic cancer stem cells (CSCs) exist in melanoma has been the focus of much controversy in recent years. A number of studies have pointed to the existence of melanoma cell sub-populations that act as CSCs and can be distinguished from other tumor cells based on specific surface marker expression or specific properties such as the capacity for extensive self-renewal. Other studies failed to identify melanoma stem cells and proposed that the potential to initiate tumors is a wide spread feature in melanoma inherent to most if not all cells of the tumor mass. As with normal stem cells, the term CSC is based on an operational definition, indicating not just a tumor-initiating cell, but also a cell with the capacity to sustain long-term tumor propagation. Therefore, the experimental set-up chosen to identify putative CSCs in melanoma is crucial: Both the method of tumor cell preparation and the procedure used to assess CSC properties *in vivo* influence the experimental outcome and hence its interpretation. In this review, we summarize our current knowledge on CSCs and the role of stem cell properties in melanoma and discuss recent findings with respect to their clinical relevance.

1. Introduction

Metastatic melanoma represents the most aggressive skin cancer, characterized by extremely poor survival. Despite decades of advances in melanoma research and drug development, the battle against this disease is still ongoing. The CSC model postulates that only a fraction of cells within a tumor has the capacity to initiate and sustain tumor growth. If correct, this model would provide a basis for more successful and advanced melanoma therapies that target specifically tumorigenic CSCs. It is believed that CSCs (sometimes also referred to as "tumor-initiating cells" or "cancer-initiating cells") share common features with corresponding tissue stem cells, namely their ability to self-renew and to give rise to hierarchically organized tissues composed of a variety of cell types. The CSC concept has recently been verified *in vivo* by three independent studies using cell fate mapping techniques in mouse models of skin, intestine and brain tumors. These studies demonstrated for the first time the existence of CSCs *in vivo* [1], [2] and [3].

Of note, however, the term CSC does not indicate that CSCs originate from the genetic deregulation of normal stem cells. As in many other cancers, the cell of origin of melanoma remains unknown, and it cannot be excluded that melanoma arise from the accumulation of genetic mutations in melanocytes acquiring stem cells features via somatic reprogramming. In addition, melanoma might derive from normal stem cells present in the skin, such as melanocyte stem cells or other adult stem cells. During development, melanocytes originate from a transient embryonic structure called the neural crest. This structure comprises cells dubbed embryonic neural crest stem cells (eNCSCs), which upon isolation can self-renew and generate a variety of neural and non-neural cells types, including melanocytes [4]. Intriguingly, cells with a very similar potential as eNCSCs have been isolated from various adult organs containing neural crest

derivatives, including skin [5] and [4]. In mice, genetic *in vivo* fate mapping showed that these cells indeed originate during embryonic development from the neural crest. Based on their potential and origin, these cells were therefore termed neural crest-derived stem cells or also adult NCSCs. Cell lineage tracing experiments further demonstrated that murine adult NCSCs are associated with the glial and melanocytic lineages in the trunk skin, while in the face they were also found in mesenchymal structures such as the dermal papilla [6], [7] and [8]. More recently, Herlyn and colleagues described a population of dermal stem cells present in the human skin and exhibiting NCSC properties [9]. Similar to adult NCSCs, these dermal stem cells express the low-affinity neurotrophin receptor p75 ($p75^{NTR}$, CD271), a marker of NCSCs, and are capable to self-renew and differentiate into mesenchymal, neuronal, and melanocytic lineages. Although genetic lineage tracing is obviously not possible in human, the similarities between murine adult NCSCs and human CD271-positive dermal stem cells suggest that these cells might indeed represent the same or overlapping cell populations, although further studies are needed to address this issue.

While our knowledge on eNCSCs is quite advanced, little is known about the biology of adult cells with NCSC properties. In particular, the physiological role of adult NCSCs *in vivo* remains to be discovered. Conceivably, however, adult NCSCs might also play a pathophysiological role, revealing their potential upon oncogenic transformation and thereby giving rise to melanoma and other tumors of neural crest origin. The idea that melanoma might derive from a cell with NCSC characteristics is consistent with histopathological findings and gene expression signatures associated with melanoma development [10]. Histologically, melanoma is a very heterogeneous tumor and, in addition to melanocytes, is often composed of cells with neuronal, glial, chondrocytic, and adipocytic features [11], [12], [13], [14], [15] and [16]. Therefore, it is plausible that melanoma-initiating cells display NCSC characteristics, sharing similar surface marker phenotypes and regulatory signalling pathways. Here, we summarize our knowledge on melanoma stem cells, markers used to identify these cells, and experimental strategies to isolate these cells and to test their tumorigenic potential.

2. Cell surface markers for the prospective identification of putative CSCs in melanoma

In 2005, Meenhard Herlyn and colleagues provided the first evidence for CSCs in human melanoma, reporting that metastatic melanoma lesions from human patients contained a cell population with stem cell-like properties [17]. In analogy to other groups, they have used *in vitro* sphere culture conditions to enrich for cells with stem cell features. Surprisingly, cells within spheres expressed the B cell marker CD20 (also known as MS4A1) [17]. The melanoma-derived spheres demonstrated not only a differentiation potential reminiscent of neural crest cells *in vitro* giving rise to melanocytes, adipocytes, osteocytes and chondrocytes, but also the ability to self-renew *in vitro* and *in vivo* (Table 1).

ABCB5 (ATP-binding cassette subfamily B member 5) belongs to the superfamily of active transmembrane transporters that act as ATP-dependent pumps to transport a variety of compounds out of the cell. ABCB5 was detected in a subpopulation of primary melanocytes (11%), cultured melanoma cells (3%), human melanoma tissue, and its expression correlates with melanoma progression [18], [19] and [20]. Furthermore, ABCB5 expression within human melanoma was observed in non-pigmented, undifferentiated cells. The ABCB5-positive population was able to sustain tumor growth when xenotransplanted into NOD/SCID mice, to serially self-renew *in vivo* giving rise to secondary tumors, and to re-establish the cellular heterogeneity characteristic of melanoma (Table 1). Strikingly, selective elimination of the ABCB5 population using a monoclonal antibody against ABCB5 in NUDE mice inhibited melanoma growth [20]. Recently, Frank and colleagues reported the identification of circulating tumor cells (CTCs) in peripheral blood mononuclear cell fractions of melanoma patients characterized by expression of ABCB5 [21]. When prospectively isolated and xenotransplanted into NOD/SCID/Il2$^{-/-}$ mice, ABCB5-positive CTCs generated metastases demonstrating that CTCs have tumorigenic potential and the ability to become invasive [21]. Interestingly, the frequency of melanoma CTCs found in the bloodstream of patients correlated with tumor progression and patient survival. ABCB5 expression on melanoma circulating cells might therefore serve as a biomarker for melanoma diagnosis and prognosis.

CD133 (Prominin-1) was identified as a marker expressed on hematopoietic stem cells, acute myeloid leukemia cells, neural stem cells and CSCs in brain tumors [22], [23] and [24]. In human melanoma biopsies, CD133 is expressed in 17% of nevi, 39% of primary melanomas and 46% of metastatic melanoma [25]. As reported by Monzani et al., CD133 expressing cells comprised less than 1 % of total melanoma cells in 7 different human metastatic melanoma, but when xenotransplanted into NOD/SCID mice, CD133-positive cells generated tumors [26] (Table 1). Furthermore, CD133-expressing cells isolated from xenografts reverted to nearly 100% positivity upon culturing. These experiments suggest that CSCs might either display plasticity, having the ability to switch the surface phenotype depending on the environment, or that changing microenvironments act selectively on CD133-positive cells.

The low-affinity neurotrophin receptor p75 (p75^{NTR}, CD271) is often used as a marker for the prospective isolation of NCSCs from tissues and for ES-based derivation of NCSCs [27], [28], [29] and [30]. Supporting the concept that melanoma stem cells might display NCSC features, CD271 has recently been identified as a surface marker for tumor-initiating cells in melanoma [31] and [16] (Table 1). As shown by Boiko and colleagues, CD271 (p75^{NTR})-expressing melanoma cells had a higher tumor-initiation capacity than CD271-negative cells and, moreover, gave rise to metastases upon transplantation, unlike CD271-negative cells [31]. As many other stem cells, CSCs are defined by their capacity to self-renew. To identify *bona fide* CSCs, serial transplantation in immunodeficient mice has, therefore, to be performed for several passages *in vivo*. Otherwise, the ability to induce tumor formation could reflect the engraftment capacity of tumor cells rather than their self-renewal *in vivo*. To address this issue, our laboratory performed serial transplantation *in vivo* using

fully immunocompromized mice and demonstrated that the CD271-positive cell fraction displayed the ability to self-renew and to repeatedly establish heterogeneous tumors even after six passages *in vivo*. In contrast, tumors generated from CD271-negative cells exhausted with time and could only be propagated *in vivo* for maximally four passages [16]. Furthermore, CD271-expressing melanoma cells were multipotent, generating tumors consisting of cells with melanocytic, neuronal, glial, and smooth muscle features, thereby consistently phenocopying the parental tumor from which they have been isolated. Thus, CD271-positive melanoma cells have a potential reminiscent of NCSCs and, by definition, are melanoma stem cells.

3. Failure to detect melanoma-initiating cells exhibiting specific surface marker expression: a matter of tumor cell preparation?

In contrast to the above mentioned studies, Morrison and colleagues challenged the concept of CSCs in melanoma using a modified assay for melanoma initiation [32] and [33]. Among other surface markers, Quintana et al. tested the capacity of ABCB5 and CD271 to discriminate between tumorigenic and non-tumorigenic cells. Although long-term tumor propagation was not assessed in these reports, there was no correlation between expression of specific surface markers and tumor initiation in a xenotransplantation assay. Another study by Bosenberg and colleagues also proposed that $p75^{NTR}$ (CD271) could not be used as a surface marker for melanoma-initiating cells in three different mouse models [34].

In an attempt to resolve the discrepancy between these papers and studies describing specific CSC populations in melanoma [34], [32], [31] and [20] we compared the methods of enzymatic digestion used for tumor cell preparation: while our study and those by Boiko et al. and Schatton et al. used collagenase or collagenase/dispase to dissociate the tumor tissue [31], [16] and [20], the Morrison group used a modification of this protocol and applied a trypsin digestion step, in addition to collagenase. Likewise, Bosenberg and colleagues applied the protocol of Quintana et al., including an additional, more extensive step of trypsin digestion [32] and [34]. Obviously, using proteases in tissue dissociation is a crucial step that can affect the yield of cells selected by surface markers. Indeed, as shown in Civenni et al. [16], the addition of trypsin during tumor cell preparation reduces the number of CD271-positive cells detectable by FACS. For example, in a particular patient sample, 14.6% of all cells were scored as CD271 positive when trypsin was omitted, while including trypsin lowered the yield of marker-positive cells to only 1.4% in the same sample. A similar difference in detectable expression was also observed for another previously described marker of melanoma-initiating cells - ABCB5 [16].

Thus, trypsin and possibly other enzymes used for tumor cell dissociation leads to partial shedding of surface markers and, therefore, to the contamination of supposedly marker-negative fractions by cells that will re-express the shedded marker, e.g. after transplantation (Fig. 1). Therefore, the chosen tumor dissociation protocol might have compromised studies that failed to detect CSCs in melanoma based on selective expression of specific surface markers. In

general it has to be considered that the destruction of a cell's surface proteome by harsh digestion procedures likely influences its interaction with surrounding tissue and hence its capacity to initiate tumors upon transplantation in vivo.

4. **Critical points to be considered when transplanting human melanoma cells into mouse models**

Does Matrigel provide an appropriate niche for tumor initiation and growth?

To assess whether a tumor cell has CSC potential, the cell's ability to establish, maintain, and propagate heterogeneous tumors has to be determined *in vivo*. For human tumor cells, such assays involve xenotransplantaion into an appropriate mouse model, which by nature of the experiment poses several problems regarding tumor-stroma and tumor-immune system interactions. To promote tumor cell survival and local growth, many groups have transplanted tumor cells embedded in Matrigel, which represents a solubilized mixture of basal membrane components, enriched in laminin, collagen IV, heparan sulfate proteoglycans. Several types of Matrigel can be used for transplantation assays, and it is likely that the type of Matrigel influences the outcome of the engrafting. Matrigel Matrix for example contains soluble growth factors such as TGFβ, EGF, IGF, and FGF, in addition to the abovementioned components. It is increasingly recognized that extracellular matrix (ECM) components might influence cellular signaling and subsequently the process of differentiation of cells (reviewed in [35]). In a recent study published by the group of Visvader and colleagues, the application of Matrigel in an *in vivo* mammary repopulating assays resulted in the promotion of a cell population with limited self-renewal capacity, distinct from *bona fide* mammary stem cells [36]. These results indicate that the addition of Matrigel in transplantation assays should be carefully evaluated. Does Matrigel resemble the natural niche for CSCs? Does it act selectively on tumor cell sub-populations? Does it promote changes in tumor cells that influence tumor take? The answers to these questions might be difficult to obtain, but are obviously relevant for a better understanding of how and to what extent endogenous, unmanipulated CSCs are involved in tumorigenesis in the patient.

Finding the right niche: Orthotopic vs heterotopic transplantation

Unavoidably, the isolation of cells from human tumors leads to the destruction of the endogenous microenvironment that might act as a niche to control growth and survival of cancer cells. Orthotopic transplantation (transplantation of a cell into its normal location in the body) provides the most accurate cellular environment by supplying a new niche for survival and proliferation. Intriguingly, for cancers proven to follow the CSCs paradigm, such as breast cancers, brain tumors, and leukemia [24], [37], [38], and [39], researchers have spent great efforts to establish orthotopic transplantation assays. In the case of breast cancer, an assay was established, in which human breast cancer cells were propagated in the mouse mammary fat pad [37]. Furthermore, in brain tumors such as glioblastoma and medulloblastoma the presence of CSCs was tested by means of injection into the brains of NOD/SCID mice [24] and [38]. Finally, in the

field of leukemia, where leukemic cells were assessed for their hierarchical organization, scientists used bone marrow transplantations [39] and [40]. In contrast to these examples, most of the studies on CSCs in melanoma rely on subcutaneous injections [17],[34],[32] and [16]. Skin is composed of two different layers, the epidermis and dermis. The subcutaneous layer, i.e. the layer beneath the skin, mostly consists of fat. However, adipocytes hardly provide the natural environment for melanoma cells, which in human originate in the epidermal-dermal junction or dermis. Therefore, normal survival, proliferation, and differentiation cues might not be provided, and signals that in a patient might never reach tumor-initiating cells might aberrantly influence the behavior of transformed cells. Transplanting melanoma cells subcutaneously could therefore lead to artifacts due to the lack of the CSC-supporting niche. Intriguingly, while most studies including those by Quintana et al. relied on subcutaneous injections of melanoma cells, Boiko et al. performed cell transplantations into the intradermal environment or even used full thickness human skin grafts to demonstrate the presence of CD271-positive melanoma-initiating cells [32] and [31] (Table 1). Given that initial steps of melanomagenesis occur within the skin and not subcutaneously, these different experimental procedures should be taken into consideration when comparing and interpreting reports on melanoma initiation.

Modulating metastasis formation in experimental models of melanoma

Similarly, the method and site of injection conceivably influences the capacity of transplanted melanoma cells to engage in metastasis. Melanoma treatment becomes difficult and survival is significantly reduced when a patient has metastatic disease (stage III and stage IV). The danger of melanoma is that a relatively small primary tumor (stage III is characterized by the size of melanoma less than 1.0 mm and not more than 4 mm) is often already associated with the presence of metastases, indicating that the main threat of this cancer is its ability to spread to distant organs. One of the important points in modeling this disease is, therefore, to develop a system, which represents more advanced stages of melanoma pathogenesis. Unfortunately, with the exception of the study by Boiko et al. [31], most of the papers on CSCs in melanoma failed to demonstrate the presence of melanoma metastases after transplantation of melanoma cells into immunocompromized mice. One of the main reasons for this might again be the injection site: While injection into a subcutaneous environment leads to growth of an encapsulated tumor mass, intradermal injections rather provide the environment where melanoma cells can invade blood vessels and form metastases in distant organs [31]. These issues are relevant for the field, as CSCs have been functionally associated with the capacity to adopt mesenchymal features, to migrate, and to form metastases [31]. Thus, model systems are required, in which the relation between ‘stemness’ and tissue invasiveness of human melanoma can be assessed in a humanized environment mimicking the natural site of tumor formation [31] and [41].

The influence of the immune system on CSCs and tumor take

Although orthotopic tumor cell injections and/or the use of a humanized environment for tumor cell transplantation allow proper tumor cell-stroma interactions, one component normally present in the patient has not been properly recapitulated in any xenotransplantation assay so far: The immune system, which appears to have a key influence on tumor initiation and growth [42]. While initial studies on CSCs in melanoma used partially immunocompromized mice [17] and [20], Quintana et al. demonstrated a broad potential for tumor initiation using a fully immunodeficient NOD/SCID/Il2$^{-/-}$ (NSG) mice lacking T cells, B cells, and NK cells [32] (Fig. 2). Weisman and colleagues also used a mouse model (Rag2$^{-/-}$ $\gamma c^{-/-}$ mice) lacking T cells, B cells, and NK cells to identify CD271 as a marker for melanoma-initiating cells [31]. In Civenni et al [16], we compared tumor initiation and maintenance in partially immunocompromized NOD/SCID and Nude mice vs. fully immunocompromized NSG mice. Intriguingly, while CD271-negative human melanoma cells failed to initiate tumors in NOD/SCID and Nude mice, unlike CD271-positive cells, both cell fractions were able to initiate tumors in NSG mice. However, even in the fully immunocompromized NSG mouse model, presence of CD271-positive cells was required for long-term tumor propagation, as shown by serial transplantation. These data indicate two important issues to be considered: a) Tumor initiation might depend on distinct mechanisms or involve distinct cell populations than long-term tumor maintenance and propagation. By consequence, a tumor-initiating cell is not necessarily equivalent to an actual CSC. In fact, it is likely that more cells in a tumor have the capacity to initiate tumor growth than there are cells able to sustain long-term tumor propagation and spreading. b) CSCs might possess specific capacities for immune system evasion or modulation. Indeed, when NK cells were depleted in NOD/SCID mice, this model system behaved like NSG mice and allowed tumor initiation also by CD271-negative melanoma cells [16]. Along these lines, ABCB5-expressing melanoma cells have recently been shown to suppress T cell activation, pointing to a specific capacity for immune system evasion by CSCs [43].

Of course, immunogenic responses associated with xenotransplantation are presumably different from anti-tumorigenic immune responses observed in a patient fighting a tumor. But the data reveal the importance of immune system components that cannot be neglected when studying the relevance of CSCs for tumor formation. To provide a humanized immune system in mouse models, NSG mice have been engrafted with human CD34-positive hematopoietic progenitor cells, a system which leads to a partial reconstitution of human immune system components in immunodeficient mice [44] and [45]. Such models might offer a more physiological environment for human melanoma cells than mice lacking an immune system. However, to successfully implement this protocol, it would be necessary to obtain hematopoietic progenitors and tumor cells from the same patient. Ultimately, how the immune system interacts with CSCs and influences processes of melanoma formation can only be addressed in an appropriate genetic mouse model.

5. Fate versus potential

It is important to note that transplantation experiments always address the potential of cancer cells rather than their role and fate under the physiological conditions that the cells encounter in the patient. But how important is it for us to know the tumorigenic potential of a single human cancer cell in a mouse body? How to estimate the relevance of a cancer cell's observed potential for future therapies? These are non-trivial questions, because a cell's intrinsic capacity to differentiate and proliferate might be influenced by the experimental approach to an extent never occurring in the patient. A cancer cell might be subject to reprogramming events by its exposure to Matrigel, *in vitro* culture conditions, or an ectopic environment. For instance, while we observed dynamic CD271 expression in human melanoma cells under certain conditions in cell culture, absence of CD271-expression was a surprisingly stable trait in tumors obtained by subcutaneous injection of CD271-negative cells into NSG mice (Kiowski and Sommer, unpublished; [16]). In colon cancer, differentiated tumor cells can acquire stem cell features by the exposure to the hepatocyte growth factor (HGF) released from myofibroblasts [46]. Likewise, hypoxia in glioblastoma promotes a switch towards a stem cell-like phenotype in the non-stem cell population, as shown by increased self-renewal and regulation of the CD133 stem cell marker [47], [48] and [49].

Although the above-mentioned experiments unambiguously demonstrate the plasticity and dynamic behavior of cancer cells *in vitro*, it is unclear whether similar processes of de- and re-differentiation occur *in vivo*. Dynamic changes in surface proteome expression and intrinsic properties could occur *in vivo*, for instance, as a consequence of an epithelial-to-mesenchymal transition (EMT) [50] or in response to the changing environment encountered by an invasive cell. Such changes likely involve epigenetic regulation on the level of e.g. DNA methylation or histone modifications. Intriguingly, expression of the chromatin-modifying enzyme Jarid1B is dynamically regulated on melanoma cells [51]. Presence of Jarid1B appears to correlate with the potential of a melanoma cell to act as a CSC, although it is unclear whether this is associated with a specific cell surface marker profile. In accordance with this, Jarid1B knock-down, and thus interference with a specific 'epigenetic status', counteracted the cells' capacity to maintain continuous tumor growth. Thus, a CSC potential might well be dynamically regulated. The question that interests the most, however, is under which circumstances this potential is realized in the patient.

In this context, the importance of recently described *in vivo* lineage tracing experiments in mouse models of skin, intestinal and brain cancers cannot be underestimated [1], [3] and [2]. In these elegant studies, researchers traced single tumor cells in their naïve environment and provided for the first time solid evidence that selected cells within a growing tumor act as CSCs that establish and maintain tumor cell hierarchies. Although CSCs remain to be discovered *in vivo* in melanoma and other cancers, these findings indicate that specific subpopulations of tumorigenic cells might indeed represent suitable targets for therapy. Moreover, the studies emphasize the significance of

assessing CSCs and their properties in *in vivo* mouse models in an undisturbed environment.

6. Targeting cancer stem cells in the clinics

The CSC hypothesis predicts that efficient therapies should eliminate CSCs, in addition to destroying the bulk tumor. Based on our knowledge on CSCs, two potential strategies emerge to target these cells in the clinics: The aim of the first approach is to suppress 'stemness' by targeting specific signaling pathways or regulatory transcriptional networks utilized by CSCs for their self-renewal and survival. A second approach consists in eliminating CSCs based on the expression of unique surface antigens. For instance, the frequency of CD271-positive cells in human melanoma samples correlates with higher metastatic potential and worse prognosis, suggesting that this cell type represents a possible target for therapy [16]. For both strategies, however, there is a risk of side effects due to potential lack of specificity. Compounds targeting CSCs should optimally be tested in genetic mouse models, in which primary tumors and metastases arise *de novo* rather than upon transplantation. Such models allow the assessment of drug efficacies without manipulating (and hence potentially changing) tumor cell properties. Moreover, in such mice, target specificity can be preclinically tested, taking into consideration undesired effects on other cell populations including normal stem cells.

A successful example of the first approach, namely targeting signaling pathways regulating "stemness" of CSCs, is our recent study revealing that reducing expression levels of the NCSC transcription factor Sox10 completely abolishes melanoma formation both in a genetic mouse model (*Tyr::Nras^{Q61K} INK4a$^{-/-}$* mice; [52]) and upon xenotransplantation of human melanoma cells [10] (Fig. 3). Sox10 was previously shown to play a crucial role in the maintenance of stem cell characteristics in NCSCs. Interfering with Sox10 activity in NCSCs results in the acquisition of mesectodermal features, reduced proliferation, and increased apoptosis [53], [54] and [55]. Similarly, reduced Sox10 levels in *Tyr::Nras^{Q61K} INK4a$^{-/-}$* mice heterozygous for a *Sox10* mutation counteracted oncogene-induced proliferation of melanoma cells. In cell culture, knockdown of SOX10 in human melanoma cell lines suppressed neural crest cell "stemness" and led to similar effects as previously observed upon Sox10 loss-of-function in NCSCs, such as a "switch" of identity from a melanocytic to a mesodectodermal program, decreased cell cycle progression, and reduced survival. In addition, the percentage of cells expressing CD271 was strongly reduced upon SOX10 knockdown *in vitro*, suggesting that SOX10 controls CSC numbers in melanoma [10]. Taken together, these data strongly indicate that blocking stem cell properties in melanoma exerts a drastic effect on tumor initiation and growth, leading to complete tumor ablation. In conclusion, drugs potentially interfering with signaling pathways or molecules controlling "stemness" of NCSCs might prevent tumor formation and expansion.

Most recently, several groups provided evidence that the alternative therapeutical approach mentioned above, namely to target CSCs based on

specific expression of surface markers, might actually be beneficial for melanoma patients [56] and [57]. Abken and colleagues achieved a targeted disruption of a CD20-positive cell population via engineering T cells with a chimeric antigen receptor (CAR), thereby redirecting specificity of cytotoxic T cells towards CD20-expressing cells [56]. Of note, the CD20-positive cell population represented less than 2% of all melanoma cells. When melanoma cells from five different human biopsies were xenotransplanted into immunodeficient mice lacking T, B, and NK cells, tumors were eradicated by application of engineered T cells. Extending CD20 experiments into the clinics, a study published by Wagner and colleagues presented the results of a pilot experiment, where they have applied rituximab (anti-CD20 antibody) treatment in a group of nine patients with metastatic melanoma at clinical stage IV [57]. After a treatment period of 2 years, and a subsequent median follow-up time of 42 months, two thirds of the patients included in this study were recurrence-free, without any signs of major side effects or toxicity [57]. CD20 is also expressed on the surface of B cells, and rituximab therapy is used to treat B-cell lymphoma [58]. Therefore, it is not surprising that anti-CD20 treatment of melanoma patients resulted not only in depletion of CD20-positive melanoma cells, but also in consistent loss of B lymphocytes in the peripheral blood. B cells are commonly activated in cancer patients and melanoma samples often contain CD20-positive lymphocytes [59] and [60], although the role of these cells in tumors still remains to be clarified. While in some cancers, depletion of B cells by using CD20 antibodies enhances tumor growth, as is the case for B16 melanoma [61], CD20 therapy may also result in the protection against tumor [62]. Furthermore, retuximab is known for its antiproliferative effect, at least partially via induction of cell cycle kinase inhibitors [63], [64] and [65]. Whatever the mechanism of rituximab's action is in melanoma, the first reports using an anti-CD20 therapy are promising. Moreover, these studies suggest that elimination of a restricted melanoma cell fraction, characterized by specific cell surface marker expression, might represent a valid therapeutical strategy. Ultimately, there will be no single therapy for the elimination of melanoma cells, and a successful therapy will most likely require the combination of several drugs in order to efficiently eradicate all cancer cells.

7. Final conclusions and remarks

Given the discrepancies and open questions existing in the field, it is valid to further test and challenge the CSC hypothesis in melanoma. Most importantly, it will be critical to assess the relevance of the CSC concept in melanoma using appropriate model systems. Given the importance of the immune system in tumorigenesis, it can be questioned whether immunocompromized mice are suitable models to study mechanisms of human cancer progression. In any case, the method of tumor cell preparation and the site of tumor cell transplantation has to be carefully chosen, and potentially artificial changes caused by tumor cell manipulation have to be taken into account. It becomes more and more apparent that the most determining proof of the CSC hypothesis is a successful targeted therapy directed against surface epitopes expressed on CSCs or against specific

stem cell properties exhibited by a selected melanoma cell fraction. Hence, the CSC theory is awaiting its final and most relevant test in the clinics.

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Figure Legends

Fig. 1. Choosing the appropriate method for tumor cell preparation to test the CSC theory. When melanoma tissue is dissociated by enzymatic digestion with collagenase or a combination of dispase and collagenase, the expression of markers on the surface of a cell is better preserved than upon treatment of melanoma cells with trypsin. Using trypsin and other harsh dissociation procedures can result in reduced numbers of marker-positive cells detectable by FACS. This can subsequently lead to the presence of false-negative cells within the "negative fraction". These cells can re-express surface markers and initiate tumorigenesis upon transplantation.

Fig. 2. Melanoma initiation and the immune system. The capacity of CD271-expressing cells to give rise to melanoma upon xenotransplantation depends on the immune system status of the recipient mouse. After tissue dissociation and FACS isolation, melanoma cells are injected into immunodeficient mice. When CD271-negative cells are transplanted into partially immunocompromized mice (Nude mice lack T cells, while NOD/SCID mice lack T and B cells), there is no melanoma formation. In contrast, fully immunocompromized NSG mice lacking T, B, and NK cells, provide a permissive environment for CD271-negative cells. Tumors generated by CD271-negative cells, however, appear to have a different cellular composition than tumors derived from CD271-positive cells and cannot be propagated extensively.

Fig. 3. Interfering with NCSC properties abolishes tumor formation in a genetic mouse model of melanoma and in human melanoma cells. a) Mice carrying mutations also found in human patients develop congenital nevi and subsequent melanoma. Oncogenic $NRas^{Q61K}$ in this model leads to upregulation of the NCSC transcription factor Sox10. Deletion of one *Sox10* allele is sufficient to counteract $NRas^{Q61K}$-dependent nevus formation and tumorigenesis. b) SOX10 expression in human melanoma cells promotes self-renewal and allows acquisition of melanocytic traits. As in normal NCSCs, suppression of SOX10 activity leads to decreased self-renewal, increased cell death, preferential acquisition of a non-melanocytic fate and loss of p75/CD271, a marker for tumor-initiating cells.

Figure(s)
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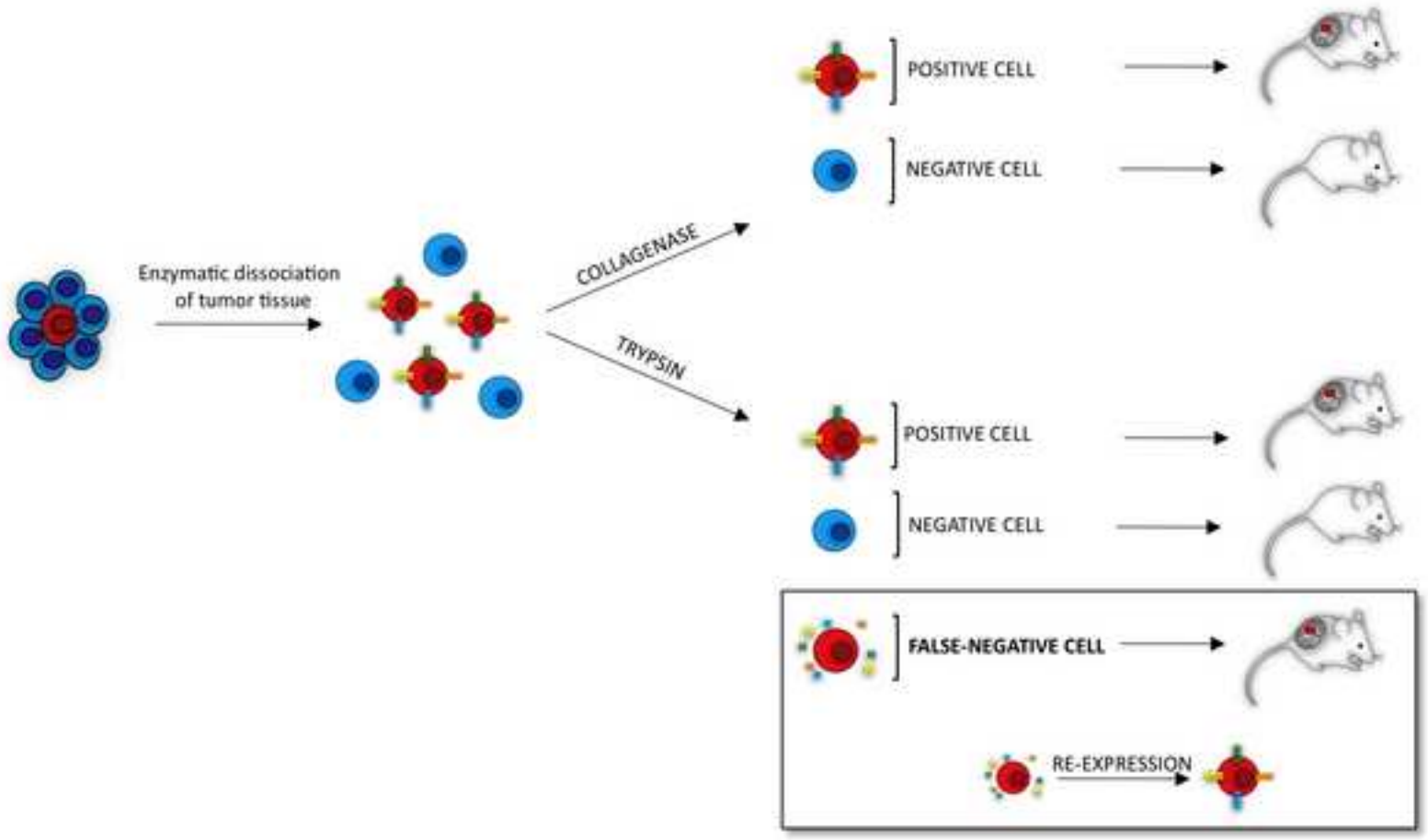

Figure 1.

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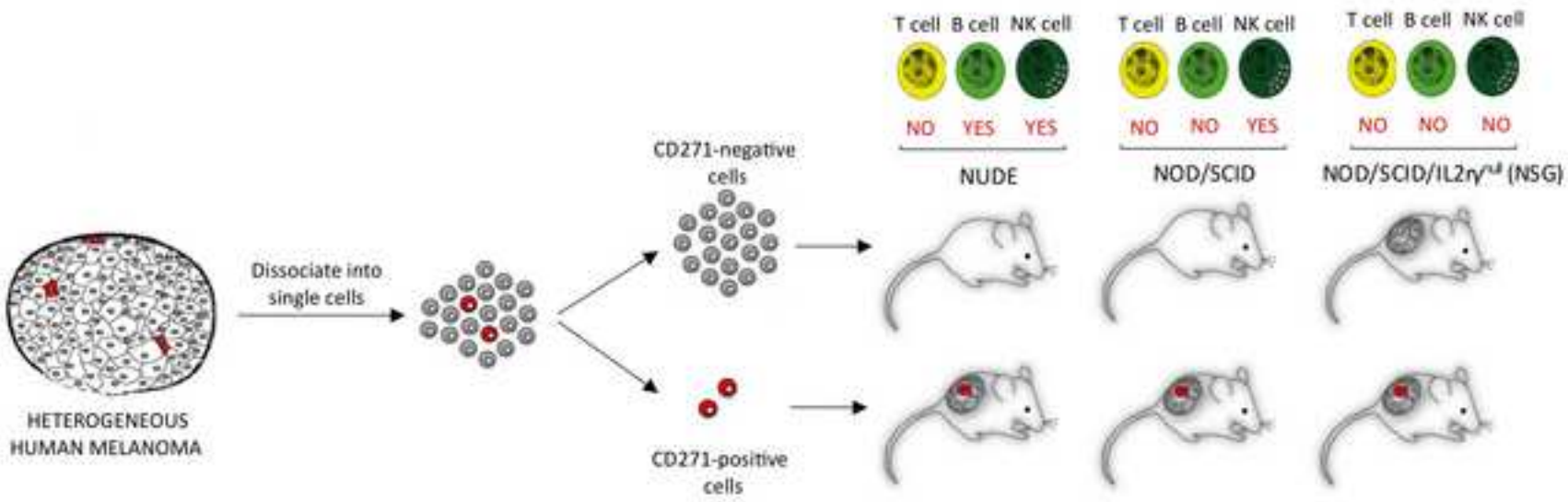

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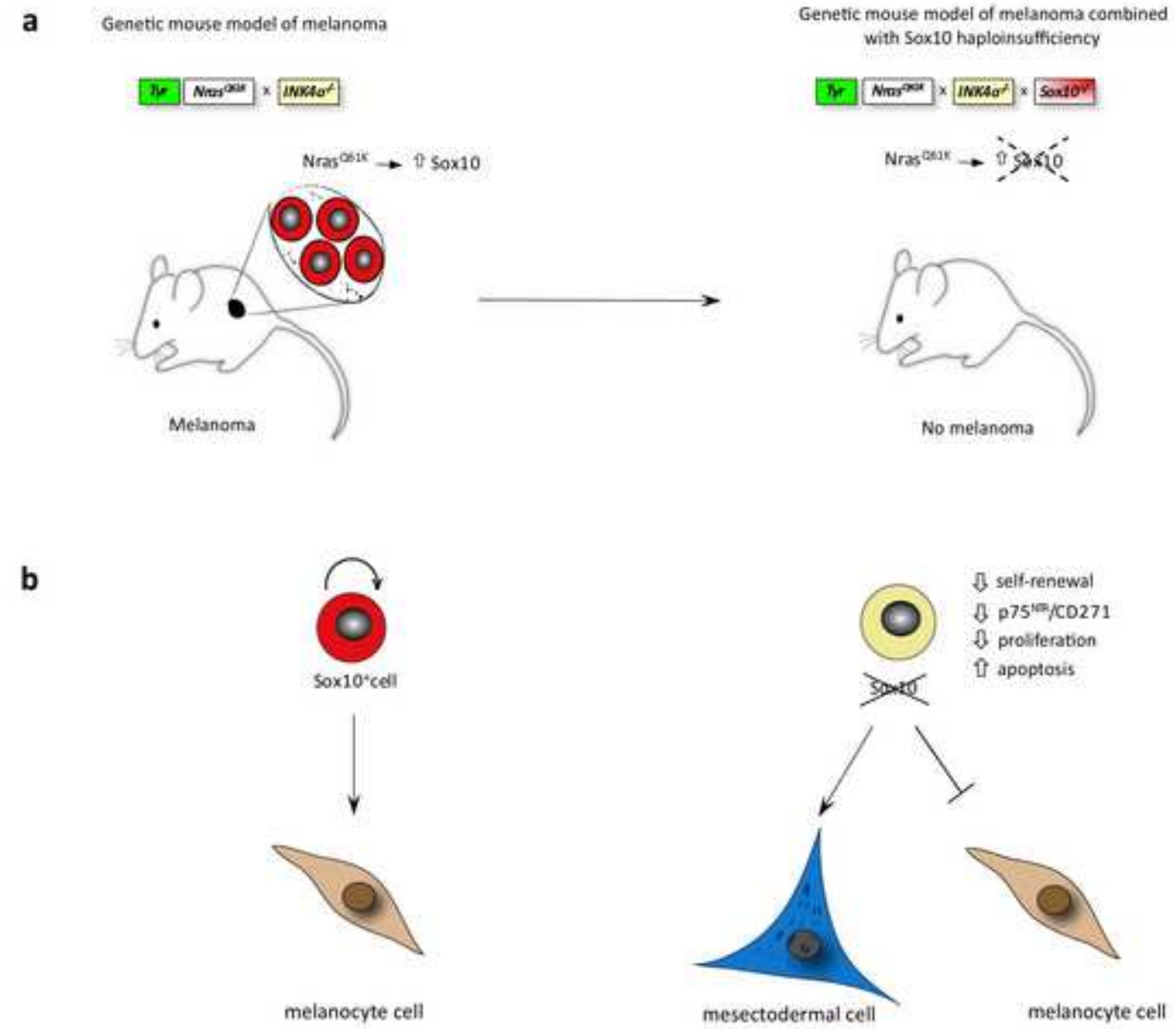

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Table 1 Surface markers expressed by cancer stem cells in melanoma

Marker	Patient's material	Enzymatic digestion	Details of transplantation experiments				Reference
			Matrigel	Injection side	Mouse model	self-renewal/ passage number *In vivo*	
CD20	metastases	collagenase	no	s.c.	SCID	n.d.	[17]
ABCB5	metastases	collagenase	no	s.c.	NOD/SCID	n.d.	[20]
CD133	metastases	collagenase	no	s.c.	NOD/SCID	n.d.	[26]
CD271 *Boiko et al.*	primary and metastases	collagenase	yes	intradermal	Rag2-/- Yc-/- NOD/SCID/IL2-/-	n=1	[31]
CD271 *Civenni et al.*	metastases	collagenase	yes	s.c.	Nude NOD/SCID NOD/SCID/IL2-/-	> 4	[16]
NO MARKER *Quintana et al.*	primary and metastases	collagenase **trypsin**	yes	s.c.	Nude NOD/SCID NOD/SCID/IL2-/-	n=2	[32,33]