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Cellular and molecular biology of cancer stem cells in melanoma: possible therapeutic implications

Short title: cancer stem cells and melanoma

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ABSTRACT

Malignant melanoma is a tumor characterized by a very high level of heterogeneity, responsible for its malignant behavior and ability to escape from standard therapies. In this review we highlight the molecular and biological features of the subpopulation of cancer stem cells (CSCs), well known to be characterized by self-renewal properties, deeply involved in triggering the processes of tumor generation, metastasis, progression and drug resistance. From the molecular point of view, melanoma CSCs are identified and characterized by the expression of stemness markers, such as surface markers, ATP-binding cassette (ABC) transporters, embryonic stem cells and intracellular markers. These cells are endowed with different functional features. In particular, they play pivotal roles in the processes of tumor dissemination, epithelial-to-mesenchymal transition (EMT) and angiogenesis, mediated by specific intracellular signaling pathways; moreover, they are characterized by a unique metabolic reprogramming. As reported for other types of tumors, the CSCs subpopulation in melanoma is also characterized by a low immunogenic profile as well as by the ability to escape the immune system, through the expression of a negative modulation of T cell functions and the secretion of immunosuppressive factors. These biological features allow melanoma CSCs to escape standard treatments, thus being deeply involved in tumor relapse. Targeting the CSCs subpopulation is now considered an attractive treatment strategy; in particular, combination treatments, based on both CSCs-targeting and standard drugs, will likely increase the therapeutic options for melanoma patients. The characterization of CSCs in liquid biopsies from single patients will pave the way towards precision medicine.

Key words: Melanoma, cancer stem cells (CSCs), CSC markers, cancer progression, drug resistance

1. Introduction

Cutaneous melanoma represents one of the most aggressive forms of skin cancer: despite it accounts for less than 5% of all skin cancers, it is the cause of the majority (75%) of the skin cancer-related deaths [1]. The high level of heterogeneity of melanomas is responsible for the lack of pan-melanoma therapies (inter-tumor heterogeneity), thus resulting in the increasingly urgent need of the patient-focused precision medicine. On the other side, the intra-tumor heterogeneity is related to the existence of distinct subsets of cancer cells in the tumor bulk, thus leading to drug resistance [2]. Encouraging therapeutic strategies have been developed in recent years, but the 10-years survival for metastatic melanoma is still less than 10%. 'Targeted therapies', such as vemurafenib (a $BRAF^{V600E}$ inhibitor), were developed in order to specifically target mutated proteins, with a response rate around 50%. However, most patients relapse within few months [3]. Similarly, the 'immune-checkpoint inhibitors', such as nivolumab (a PD-1 inhibitor) and ipilimumab (a CTLA-4 inhibitor), were developed to improve the endogenous antitumor immune response. However, they are effective on only a subset of patients, with a primary resistance higher than 50% and the development of acquired resistance in initially responders [4].

Two models have been used to explain melanoma heterogeneity. In the stochastic model, all tumor cells are equivalent and characterized by subclonal differences that result from differential genetic and epigenetic alterations during the development of cancer. In the hierarchical model, a small, slow-cycling subpopulation of cells called 'cancer stem cells' (CSCs) resides at the top of the hierarchy [5, 6]. The classical, idealized notion holds that CSCs are characterized by their limited number and their ability for self-renewal through asymmetric cell division, thus behaving as normal stem cells: on one side, a differentiated and rapidly proliferating daughter cell is generated, so that the tumor mass is consecutively formed, but on the other side, a new stem cell is renewed, providing the source for the continuous repopulation of the tumor bulk [7]. The two models are not mutually exclusive, because the progeny of differentiated cells could undergo to clonal evolution [5]. If the existence of a stemness state appears not to be questioned, the hierarchical organization of the tumor is a matter of debate. Recent evidences suggest that every cancer cell gains the potential to shift to a stem cell state, and this acquired phenotype could be reverted again toward a non-stem state, due to the high level of plasticity of the tumor cell [5]. This appears to be true especially for melanomas, where the existence of CSCs is well documented, as well as their role in tumor relapse after administration of chemotherapy and targeted therapies [8].

This review will discuss the standard definition and characterization of melanoma stem cells, pointing to the skepticism related to their biomarkers. Their functional features and phenotypic plasticity will be presented, together with the consequent therapeutic implications.

2. Melanoma CSCs biomarkers

Accordingly to the classical definition of CSCs, *i.e.*, a rare subpopulation of cells endowed of self-renewal capacity and tumor-generating potential, the definitive demonstration of their existence resides in their ability to give rise to a tumor mass when serially transplanted at limited dilutions *in vivo*: a plethora of studies demonstrated the high tumorigenicity of CSCs in different types of cancers [9-13]. In order to reach such proof-of-concept, different methods have been developed and are currently exploited to isolate CSCs from patient-derived tumors or cancer cell lines *in vitro*. In particular, it has been demonstrated that a CSCs-enriched culture can be obtained through culturing the bulk of tumor cells in a stem cell-specific medium, thus allowing their selection and growth as

spherical colonies in suspension [12, 14]. A finest isolation can be obtained through fluorescence-activated cell sorting (FACS), exploiting the expression of CSC-specific markers, leading to a pure population of CSCs [12, 15-17]. In this context, specific patterns of biomarker that identify CSCs have been defined for some tumors (*i.e.*, $CD44^+/CD24^{-/low}$ for breast cancer, $CD44^+/CD24^+/ESA^+/CD133^+$ for pancreatic cancer, $CD133^+$ for glioma). Differently, the existence of a unique and specific biomarker signature for melanoma stem cells is still controversial, because of the high degree of plasticity of this tumor, and possibly of the existence of multiple mechanisms that lead to melanoma progression [18].

In the last decade, different biomarkers have been proposed for the identification of melanoma stem cells, but most of them were found to be expressed on different subclones, rather than restricted to the highly tumorigenic population of cells. An elegant study was performed by Quintana *et al*., where the tumorigenic potential of patient-derived tumor cells was analyzed in NOD/SCID $IL2R\gamma^{null}$ mice, dependently on the differential expression of 22 putative melanoma stem cells markers. The authors demonstrated that none of them was able to significantly identify the stem cell subpopulation, since cells lacking those markers retain the tumorigenic ability [5].

The markers commonly used are related to a melanocyte undifferentiation state (CD271, CD133), to mechanisms of drug resistance (ATP-binding cassette transporters, ABCs), or to metabolic features (ALDH activity) [19-21]. Other studies revealed the expression of embryonic stem cell markers, such as Sox2, Klf4, Oct4 and Nanog, as a common feature of CSCs derived from different tumors [22].

2.1. Surface markers

Among the surface markers commonly and more consistently used to identify melanoma stem cells, CD271 (also known as p75) represents the most controversial. Since the low affinity receptor for the neurotrophin NGF (nerve growth factor) is mainly expressed in the neural crest during embryonic development, the expression of CD271 on melanoma cells could reflect a certain degree of undifferentiation, a typical feature of CSCs, beside their ability of multilineage differentiation under appropriate conditions. However, a very high variability is observed in the expression of this marker: it has been calculated that it can mark a subpopulation of melanoma cells ranging from 2.5% to 40% [20], and conflicting results were obtained about the reliability of this marker [5, 23-26]. However, other recent studies highlight how CD271 could be functionally involved in the aggressive features of melanoma cells [27-30] and in melanoma progression and prognosis [31, 32]. CD133 (Prominin-1) is a membrane protein originally identified in murine neuroepithelial stem cells and associated with primitive human cell differentiation, but its role in cancer cells is still unclear. It seems to be related to pathways of tumor progression and aggressiveness (epithelial-to-mesenchymal transition, EMT; Wnt signaling pathway) [33] and, as such, it has been associated with CSCs in different tumors [34]. However, conflicting results have been reported regarding its role in defining the CSC population in melanoma tumors [21, 35, 36].

2.2. Markers associated with drug-resistance

The ATP-binding cassette (ABC) transporters are known to mediate multidrug resistance through drug efflux in cancer cells, a phenomenon usually associated with CSCs [37]. In particular, ABCB5 was found to be a marker of melanoma stem cells, due to the higher tumorigenic ability of $ABCB5^+$

cells, to the correlation with tumor progression in melanoma patients and to the co-expression with other putative melanoma stem cell markers such as CD133 [38-41]. Similar results have been obtained for ABCG2 [42, 43], another ABC transporter associated with stemness in different tumors such as pancreatic cancer, melanoma and breast cancer. The identification of CSCs through ABCG2 is achieved by the expression of the transporter itself or by the analysis of its functionality: ABCG2 is able to favor the efflux of fluorescent dyes such as rhodamine 123, so that the cells expressing the transporter result negatively stained, and visualized on a flow cytometry plot as a "Side Population" adjacent to the $ABCG2^-$ cells [37]. Moreover, it has been demonstrated that the population of ABCG2-expressing cells is endowed of higher autofluorescence compared to the $ABCG2^-$ population, due to the accumulation of riboflavin in intracellular vesicles, in pancreatic cancer and in melanoma, so that the autofluorescence level itself could be exploited as a CSC marker [26, 44-46].

2.3. Intracellular markers

CSC intracellular markers comprise enzymes such as aldehyde dehydrogenases (ALDH), and transcription factors such as Sox2 and Klf4.

ALDHs are key enzymes involved in detoxification, converting aldehydes in their respective carboxylic acids. ALDH isozyme 1 (ALDH1) have been found to be overactivated in CSCs of different types of cancer, including melanoma, where the isoforms 1 (ALDH1A1) and 3 (ALDH1A3) are correlated with CSC functions [47, 48]. It has been demonstrated that retinoic acid (RA), produced through the conversion of retinaldehyde, can lead to the expression of genes involved in CSC functions, mediated by its binding to the nuclear receptors RAR (retinoic acid receptor) and RXR (retinoic X receptor) [47]. Methods for the identification of CSCs on the basis of their ALDH activity include Aldefluor® assay: BODIPY-aminoacetaldheyde (BAAA) is a fluorescent substrate that diffuse into viable cells and is converted to BOPDIPY-aminoacetate (BAA) by ALDH. BAA is negatively charged and retained inside the cell: the brightest signal ($ALDH^{br}$) is detectable by flow cytometry and is representative of the higher ALDH activity.

The embryonic transcription factors Sox2 and Klf4 are closely associated with CSC functions, reflecting their similarity with normal stem cells. They are considered as pluripotency markers, and their overexpression in melanoma cells drives the reprogramming toward a cancer stem cell phenotype, in terms of dedifferentiation, induction of cell proliferation, inhibition of apoptosis, tumor initiation and drug resistance [49-51].

It is noteworthy that other putative markers, that could be generalized to different types of cancer, have been proposed for melanoma stem cells. However, they are more related to aggressiveness and adaptive/plasticity features of the whole cancer cell population (*i.e.*, metabolic switch, EMT) rather than to specific CSC features such as undifferentiation or drug resistance.

3. Melanoma CSCs and tumor progression

Tumor progression is often accompanied by the ability of cancer cells to give rise to metastases, responsible for the majority of cancer associated deaths. According to the 'seed and soil' hypothesis [52], metastasis is a multi-step process in which a heterogeneous group of cells derived from the primary tumor mass (seed) acquires the ability to: escape from the original cancer tissue, spread into the surrounding tissue by invading the extracellular matrix, enter (intravasation) and travel through

the bloodstream (now called circulating tumor cells, CTCs) from which they finally exit (extravasation) and colonize distant sites (soil). To this purpose, it must be underlined that most of melanoma metastases involve lymph nodes and lungs [53].

Before disseminating, primary tumor cells undergo a phenotypic conversion characterized by the acquisition of EMT features. This process is characterized by peculiar changes of the expression levels of cell-matrix or cell-cell adhesion molecules (*i.e.*, integrins, cadherins, EpCAM, cytokeratins) [54]. Once reached the secondary site, disseminated cancer cells must undergo a reversal of the EMT toward a MET (mesenchymal-to-epithelial transition) phenotype in order to give rise to a manifest metastasis; at the metastasis level, CTCs also enter a dormancy state likely associated with the quiescent characteristic of CSCs [8, 55].

The presence of CTCs in liquid biopsies, such as blood, of cancer patients plays a key role in tumor diagnosis and, therefore, in the prevention of cancer progression [56-58].

During the last years, growing evidence from the literature has strongly demonstrated that CSCs are deeply involved in the growth and dissemination of tumors, including melanoma [8, 58-61]. This evidence is mainly based on the presence of cells expressing stemness markers both in the circulation and at the metastatic sites. First, it has been shown that a multi-marker analysis can increase the isolation of CTCs from the blood of melanoma patients, although a high heterogeneity exists among these cells in terms of specific marker expression; importantly, CTCs with different markers expression differentially respond to standard treatments [62].

As discussed above, the presence of surface markers (CD271, CD133), of markers of drug resistance (ABCB5, ABCG2) and of intracellular markers (ALDH isozymes) was widely shown to be associated with increased tumorigenic properties of melanoma cells as well as with a high incidence of melanoma metastases and resistance to standard targeted therapies (*i.e.*, vemurafenib) [8, 27, 32, 63-66].

Knockdown of stemness markers in melanoma cells suppresses their metastatic ability *in vitro* and *in vivo* (nude mice bearing melanoma xenografts) and potentiates the anti-proliferative and anti-metastatic activity of chemotherapeutic drugs [40, 67]. In line with these observations, Gray *et al*. demonstrated that the presence of cells expressing stemness markers in the blood of patients treated with standard targeted therapies is prognostic of a shorter progression-free survival [68].

As stated above, the process of EMT is a cell-biological program that plays a key role in tumor progession. Recent evidence strongly supports that two different EMT programs are activated in cancer progression. According to the classical theory, during the transition from the epithelial to the mesenchymal phenotype, neoplastic cells acquire specific mesenchymal features that allow them to loose their cell-matrix/cell-cell adhesion properties and to acquire migratory and invasive properties [69, 70], leading to the dissemination to distant sites and the initiation of macrometastases. On the other hand, in the last years it has become increasingly clear that the EMT is associated with a second crucial program based on the acquisition, by a subpopulation of the bulk of tumor cells, of the classical morphological, biological and functional CSC-like traits, such as stem cells superficial markers and self-renewal capacity, thus leading to the generation of CSCs [17, 71-74]. Thus, EMT is responsible not only for the acquisition of mesenchymal features by carcinoma cells but also for the de-differentiation of tumor cells into cells with CSCs traits.

It is now established that both EMT programs are triggered by different signals from the tumor microenvironment. Specifically, in cancer cells, biological factors secreted by tumor-associated stromal cells play a key role in inducing the expression of transcription factors that direct the EMT

programs (EMT-TFs) through activation of different intracellular signaling pathways [17]. Interestingly, cancer cells were shown to acquire CSC traits according to the specific EMT-TFs that are activated by these signals [17, 75]. In particular, the transcription factors Slug, Snail, Zeb, Twist, Sox, MITF (Melanogenesis-associated Transcription Factor) and ESRP1 (Epithelial Splicing Regulatory Protein1) were reported to be involved in the acquisition of CSC traits by melanoma cells. Specifically, Twist1 and Zeb1 (but not Twist2 and Zeb2) act as promoters of the acquisition of a CSC phenotype in melanoma cells, while the tumor levels of ESRP1 inversely correlate with the EMT transition in these cells [74-77].

In line with these observations, Zeb1 expression positively correlates with the expression levels of the CSCs markers CD133 and CD44 in murine melanoma cells (B16F10); on the other hand, silencing of this EMT-TF reduces the metastatic potential of melanoma stem cells. Moreover, by binding to the GLI2 transcription factor, Zeb1 suppresses the expression of the epithelial marker E-cadherin in both murine and human melanoma cells [78, 79]. Moreover, silencing of the oncosuppressor Zeb2 significantly increases the expression of Zeb1 together with that of proteins related to the mesenchymal traits and metastatic behavior of melanoma cells, such as vimentin and fibronectin [80].

In addition to these transcription factors, IGF-1 (insulin-like growth factor-1) expression has been shown to correlate with EMT transition and stemness traits in different cancer cells. IGF-1 is produced by both melanoma cells and melanoma microenvironment. Silencing of IGF-1 in murine B16F10 melanoma cells was found to decrease the expression levels of Zeb1, mesenchymal markers and stem cells markers (CD133, Sox2, Oct3/4): this effect was accompanied by a decrease in functional stemness markers (sphere formation, ALDH1 activity) [81]. Moreover, IGFBP5 (Insulin-like Growth Factor Binding Protein 5) was reported to decrease the expression levels of stem cell markers in human melanoma cells (CD133, Sox2, Oct4) [82].

Taken together, these data strongly support that: 1) CSCs are critical for the process of melanoma growth, dissemination and metastasis, and 2) cells with morphological, biological and functional traits of CSCs can be generated during the EMT transition through the de-differentiation of a subpopulation of the bulk of melanoma cells (**Figure 1**).

It is now well known that melanoma progression can also be associated with the so called 'mesenchymal-to-ameboid' transition (MAT). During this process, melanoma cells acquire specific stemness markers together with an increased metastatic potential; this process seems to be strictly related to the expression of EphA2 (ephrin type-A receptor 2, a member of the Eph family of receptor tyrosine kinases) and RacN17 (a dominant-negative form of Rac1) [83].

4. Melanoma CSCs and angiogenesis

The process of angiogenesis (*i.e.*, the formation of new blood vessels) is a hallmark of the growth and progression of tumors, including melanoma [84]. Blood vessels provide oxygen and nutrients to tumor cells and, at the same time, facilitate their dissemination to distant sites in order to give rise to metastases. Different factors critically involved in the formation/maintenance of angiogenesis were shown to be expressed in melanomas, such as vascular endothelial growth factor (VEGF), particularly the isoforms $VEGF_{165}$ and $VEGF_{121}$, VEGF receptors (VEGFR), basic fibroblast growth factor (bFGF) and its receptors, platelet derived growth factor (PDGF) receptors, interleukins, matrix metalloproteinases, urokinase plasminogen activator, as reviewed by Jour and

coworkers [85]. Moreover, highly aggressive melanomas were reported to be characterized by the acquisition of an endothelial phenotype by the tumor cells themselves, enabling their participation in new vessel formation (*i.e.*, vascular or vasculogenic mimicry).

Melanoma cells with stem cell-like traits, particularly located at the margin of the tumor (*i.e.*, melanoma initiating cells), express and secrete in the microenvironment several factors associated with angiogenesis. In these cells, Lee and coworkers reported that $\alpha_5\beta_1$ integrin, through its downstream target ERK, triggers angiogenesis and neovascularization by activating the STAT and HIF1α pathways [86]. Moreover, melanoma cells expressing the CD133 and ABCG2 stem cells markers were shown to overexpress proangiogenic proteins, such as VEGF and its receptor VEGFR-2, Tie2 and angiopoietin [42]. In line with these observations, it has been recently reported that melanoma stem cells positive for CD133 can induce neoangiogenesis, through the Notch1/MAPK signaling pathway and the subsequent increased expression of VEGF and matrix metalloproteinases MMP-2/-9 [87].

Interestingly, CSCs (including melanoma stem cells) are characterized by a high level of differentiation plasticity that makes them able to trans-differentiate, thus acquiring an endothelial phenotype and inducing vessels formation. Based on this property, it is now accepted that these cells can directly contribute to the 'angiogenic switch' by forming an alternative microvascular circulation that allows tumor dissemination and metastasis. This process in known as 'vascular or vasculogenic mimicry' (VM) and is independent of endothelial cell-related angiogenesis [8, 88-90]. Melanoma CSCs positive for ABCB5 express specific endothelial and proangiogenic factors as well as VE-cadherin, Tie2, VEGF and its receptors, specific markers of VM [91]. In addition, $CD133^+$ melanoma CSCs, shown to express proangiogenic factors, were found to trans-differentiate into endothelial-like cells and to be endowed with tube formation ability, the typical feature of vascular endothelial cells [87]. Similarly, $CD271^+$ melanoma CSCs were reported to be associated with VM, through activation of the VEGR receptor/PKC signaling pathway [92, 93]. Syndecan-1, a cell surface heparan sulphate (HS) and chondroitin sulphate (CS) proteoglycan, has been shown to be deeply involved in the VM process of melanoma stem-like cells [94]. Melanoma cells made resistant to DNA-alkylating agents and expressing high levels of ABC transporters acquire an endothelial phenotype and trigger the VM process [95]. These results were further supported by data from *in vivo* studies. In particular, Zimmerer and coworkers, using *in vivo* fluorescence microscopy, demonstrated that $CD133^+$ melanoma D10 cells, xenografted into nude mice, are able to trigger a significant angiogenesis process [96]. Very recently, VM induced by CSCs has been reported to be an interesting biological target of anticancer compounds (*i.e.*, the natural phytochemical lupeol) in melanoma cells, both *in vitro* and *in vivo* [97].

The classical angiogenesis process, together with the vasculogenic mimicry associated with cells displaying stemness traits, are recognized as the key events allowing intravasation of tumor cells and their dissemination to distant sites. Interestingly, in addition to the intravascular dissemination, a different mechanism has been recently proposed to play a key role in the spread of tumor cells, including melanoma cells. This process, known as 'angiotropism', is based on the ability of cancer cells to migrate moving along the abluminal vascular surface (extravascular vasation migratory metastasis, EVMM) without intravasation. In this way, tumor cells migrate in a pericytic-like manner ('pericytic mimicry') along the surface of blood vessels that represent a biological track for their long 'trip' towards distant metastatic sites [98, 99]. Interestingly, Lugassy and coworkers demonstrated that angiotropic melanoma cells moving along the abluminal vascular surface express

markers of stemness and resistance to therapy, such as the Platelet-Derived Growth Factor β (PDGFB) and its receptor [98].

The involvement of melanoma CSCs in tumor angigenesis is summarized in **Figure 1**.

Further studies are needed to confirm the involvement of melanoma CSCs in these mechanisms.

5. Immunogenic properties of melanoma CSCs

It has been demonstrated that CSCs are endowed of immunogenic features that lead to immune escape. The concept of tumor immunosurveillance arose in early 1900, when Paul Ehrlich and subsequently Lewis Thomas postulated that immune cells could recognize cancer cells and prevent tumor growth [100]. Supporting evidence is represented by the negative correlation existing between immunocompetence and cancer incidence: immunosuppressive states (*i.e.*, following HIV infections and organ transplantations) have been closely associated with an increased risk of cancer development, independently of the specific type of tumor [101]. Immune-escaping events could bring cancer cells to evade the elimination mechanisms exerted by the host's immune system. As long as the balance between evasion and elimination keeps the tumor in a dormancy state, tumor outgrowth is prevented. Additional escaping mechanisms, immune exhaustion, and the consequent expansion of evading clones lead to tumor initiation and progression [102, 103].

Immune cells can be activated against cancer cells due to the expression of tumor associated antigens (TAAs), that have been recognized and inversely correlated with the expression of stemness markers. TAAs can be classified into two categories:

1. Differentiation antigens are usually expressed on normal cells, but are overexpressed by cancer cells, irrespectively of their lineage-specificity. MART-1 (Melanoma Antigen Recognized by T cells), tyrosinase, gp100 are examples of melanoma-associated antigens linked to the melanocytic lineage. On the other hand, cancer-testis antigens such as NY-ESO1, MAGE-A3, MAGE-A4 are typically restricted to germ cells and placenta but their expression has been linked to melanoma.
2. Neoantigens are restricted to tumor cells, because they originate from mutation events that generate new epitopes, such as the BCR-ABL fusion protein in leukemia.

Melanoma is generally recognized as a high immunogenic tumor, and this means that it can easily elicit antitumor immune responses and it is characterized by an important immune infiltrate involving innate cells in early phases and adaptive cells in late phases. However, the immune system is not effective in fighting tumor growth. This could be due to the existence of a subpopulation of cells in the tumor bulk endowed of low-immunogenic profile and/or of immunosuppressive abilities. These cells may be positively selected based on their immune-privileged state and favor tumor progression [102-104].

CSCs show a low-immunogenic profile, since they are characterized by the downregulation of TAAs: it has been demonstrated that $ABCB5^+$ melanoma cells do not express the immunogenic differentiation antigen MART-1 at significant levels [105], as well as they do not express cancer testis antigens [106]; similarly, $CD271^+$ melanoma cells do not show the expression of both differentiation and cancer testis antigens [107, 108]. However, conflicting results do exist, since other studies demonstrated that $CD133^+$ melanoma cells are able to induce a significant T cell response due to the overexpression of the NY-ESO1 antigen [109] and that melanoma stem cell-based vaccination is able to elicit effective antitumor immune responses in murine melanoma models [110, 111].

The ability to escape the immune system could be achieved by defective antigen processing/presentation, despite the presence of immunogenic antigens. It has been demonstrated that melanoma stem cells show a 'defective' expression of molecules related to antigen presentation such as MCH class I molecules (Major Histocompatibility Complex-I) [105, 106, 112]. Even if antigens are presented correctly, in order to permit the full T cell activation, a costimulatory signal is necessary. On the contrary, T cell downregulation is achieved by a negative costimulation: melanoma stem cells can thus express molecules involved in the negative modulation of T cell activation, such as PD-L1, PD-1 and CD86, escaping the antitumor immune response [111, 113-115]. Similarly, a protumor environment is promoted by the secretion of immunosuppressive molecules by cancer cells, or by the recruitment of protumor immune cells.

Cancer cells may directly induce T-, B- and NKs cell death (*i.e.* through FasL secretion). T cell inhibition is also favored by the secretion of selected cytokines such as IL4, IL10, TGFβ: it has been demonstrated that $ABCB5^+$ melanoma cells are characterized by the upregulation of TGFβ-related proteins [38]. Moreover, cancer cells can selectively recruit immune cells endowed of tumor-promoting abilities, especially favoring the expansion of cells belonging to the myelocytic lineage (myeloid-derived suppressor cells, granulocytes and macrophages), and their infiltration within the tumor mass [116]. The recruitment of T cells endowed of immunosuppressive properties such as regulatory T cells (T regs) and T helper cells (Th17) represent an additional mechanism by which melanoma cells can take advantage in order to shape the immune microenvironment toward a protumo setting. Whether the recruitment of these cells is related to melanoma stem cells rather than to a highly plastic subpopulation of cells is not clear yet, but intricate interconnections between melanoma cells and immune cells have been documented, together with the recruitment of specific protumor immune cell subsets and their role in resistance to immunotherapies [117-119].

The immunogenic properties of melanoma CSCs are shown in **Figure 2**.

6. Melanoma CSCs and metabolic reprogramming

It is now well accepted that metabolic reprogramming represents a hallmark of neoplastic transformation in most types of cancer, including melanoma [120-122]. Cancer cells need high amounts of energy as well as of building blocks for the synthesis of new cellular components to support their growth and survival. In particular, cancer cells display increased activity of the glycolysis pathways, also in the presence of oxygen, with formation of pyruvate that is further converted into lactate. This phenomenon, accompanied by high levels of glucose uptake, was first described by Otto Warburg and is now referred to as the 'Warburg effect' [123]. On the other hand, in contrast to previous theories, it is now accepted that cancer cells can also possess functional mitochondria with increased oxidative phosphorylation (OXPHOS) with elevated expression/activity of the respiratory chain proteins and ATP production, accompanied by high oxygen consumption [124, 125]. Another aspect of the metabolic reprogramming in cancer cells is the high rate of fatty acid synthesis and of glutaminolysis, leading to the conversion of glutamine into glutamate to fuel the TCA cycle (tricarboxylic acid cycle) and into intermediates necessary for aminoacids and nucleotides synthesis [126, 127]. These pathways of metabolic rewiring were reported also in melanoma cells in which they were shown to be linked to the specific genetic mutations, based on the characteristic heterogeneity of this tumor [127-131].

CSCs need a high amount of energy for their functions, such as proliferation and evasion from the antitumor activity of chemotherapy; based on this observation, metabolic rewiring has been proposed as a hallmark also of CSC functions [125, 132, 133]. According to some reports, CSC metabolism is characterized by a high glycolytic profile, together with increased production of lactate; this allows CSCs to obtain ATP for their high energy demands as well as different metabolites necessary for the synthesis of novel molecules (aminoacids, lipids, nucleotides), thus supporting their survival and propagation. Stem cells from some tumor types (breast, hepatocellular carcinoma) were reported to express high levels of the glucose transporter GLUT1 as well as of the glycolytic enzymes together with low levels of enzymes involved in the gluconeogenic processes. On the other hand, increasing experimental evidence demonstrates that different cancer cells (ovarian, lung, pancreatic, prostate cancers) preferentially rely on mitochondrial OXPHOS to produce enough levels of ATP for their self-renewal and survival [134-139]. This increased OXPHOS activity was shown to be associated with increased mitochondrial mass (biogenesis) through increased expression of PGC1α [140, 141]. Taken together, these observations suggest that the dependence of CSCs on the glycolytic pathway *vs.* the OXPHOS metabolism may be related to the specific types of tumor cells.

Alterations of lipid metabolic pathways (*i.e.*, increased intracellular lipid levels) was also reported to be associated with stemness features in tumor cells and to provide them the adequate amount of energy and metabolic intermediates necessary for their growth/survival [139, 142, 143]. For instance, increased activity of fatty acid synthase (Fasn), the key enzyme in the process of *de novo* lipid synthesis, as well as of enzymes involved in the mevalonate pathway (essential for cholesterol synthesis) were observed in different types of CSCs [144, 145]. On the other hand, CSCs were shown to rely on fatty acid oxydation (FAO) to obtain sufficient amounts of acetyl-CoA and NADH to fuel the subsequent production of ATP [146].

It is known that CSCs can obtain lipids from their microenvironment. Fatty acids can enter into CSCs, through the binding to their specific CD36 transporter expressed on their membranes, where they are broken down by β-oxidation to produce ATP [147]. In agreement with these findings, CSCs expressing the CD36 receptor and enzymes involved in lipid metabolism, were found to be characterized by increased fatty acids (*i.e.*, palmitic acid) uptake together with increased self-renewal/proliferation and metastatic behavior; the presence of CD36 positive cells is now considered a poor prognosis for different types of tumors [148].

In CSCs, lipids (fatty acids and cholesteryl esters) are stored into organelles known as 'lipid droplets' (LD), originating either from the Golgi or the endoplasmic reticulum membranes [144]; a positive correlation has been reported between lipid droplets content and stemness traits in CSCs from different types of cancers (colorectal, breast, ovarian); moreover, being released from cancer cells, the amount of lipid droplets in patients liquid biopsies (*i.e.*, blood) is now considered an effective marker of tumor aggressiveness [149].

CSCs also rely on glutamine (Glu) metabolism to sustain their energy formation and self-renewal. Extracellular glutamine is transported into the cells by its specific transporter ASCT2 (SLC1A5) and then undergoes different metabolic pathways, providing both carbon and nitrogen for the synthesis of novel aminoacids, nucleotides and lipids [143, 150]. For instance, Glu is metabolized into glutamate which, in turn, can be converted in α-ketoglutarate to fuel the TCA cycle and subsequent ATP production. The Glu-aspartate-oxaloacetate metabolic pathway was also reported to be activated in CSCs [143, 151].

High expression levels of ASCT2 were found to be associated with stemness traits in cancer cells, while the knockdown of this receptor reduces the proportion of CSCs in tumors, such as colon cancer. An altered glutamine metabolism was observed in colon CSCs resistant to the activity of antitumor compounds, such as metformin; on the other hand, inhibition of glutamine metabolism restored the sensitivity of the stem cells to the drug [152]. Both glutamine synthetase and glutaminase 1 levels were reported to be higher in cancer stem cells when compared with the non-stem cancer cells counterpart [153, 154].

A metabolic reprogramming was reported also for melanoma CSCs. We have previously demonstrated that melanoma cells escaping the antitumor activity of vemurafenib (the selective mutant-BRAF inhibitor) are characterized by stem cells features, such as the ability to form melanospheres when cultured in appropriate conditions [26, 45, 46, 155]. Melanoma cells that survive to the antitumor activity of BRAF and MEK inhibitors were shown to display an increased expression/activity of PGC1α, the factor well known to be responsible for increased mitochondrial biogenesis and subsequent mitochondrial OXPHOS activity [156-158]. Prolonged inhibition of BRAF was also reported to be associated with increased mitochondrial fusion (associated with decreased mitochondrial fission) and oxydative phosphorylation [158, 159]. Moreover, BRAF and MEK inhibitors are known to trigger the endoplasmic reticulum (ER) stress pathways leading to Ca^{2+} release from ER and its subsequent entry into mitochondria where it activates the enzymes specifically involved in the OXPHOS mechanism [158, 160]. Thus, according to these studies, cells resistant to BRAF and MEK inhibitors display enhanced mitochondrial biogenesis and fusion together with an increased ER-mitochondria Ca^{2+} axis; altogether, these mechanisms are responsible for an increased mitochondrial respiration in these cells. In line with these data, it was shown that melanoma cells resistant to vemurafenib display high OXPHOS associated with increased ROS production [128, 160]. Based on these observations, Sotgia and coworkers recently proposed the clinical utility of a 'mitochondrial based oncology platform' to specifically target cancer cell cells in different tumors, including melanoma [141].

On the other hand, by means of a metabolomic analysis, melanoma cells expressing the stem cell marker ABCB5 were shown to display a high activity of the glycolytic pathway, together with increased membrane phospholipid metabolism and glutaminolysis [161]. Moreover, Luo and coworkers [162] reported that mitochondrial phosphoenolpyruvate carboxykinase 2 (PKC2) is downregulated in melanoma stem cells, leading to citrate release from the mitochondria and subsequent activation of the oxalacetate-malate-pyruvate (increased glycolysis) and acetyl-CoA.fatty acid (fatty acids synthesis) pathways, associated with decreased mitochondrial OXPHOS. The same authors also reported that cytosolic PKC1 is upregulated in melanoma stem cells and this is linked to glucose consumption and activation of the glycolytic pathways [163]. A reduced mitochondrial activity, mediated by the β3-adrenoreceptor, associated with increased glycolysis in melanoma cells was also reported by Calvani and coworkers [164].

Taken together, these observations strongly support that the specific involvement of the glycolytic *vs.* the mitochondrial pathways in the metabolic reprogramming of melanoma stem cells is still a matter of debate.

Melanoma stem cells also display an altered lipid metabolism. First of all, melanoma cells with a high lipid content were reported to possess a high metastatic behavior [165]. Melanoma initiating cells were shown to express the membrane receptor CD36, responsible for fatty acid uptake from the tumor microenvironment, and to obtain energy (ATP) by means of the lipid β-oxidation

pathways to support their self-renewal and metastatic properties [148]. $CD133^+$ melanoma cells were demonstrated to have a high lipid content, specifically triglycerides and cholesteryl esters, together with accumulation of LD and increased synthesis of lipogenic genes, such as SREBP-1 (sterol regulatory element-binding protein-1) and PPAR-γ (peroxisome proliferator-activated receptor-γ) [166]. These metabolic changes were found to be associated with a reduced autophagic axis [167].

CSCs are also characterized by high levels of monounsaturated fatty acids, produced by the key enzyme SCD1 (stearoyl-CoA desaturase 1). SCD1 was reported to be overexpressed in melanoma stem cells. Specifically, in melanoma cells resistant to MAPK inhibitors, the increased levels of SCD1 was associated with an upregulation of the Hippo pathway, through the activation of the nuclear adaptor factors YAP/TAZ known to be involved in the biological features of CSCs; inhibition of SCD1 reduced the percentage of MAPK-resistant stem cells by downregulating this pathway[168].

Finally, the data so far available on the role of the glutamine metabolism in the biological features of melanoma stem cells are still very scanty and further studies are needed to definitively clarify this issue [131, 169, 170].

Taken together, the results here summarized suggest that metabolic alterations in cancer stem cells might represent a novel interesting therapeutic target for most aggressive melanomas.

7. Intracellular signaling pathways in melanoma CSCs

As discussed above, CSCs are deeply involved in melanoma development and progression, EMT and angiogenesis/vasculogenic mimicry. Based on these biological properties, CSCs act as a key player in the development of drug resistance [8, 17, 171]; thus, the elucidation of the molecular pathways involved in melanoma CSCs protumorigenic activity might help increase the identification of novel molecular targets of new effective therapeutic strategies.

Different signaling pathways have been so far reported to be involved in melanoma CSC biological functions, such as TGFβ, Notch, Wnt/β-catenin, Hedghog (HH) [8, 171-174]; these pathways trigger the expression of EMT-TFs, responsible for the EMT phase and acquisition of stemness traits by cancer cells.

TGFβ (transforming growth factor beta) is known to be involved in the mechanisms of cell growth and differentiation and to play a key role in EMT and stemness in different types of cancers [175-177]. The binding of TGFβ to its receptors (TGFβ receptors I and II) induces their dimerization, leading to the phosphorylation of Smad2 and 3 and their association with Smad4; the transcription factors Smad2/3-Smad4 complexes translocate into the nucleus where they regulate the expression of genes involved in tumor growth and progression. TGF β also interacts with different signaling pathways, such as p38 MAPK kinase, ERK, PI3K and Wnt/β catenin [178-180] and has been shown to be the most important paracrine factor produced by stroma cells in the cancer microenvironment [179, 181, 182] where it acts as a tumor-promoting factor [181]. TGFβ also triggers the switching from a non-stem to a cancer stem cell condition [183].

In line with these observations, Lv and coworkers demonstrated that TGFβ secreted by mesenchymal stem cells induces B16 mouse melanoma cells metastatic behavior and this activity is mediated by the transcription factor Snail [184]. TGFβ was reported to induce EMT in melanoma cells accompanied by a decreased expression of MITF (a melanocyte differentiation marker) and an

increased expression of N-cadherin; TGF β treatment was also found to stimulate melanoma cells migratory and invasive behavior through activation of PDGF and its downstream signaling pathways (PI3K/Akt/PKB) [185]. Cantelli and coworkers reported that TGFβ and its downstream functional axis Smad2 and CITED1 (the Smad 2 adaptor) promote melanoma cell ameboid migration and attachment to endothelial cells, markers of cancer cell aggressiveness independent of EMT [186].

Tumor associated macrophages (specifically the M2 subtype) sustain the proliferation and the stemness behavior of melanoma initiating cells and this effect is mediated by macrophage-secreted TGFβ [187]. Moreover, Nodal, a member of the TGFβ family, was shown to be activated in chemoresistant melanoma cells and to escape the antitumor activity of targeted therapies (*i.e.*, BRAF inibitors) [188]. In metastatic melanoma cells, Nodal could also induce vasculogenic mimicry (a marker of melanoma cells plasticity) through the binding to and activation of TGFβ receptor II [189].

IGF-1 is another growth factor deeply linked to cancer progression and expression of stemness traits in different types of tumors, including melanoma. IGF-1 can be produced by cancer cells or by cells of the tumor microenvironment, to act as either an autocrine or a paracrine stemness promoting factor [190]. Blockade of IGF-1 was shown to prevent melanoma cell metastatic behavior and the EMT process through downregulation of mesenchymal (Zeb1, CD44, CD29, N-cadherin) and stemness (Sox2, Oct3/4, CD133) markers as well as of features of the functional behavior of stem cells (ALDH activity, sphere formation) [81]. In line with these observations, Wang and coworkers demonstrated that overexpression of IGFBP5 inhibits melanoma cell metastatic features and this is accompanied by the suppression of stem cell markers, such as Nanog, Sox2, Oct4 and CD133. IGFBP5 also interferes with the activation of the IGF1 receptor and its downstream signaling pathways (ERK and p38-MAPK) as well as with the expression of HIF1α, MMP9 and VEGF, known to be crucial players in the EMT process in melanoma cells [82].

The Notch receptors family comprises a group of cell surface receptors that are highly activated in cancer cells and specifically involved in the tumorigenic functions of CSCs. The expression of these receptors has been widely found to correlate with the expression of stem cell markers (*i.e.*, ALDH1) in different types of cancer cells [173, 174, 191, 192]. It is well known that the Notch receptors signaling is dependent on the activity of the γ-secretase (GSI), a protease complex that is responsible for the cleavage of these receptors, thus releasing their active intracellular fragment (NCID, Notch Intracellular Domain). NCID then translocates into the nucleus to regulate the expression of different genes involved in CSCs features and functions [174].

Notch1 signaling has been reported to be overactivated in melanoma cells, acting as a driving factor in their metastatic progression; its expression correlates with the expression levels of nestin. Murtas and coworkers reported that Notch1 activation is involved in the 'maintenance of melanocyte stem cells and associated vasculogenesis' [193]. Notch1 activity was found to correlate with the expression of the CD133 stem cell marker in melanoma cells; in these cells, the expression of CD133 was shown to be regulated by NICD1. Interestingly, the Notch1/CD133 axis modulates the expression level of proteins involved in melanoma progression and metastasis, such as MMPs and VEGF, through the p38-MAPK pathway [87].

Hsu and coworkers reported that Notch3 signaling increases the plasticity of melanoma cancer stem cells; in these cells, silencing of Notch3 induces a downregulation of CSC markers, such as CD271 and CD133 [194]. Finally, Notch4 was reported to be highly expressed in melanoma stem-

like cells and to trigger metastasis through activation of the Twist/VE-cadherin/E-cadherin signaling pathway [195].

Several data from the literature support that the Wnt signaling pathway is deeply involved in the regulation of CSC features. Specifically, Wnt proteins are a family of 19 secreted glycoproteins that are involved, either in an autocrine or in a paracrine way, in the control of CSC expansion, self-renewal, resistance, movement and polarity. These proteins were shown to act through the activation of both a canonical (β-catenin-mediated) and a non-canonical (β-catenin-unrelated) signaling pathway [171, 196-198]. The prominent role of the β-catenin-mediated pathway in cancer resistance and stemness, with respect to the non-canonical pathway, is now widely accepted.

Secreted Wnt (particularly Wnt2B and Wnt3 in CSCs) binds to the Frizzled receptor (Frz) which is complexed with its co-receptor LRP-5/6 (low density lipoprotein receptor-related 5/6). In the absence of Wnt ligand, β-catenin (bound to axin) is phosphorylated by CK1 (casein kinase 1) and GSK3 (Glycogen Synthase Kinase 3), resulting in its ubiquitination and proteasomal degradation. [199]. When Wnt binds to the receptor complex, axin is recruited to the membrane together with LRP5/6 and Disheveled, thus interrupting β-catenin degradation. Stabilized β-catenin can now enter the nucleus where it displaces co-repressor proteins and transcription factors, such as Groucho and TCF/LEF, triggering the transcription of its target genes. Genes activated by β-catenin are deeply involved in the EMT program and development of resistance and include Twist, Slug, Snail, vimentin, and the transporter ABCG2 [171, 196-198]. However, it must be underlined that a positive correlation of this pathway with melanoma progression has been recently questioned [200].

Hedgehog (HH) is another signaling pathway involved in EMT and CSC features. [201]. In the absence of the ligand, HH receptors are linked to the membrane protein Smoothened (Smo). In this condition, the HH effector proteins (Gli, Glioma-associated oncogene homolog) are phosphorylated and subsequently degraded at the proteasomal level. After the binding of HH protein to the receptor, Smo is activated and triggers the activity of Gli, especially Gli1, responsible for the induction of stem cell and of EMT markers [202]. Santini and coworkers reported that the HH pathway is highly activated in melanoma stem cells; on the other hand, targeting Smo or Gli1 by means of specific antagonists results in decreased stem cell features, such as melanosphere formation and ALDH expression [203].

The embryonic stem cell marker Sox2 is highly expressed in melanoma stem cells and is deeply involved in their stemness behavior and features; in these cells, Sox2 was found to be upregulated by Gli1 and Gli2 at the transcriptional level [204]. Thus, the HH signaling pathway may represent an additional molecular target for anticancer compounds (including natural compounds) specifically acting by depleting the stem cell subpopulations in tumors [205].

8. Melanoma CSCs as novel therapeutic strategies

A major challenge of the current therapies for solid tumors, such as chemotherapy and radiotherapy, is represented by the development of resistance to drugs and tumor recurrence. Different molecular mechanisms were proven to be involved in therapeutic resistance, such as: increased drug metabolism and drug efflux (mainly related to increased expression of drug transporters); enhanced repair capacity of damaged DNA; reactivation of drug targets; overactivation of growth and survival signaling pathways; amplification of genetic mutations; impaired activity of the apoptosis/autophagy pathways [206, 207]. Tumor heterogeneity is another well accepted key driver of drug resistance in cancer patients [208, 209]. In particular, in contrast to

the bulk of tumor cells, the subpopulation of CSCs has been widely shown to possess specific features that allow them to escape the antitumor activity of cytotoxic therapies in different types of cancers [58, 210-213].

CSCs, at their membrane level, express different members of the family of 'ATP-binding cassette (ABC) transporters' (ABCB5, ABCG1, ABCG2, ABCG5), a family of 'efflux pumps' that are able to bind and extrude drugs (such as chemotherapeutics) from the cells into their microenvironment. These proteins were reported to be expressed in CSCs and to be involved in the mechanisms of their survival to cytotoxic agents [214-216].

In addition to the ABC transporters, high levels of ALDH activity were observed in cells resistant to classical antitumor drugs, such as taxanes, cyclophosphamide, doxorubicin; mechanistically, ALDH was reported to catalyze the metabolism of these drugs into inactive compounds, thus making the cells resistant to treatments [212, 217, 218].

CSCs were shown to resist to chemotherapy by overexpressing factors involved in the regulation of the apoptosis pathways, such as the anti-apoptotic protein Bcl-2, through activation of the Notch/Hedgehog signaling [219, 220]. Moreover, some environmental conditions, such as tumor-associated vasculature and hypoxia, can selectively promote CSC survival and proliferation [210].

CSCs were also shown to be resistant to radiation therapy, mainly based on their ability to repair damaged DNA; these observations derive from studies performed in glioblastoma cells, since radiation therapy is the treatment of choice for this type of tumor. It was found that glioblastoma cells expressing the stem marker CD133 can efficiently repair radiation-induced DNA damage, through activation of checkpoint kinases CHK1 and CHK2; moreover, radiation treatment selectively kills the bulk of tumor cells while sparing CD133 positive cells in *in vitro* as well as in *in vivo* (xenografts derived from patients cells) [221]. In addition, radiation therapy can induce the formation of reactive oxygen species (ROS), responsible for DNA damage and apoptosis. CSCs escape these effects by inducing a constitutive up-regulation of ROS scavengers [222].

Taken together, these observations strongly support that CSCs represent a very attractive target for novel treatment strategies; the combined use of standard therapies directed against the bulk of tumor cells and compounds that specifically target the CSCs subpopulation might significantly improve the therapeutic opportunities for cancer patients.

CSCs were widely reported to be involved also in the development of resistance to standard therapies ('targeted therapies', such as vermurafenib, dabrafenib, binimetinib; chemotherapies, such as dacarbazine, taxanes) in melanomas. We recently showed that treatment of BRAF-mutated A375 melanoma cells with vemurafenib dose-dependently inhibits cell viability; interestingly, we found that cells escaping the anticancer activity of the compound showed a high ability to form melanospheres [26, 45, 46], a specific CSC feature. In a series of BRAF-mutated melanoma cells, resistance to BRAF and MEK inhibitors was demonstrated to be associated with increased expression of the transcription factor Sox2, known to be involved in cancer cell stemness, and its downstream target CD24, a protein associated with cancer cells aggressiveness and that specifically localizes in membrane lipid rafts where it interacts and activates Src-kinases [49]. BRAF-mutated melanoma cells escaping the antitumor activity of vemurafenib and binimetinib were reported to be associated with enhanced expression of the YAP/TAZ stem cell markers [168]. Cordaro and coworkers demonstrated that melanoma cells made resistant to dabrafenib were found to exhibit stemness features, associated with activation (nuclear translocation) of the transcription factor Oct4 and subsequent increased expression of CD20, a specific melanoma stem cells marker [223].

Similar observations were reported in melanoma cells resistant to chemotherapeutic drugs. For instance, taxol was found to increase the activity of the ERK pathway in $CD133^+$ (but not $CD133^-$) melanoma cells [224]; moreover, in mouse B16F10 melanoma cells, resistance to paclitaxel was shown to be associated with the expression of ABCB5; a combination treatment with paclitaxel and a short-interfering RNA against ABCB5 significantly suppressed tumor growth escaping the development of resistance [225]. Interestingly, in the same murine cell line, the chemotherapeutic dacarbazine induced upregulation of the subpopulation of CD133 positive cells and increased the 'vasculogenic mimicry' property of these cells [97] Recently, integrin β3 has been suggested as a novel marker of cell stemness. Zhu *et al*. reported that, in both murine and human melanoma cells, development of resistance to doxorubicin and methotrexate is associated with increased expression of integrin β3; cotreatment of the cells with chemoterapeutic drugs and an inhibitor of this integrin was able to kill melanoma cells and to reverse drug resistance [226].

Based on these observations, targeting the specific biological features of melanoma stem cells (*i.e.*, expression of stemness markers, molecular pathways involved in self-renewal, metabolic alterations) might represent an effective therapeutic strategy to eradicate this aggressive cell subpopulation and overcome drug resistance.

CD20 is a well known cell surface marker of melanoma cells stemness. Schmidt and coworkers reported that specific targeting of $CD20^+$ melanoma cells induce a complete eradication of tumor lesions *in vivo* [227]. In melanoma patients resistant to chemotherapeutic drugs, intra-tumoral injections of rituximab, the specific antibody against CD20, promoted a regression of tumors growth, accompanied by a significant decrease of serum inflammatory markers [228]. Interestingly, liposomes conjugated with CD20 antibodies and loaded with vincristin, a well known anti-melanoma drug that specifically targets the bulk of tumor cells without affecting the CSCs subpopulation, were found to significantly decrease the growth of melanospheres *in vitro* and *in vivo* in xenografted mice [229]. More recently, Zeng *et al.* developed salinomycin-loaded lipid polymer nanoparticles linked to anti-CD20 aptamers. The aim of this drug design was to specifically target salinomycin, a compound characterized by a poor solubility, to CD20-positive melanoma stem cells. These aptamers were efficiently uptaken into $CD20^+$ melanoma cells, exerting a significant cytotoxic activity against these cells and reducing their ability to form melanospheres. These results were further confirmed in preclinical studies (nude mice xenografted with melanoma cells and treated with the nanoparticles) [230].

By short interfering RNA, in *in vitro* and *in vivo* experiments, it was observed a downregulation of $CD133^+$ in FEMX-I highly metastatic melanoma cells associated with a significant decrease in cell proliferation, metastatic behavior and ability to form melanospheres. Similar results were obtained with antibodies specifically targeting CD133 [231]. $CD133^+$ melanoma cells were reported to resist to the antitumor activity of the bioactive compound CAPE, caffeic acid phenylester, through an upregulation of the drug transporter ABCB5; silencing this membrane pump resensitized the cells to the antitumor activity of CAPE [232]. Bostad *et al.* utilized the 'phytochemical internalization' technique to further support the role of CD133 as a molecular target of antitumor strategies specifically targeting CSCs [233]. The AC133-saporin is an immunotoxin formed by a complex formed by a biotinylated monoclonal antibody against CD133 (AC133) linked to saporin. In their work, Bostad and coworkers entrapped this complex into endocytic vesicles that were specifically uptaken into FEMX-1 melanoma cells expressing CD133 to form endosomes. By using the 'phytochemical internalization' technique, they could induce the disruption of the endolysosomal

membranes, thus leading to the release of the free cytotoxic compound in the cytoplasm. AC133-saporin specifically inhibited the proliferation of the subpopulation of melanoma cells expressing the surface $CD133^+$ marker [233]. Melanoma cells expressing this marker were also reported to express the VEGF receptor (VEGF-R2). Treatment of these cells with both the cytotoxic drug etoposide and the antiangiogenic compound bevacizumab significantly reduced their ability to form melanospheres *in vitro* and to give rise to lung metastasis *in vivo* [234]. More recently, the polyphenol morin, isolated from members of the *Moraceae* family of plants and extracted from woods, was shown to decrease the growth and sphere formation ability as well as the expression of different surface markers of stemness (CD133, CD20 and CD44) in melanoma cells by inducing the expression of miR-216a and subsequent downregulation of Wnt-216a expression levels [235].

Nanoparticles coated with hyaluronic acid, a specific ligand of the marker CD44, and loaded with the chemotherapeutic drug paclitaxel, significantly inhibited $CD44^+$ stem cells by inducing apoptosis, *in vitro* and *in vivo* [236]. It is known that some tumor cells, at their membrane level, overexpress CD47, an integrin-associated protein which can bind to a specific receptor expressed on macrophages, to counteract their phagocytic activity. Interestingly, a cotreatment with antibodies targeting CD47 and the stemness marker CD271 significantly counteracts tumor metastasis formation in melanoma patient-derived cells xenografted in nude mice [64].

As discussed above, the reliability of surface markers has been recently questioned, stimulating the search for novel markers of stemness in cancer cells. In a recent paper, we demonstrated that melanoma cells with stemness features are characterized by high fluorescence [26], possibly due to the presence of riboflavin in vesicles coated with the transporter ABCG2 as previously reported by Miranda-Lorenzo and coworkers [44]. In this paper, we also demonstrated that the natural compound delta-tocotrienol (δ-TT) triggers the disaggregation of melanospheres and counteracts the ability of melanoma stem cells to form melanospheres, by decreasing the expression of ABCG2 [26].

Another effective marker of stemness is ALDH. In a recent paper by Sarvi *et al*., it has been shown that ALDH1 is able to bio-activate nifuroxazide, belonging to the family of 5-nitrofuranes; interestingly, activated nifuroxazide specifically targets the $ALDH1^{high}$ melanoma cells by reducing their stemness properties [237].

Both synthetic and natural compounds were reported to affect the survival and self-renewal of melanoma CSCs by targeting stemness-associated transcription factors, such as Nanog, Sox2 and Oct4 [238-240] as well as intracellular signaling pathways involved in CSC growth (such as Notch and Wnt/β-catenin) and apoptosis (such as Bcl-2 and Bad) [197, 241-245]. A high efficiency in killing CSCs was observed by treating melanoma cells with Bcl-2 inhibitors together with a retinoid derivative (fenretinide) [246] or with an inhibitor of γ-secretase (GSI) [247].

A key role of the Wnt pathway has been described in melanoma CSCs [248]. This pathway was shown to serve as an effective molecular target of different natural or synthetic compounds. The polyphenol morin (3,5,7,2',4'-pentahydroxyflavone) was reported to inhibit the proliferation, self-renewal and sphere formation ability of melanoma cells expressing stem cells markers (CD133, CD44, CD20) by downregulating Wnt-3 through increased expression of MiR-216a [235]. Pimozide is an antipsychotic compound shown to possess also anticancer activity in different types of tumors, including melanoma [249]. Pimozide has been shown to exert its activity by targeting the Wnt/ β-catenin signaling pathway [250]. Liu and coworkers recently demonstrated that the compound 35b, a derivative from the natural chemical scaffold of symplostatin, affects the stem

cells traits (ALDH, sphere formation ability) of melanoma cells by interfering with the activity of the Wnt/β-catenin pathway [245].

As stated above, a rewiring of mitochondrial biogenesis and activity is a key characteristic of melanoma CSCs. Lamb and coworkers demonstrated that 'mitochondrial-targeted antibiotics (tetracyclins, erythromycins, glycylcyclines), reported to be devoid of side effects in normal cells, specifically eradicate CSCs supporting their efficacy for preventive strategies. These results were further supported by clinical trials [251]. In line with this observation, Sotgia *et al.* recently suggested the relevance of the treatment of melanoma cells with 'mitochondrial-based oncology platforms, MITO-ONC-RX' to specifically target the CSC subpopulation in tumors [139, 141].

Taken together, from the data so far available the literature it is clear that targeting the CSC subpopulation represents an attractive strategy to increase the therapeutic options for melanoma patients, independently of their genetic mutations.

9. Concluding remarks

Cutaneous melanoma still represents the cause of the majority of cancer-related deaths among skin cancer patients. It is characterized by a high cell heterogeneity, responsible for its frequent development of resistance to standard therapies. CSCs are a small, slow-cycling subpopulation of cells present in different tumors, including melanoma. These cells are characterized by self-renewal capacity and a high ability to proliferate and differentiate thus sustaining the initiation and the growth of the tumor mass. CSCs were widely reported to play a key role in the mechanisms of drug resistance; for this reason, the identification/isolation of CSCs and the characterization of their molecular, biological and functional features is now considered a crucial step for the improvement of anticancer strategies.

From the molecular point of view, melanoma CSCs are characterized by the expression of: surface markers of indifferentiation state (CD271 and CD133), ABC transporters involved in drug efflux, intracellular metabolic markers (ALDH enzymes) and embryonic stem cell markers (Sox2, Oct4, Nanog). However, it must be underlined that the reliability of surface markers has recently become a matter of debate. To overcome this problem, additional markers of stemness were investigated. It was found that a subpopulation of cells associated with autofluorescence, and the expression of the ABCG2 transporter, is present in melanoma cell lines, pointing out an additional potential stemness marker. Melanoma CSCs are able to form spheres when cultured in appropriate conditions and to give rise to the original tumor mass when xenografted in nude mice.

From the biological point of view, melanoma CSCs are associated with different functional features. They play a critical role in the process of tumor dissemination, based on the observation of their presence both in the blood and at the level of metastatic tissues of melanoma patients. Moreover, the tumor progression toward the EMT stage is characterized by the de-differentiation of tumor bulk cells into cells with stemness traits. Melanoma CSCs are also deeply involved in the process of angiogenesis through different mechanisms: expression of proangiogenic factors, trans-differentiation into endothelial cells (vascular or vasculogenic mimicry) and migration moving along the abluminal vascular surface (pericytic mimicry). Similarly to other tumors, melanomas are often associated with escape from the immune system, due to a low-immunogenic profile and/or immunosuppressive properties. Specifically, melanoma CSCs were shown to be associated with a low-immunogenic profile, due to a very low expression of tumor associated antigens, and with the

ability to evade the immune system, by expressing factors involved in the negative regulation of T cell activation and by secreting immunosuppressive molecules. These cells also display a specific metabolic reprogramming.

Both these molecular and functional traits of melanoma CSCs, associated with specific intracellular signaling pathways, are known to be deeply involved in the key role of this subpopulation of tumor cells in the development of drug resistance. Based on these observations, CSCs are now considered an attractive target for innovative therapeutic interventions. Specifically, combination treatments based on both CSCs-targeting drugs together with standard treatments may offer the opportunity to definitely eradicate all the tumor cells subpopulations. Moreover, further studies aimed at increasing the opportunity to isolate and characterize melanoma CSCs in single patients are urgently needed to improve both the prognostic and the therapeutic strategies in terms of personalized medicine.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions:

Figure 1. Melanoma CSCs and tumor progression. CSCs are deeply involved in melanoma dissemination, as demonstrated by their presence both in the patients circulation and at metastatic sites. CSCs are also involved in the processes of epithelial-to-mesenchymal transition (EMT) and angiogenesis. It is now established that two different EMT programs are activated during tumor progression. According to the classical theory, tumor bulk cells can acquire mesenchymal traits that allow them to detach from the tumor mass and give rise to dissemination. In addition, a subpopulation of tumor cells can de-differentiate into cells with CSCs features; these cells invade the extracellular matrix, enter (intravasation) the blood vessels from which they exit (extravasation) to colonize distant organs. This second program of EMT in melanoma is schematized in this figure. Melanoma CSCs also play a key role in the process of angiogenesis through different mechanisms: 1) they express and secrete the main proangiogenic factors (*i.e.*, VEGF) in the tumor microenvironment; 2) they can trans-differentiate into endothelial-like cells giving rise to an alternative microvascular circulation (vascular or vasculogenic mimicry); 3) they migrate moving in a pericytic-like manner along the surface of blood vessels (extravascular vasation migratory metastasis, EVMM), thus reaching distant metastatic tissues.

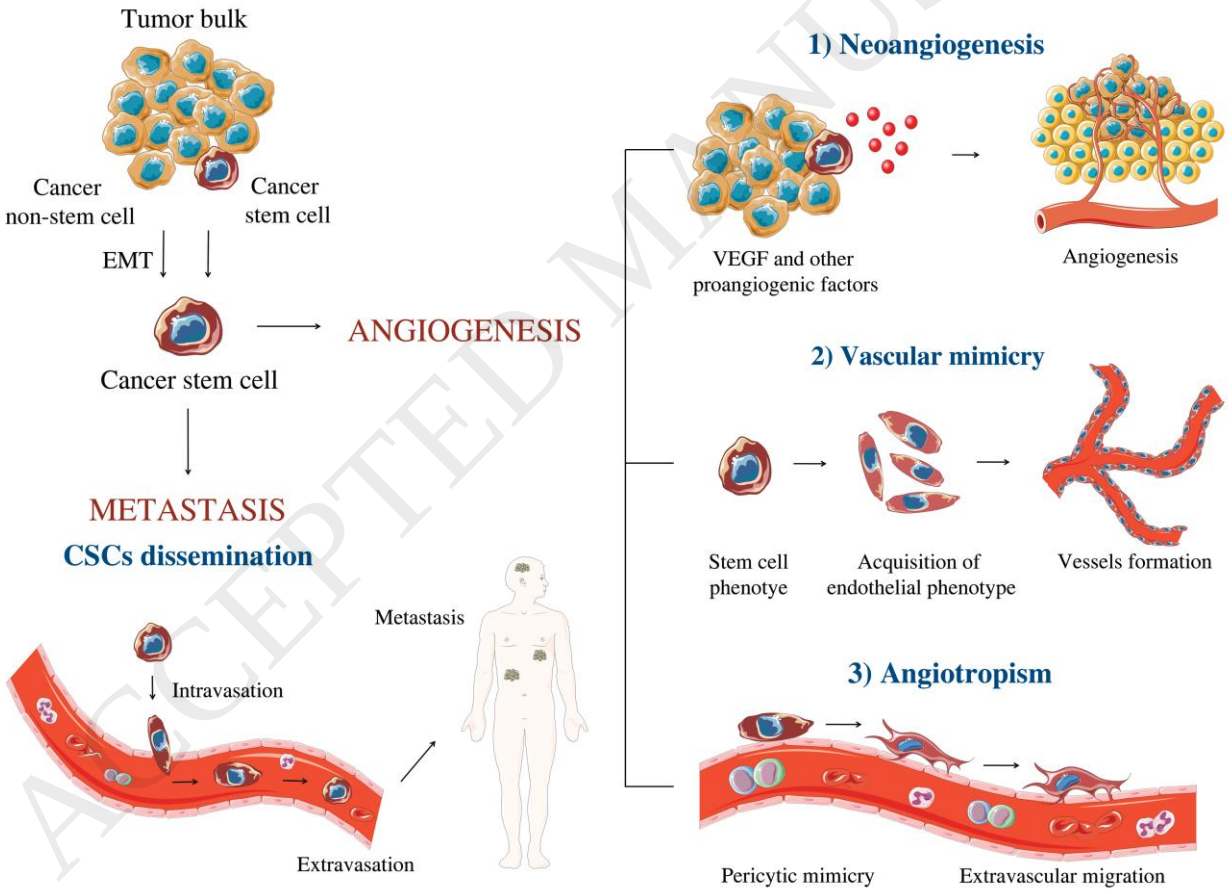

Figure 2. Immunogenic properties of melanoma CSCs. Melanoma cells can elicit antitumor immune responses by recruiting innate (early phases) and adaptive (late phases) immune cells. The secretion of cytokines and chemokines by cancer cells leads to the infiltration of antitumor macrophages (M1) and neutrophils (N1). The overexpression of tumor-associated antigens (TAAs)

leads to the recruitment of professional antigen-presenting cells (dendritic cells), that in turn activate effector B and T cells against tumor cells. The expression of TAAs can be accompanied by the expression of MHC-I (Major Histocompatibility Complex-I) on melanoma cells, that leads to the direct recruitment of T cells. On the other hand, melanoma CSCs are able to evade immunosurveillance through the secretion of cytokines involved in the recruitment of protumor immune cells, through the overexpression of immunomodulatory molecules such as checkpoint proteins involved in the negative regulation of T cells (PD-L1), and through the secretion of proapoptotic factors such as FasL. MDSC, myeloid-derived suppressor cells; Treg, regulatory T cells; Th17, T helper 17 cells.