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Insight into Melanoma Stem Cells: The Role of the Hedgehog Signaling in Regulating Self-Renewal and Tumorigenicity

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8.1 Introduction

Cutaneous melanoma is among the most aggressive types of human cancer, and if untreated, has the potential to metastasize. While patients with low tumor thickness can be cured in 90% of cases by surgery, the majority of patients with metastatic disease die because of the inefficiency of current systemic treatments to produce long-term effects.

Cutaneous melanoma is defined as a malignant tumor derived from the transformation and proliferation of epidermal melanocytes, enabling a stepwise progression from common melanocytic nevus to radial growth phase melanoma, vertical growth phase melanoma and, finally, metastatic disease [1]. However, recent data suggest that a considerable number – around 60% to 75% – of melanomas develop *de novo*, without any precursor lesions. On the basis of these observations and according to repeated findings on melanoma heterogeneity, an alternative hypothesis has been proposed in light of the emerging cancer stem cell (CSC) concept. Mounting evidence suggests that melanoma may arise from a multipotent CSC that is able to self-renew, differentiate into diverse progenies and drive continuous growth [2]. In this context, the term *melanoma stem cell* represents an operational definition, indicating a multipotent tumor-initiating cell subset that – although monoclonal in origin – can give rise to a heterogeneous progeny that recapitulates the tissue of origin.

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8.2 Evidence for the Existance of Melanoma Stem Cells with Self-Renewing and Tumorigenic Properties

During the past decades, indirect evidence has supported the presence of melanoma stem cells. First, melanomas show phenotypic heterogeneity both *in vitro* and *in vivo*, suggesting an origin from a cell with multilineage differentiation abilities. Melanomas retain their morphologic and biological plasticity, despite repeated cloning. Second, melanoma cells often express developmental genes such as Sox10, Pax3, Mitf and Nodal. Melanomas also express the intermediate filament Nestin, which is associated with multiple stem cell populations. Third, melanoma cells can differentiate into a wide range of cell lineages, including neural, mesenchymal and endothelial cells.

Next to these indirect findings, recent studies have provided direct evidence for the existence of melanoma stem cells [reviewed in 2]. Applying growth conditions suitable to human embryonic stem cells, Fang and colleagues [3] found a subpopulation of melanoma cells propagating as non-adherent spheres in approximately 20% of metastatic melanomas, whereas in standard media only adherent monolayer cultures developed. Sphere formation *in vitro* has been proposed by different groups as a common growth feature of stem cells, including neural crest–derived stem cells. The authors showed that melanoma spheres can differentiate under appropriate culture conditions into multiple lineages, such as melanocytes, adipocytes, osteocytes, and chondrocytes, recapitulating the plasticity of neural crest stem cells [3]. Multipotent melanoma spheroid cells persisted over several months after serial cloning *in vitro* and transplantation *in vivo*, indicating a stable capacity to self-renew. Interestingly, sphere cells were more tumorigenic than their adherent counterparts when grafted into mice. Finally, the authors found that the stemness criteria were significantly enriched in a small CD20-positive subpopulation, indicating that CD20 might be a suitable surface marker for the identification of melanoma stem cells [3].

Additional support for the existence of melanoma stem cells came from the finding that the surface marker CD133, a stem cell marker previously applied to neural stem cells, could be employed to isolate a subset of stem-like melanoma cells from patient biopsies [4]. Using fluorescence-activated cell sorting (FACS) from freshly isolated melanoma cells, the authors demonstrated that the CD133-positive subpopulation represents less than 1% of the total tumor mass of melanoma, a finding consistent with designated stem cell subpopulations from other tissues. Like the CD20-positive population defined by Fang and colleagues [3], CD133-positive melanoma cells revealed an

increased tumorigenicity when injected into non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice [4]. However, because of stringent experiments on self-renewal and transdifferentiation capacity of CD133-positive melanoma cells were not performed, the role of CD133 as a potential melanoma stem cell marker remains elusive. Of interest however, the marker CD133 was highly co-expressed with ABCG2, a member of the ATP-binding cassette (ABC) transporter family [4].

Another common characteristic of stem cells is the ability to efflux Hoechst 33342 dye and thus to exhibit low fluorescence in FACS analysis. Using flow sorting of three melanoma cell lines isolated from lymph node metastases, Grichnik and colleagues detected a tiny subpopulation (also called the side population) of small-sized cells with Hoechst 33342 efflux and with the ability to give rise to cells of different morphology when cultured *in vitro* [5]. Cells from this sub-population additionally showed a low proliferation rate but a high ability to self-renew.

Later, an important study showed that ABCB5 is a functional biomarker of melanoma stem cells [6]. In contrast to previous reports, this study examined *i)* serial xenotransplantation of prospectively isolated subpopulations of melanoma cells, *ii) in vivo* genetic lineage tracking and *iii)* targeting of melanoma cells using a monoclonal antibody to ABCB5, to demonstrate the existence of ABCB5-expressing cells. ABCB5+ melanoma cells, but not their ABCB5- counterpart, not only proved capable of tumorigenesis, but also of self-renewal and differentiation into a heterogeneous population. Interestingly, the authors established also a clinical correlation between ABCB5 expression and disease progression, suggesting that ABCB5 may be a biomarker of melanoma progression [6]. Most importantly for therapeutic purposes, administration of anti-ABCB5 monoclonal antibodies impaired growth of melanoma xenografts in nude mice [6].

However, soon after the identification of ABCB5 as a melanoma stem cell marker, the existence of cancer stem cells in human melanoma was questioned. In fact, two studies using a severely immunocompromised mouse model (interleukin-2 receptor γ chain-null NOD/SCID) suggested high frequency of tumor-initiating cells in melanoma [7, 8]. These studies failed to find any correlation between a specific phenotype and tumor-initiating ability and led to questioning the existence of melanoma stem cells [7, 8]. Much of the controversy might be found on the use of dissimilar methodologies to study melanoma stem cells and on differences in the definition of cancer stem cells and their abundance.

Additional evidence that melanoma follows a cancer stem cell model came from recent studies. Melanoma cells from primary and metastatic tumors that expressed CD271 (nerve growth factor receptor), a surface marker of neural crest stem cells, displayed a marked capacity for self-renewal and differentiation plasticity when compared with CD271- melanoma cells. Furthermore, when tumorigenesis was examined CD271+ melanoma cells, but not CD271-, formed tumors and metastases [9, 10]. Interestingly, CD271 and ABCB5 were recently found to be co-expressed in clinical human melanoma samples.

In another study, a sub-population of melanoma cells identified by higher aldehyde dehydrogenase (ALDH) activity displayed enhanced tumorigenicity and capacity to self-renewal when compared to ALDH-negative cells [11]. Melanoma cells that express the receptor activator of NK-kB (RANK) demonstrated increased tumorigenicity compared with RANK-negative melanoma cells. Moreover, RANK was coexpressed with ABCB5 and CD133 in melanoma cells, and preferentially expressed by peripheral circulating melanoma cells [12]. Recently, a temporary distinct subpopulation of slow-cycling cells characterized by the expression of the H3K4 demethylase JARID1B has been shown to be required for continuous melanoma growth [13].

8.3 The Hedgehog Signaling Pathway

A handful of morphogenetic signaling pathways regulating developmental processes, organ homeostasis and self-renewal in normal stem cells, plays also a critical role in tumorigenesis. Among them, Hedgehog (Hh) is crucial for determining proper embryonic patterning and controlling growth and cell fate during animal development. Similarly, in the adult, is involved in tissue maintenance and repair, regulating stem cell behaviour.

Activation of the Hh signaling is initiated by the binding of Hh ligands (Sonic, Indian and Desert) to the trans-membrane protein Patched (Ptch), which, in absence of the ligands, represses the pathway by preventing the activation of the essential trans-membrane protein Smoothened (Smo). Binding of Hh to Ptch allows activation of Smo, leading to the formation of activating forms of the Gli zinc finger transcription factors Gli1, Gli2 and Gli3 [14, 15] (Figure 8.1). Direct transcriptional activation of Gli1 by Gli2/3 enhances the level of Gli activators and high level expression of Gli1 is considered a reliable indicator of Hh pathway activity. Gli1 and Gli2 act as main mediators of Hh signaling in cancer by controlling the expression of target genes involved in proliferation, metastasis, survival and

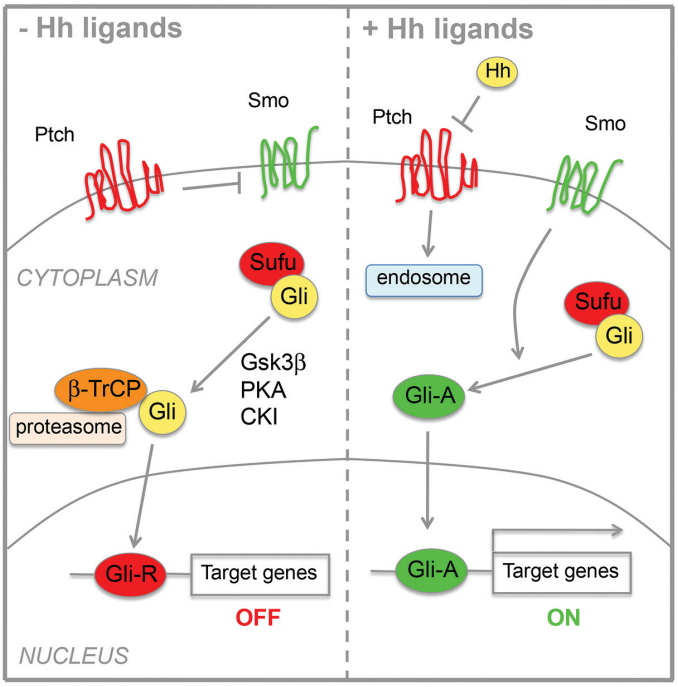

Figure 8.1 Schematic diagram of the Hh signaling pathway. In the absence of the ligand (left), the Ptch receptor suppresses the function of Smo. Full-length Gli proteins (Gli, yellow) are converted to a C-terminally truncated repressor form (Gli-R, red). Formation of the Gli-R is promoted by sequential phosphorylation of full-length Gli by GSK3 β, PKA and CKI, which creates binding sites for the adapter protein β-TrCP, becoming subject of ubiquitination. The Gli-R mediates transcriptional repression of target genes. In presence of the ligands (right), binding inhibits Ptch's function, which results in activation of Smo. Active Smo promotes the activation of full-length Gli proteins (Gli, green), which enters the nucleus and promotes transcription of target genes.

stemness [14, 15]. The activity of the three Gli proteins is tightly controlled. First, nuclear-cytoplasmic shuttling is tightly regulated by protein kinase A (PKA) and by Suppressor of Fused (Sufu), which not only prevents their nuclear translocation, but also inhibits Gli1-mediated transcriptional activity. Second, ubiquitination and protein degradation of Gli proteins are regulated by several distinct mechanisms, including β-TrCP-, cul3/BTB-, Numb/Itch- and acetylation-mediated ubiquitination. Third, Gli3 and, to a lesser extent, Gli2, can be processed into transcriptional repressors [reviewed in 16] (Figure 8.1).

8.4 Role of the Hedgehog Signaling in Regulating Self-Renewal and Tumorigenicity of Melanoma Stem Cells

In physiological conditions, Sonic Hh promotes the development of multipotent neural crest progenitors [17], from which melanocytes derive, and regulates proliferation of human melanocytes [18]. Similarly, it regulates brain stem cell lineages [e.g. 19] and skin stem cells [e.g. 20]. Aberrant activation of the HH-GLI signaling has been demonstrated in several types of human cancers [16]. The HH pathway is active and required for melanoma proliferation and growth *in vivo* of human melanoma xenografts [18] and GLI2 has been shown to drive melanoma invasion and metastasis [21]. In particular, our group has showed that HH signaling is active in the matrix of human hair follicles and that it is required for the normal proliferation of human melanocytes in culture. HH signaling additionally regulated the proliferation and survival of human melanomas. The growth, recurrence, and metastasis of melanoma xenografts in mice were prevented by local or systemic interference of HH function. Moreover, we presented evidence that oncogenic RAS-induced melanomas in transgenic mice express Gli1 and require Hh signaling *in vitro* and *in vivo* [18].

HH signaling has been shown to regulate CSC survival and expansion in several types of cancer, such as glioma, colon and gastric cancer, multiple myeloma and myeloid leukemia [22–26].

In a recent work, we have used the sphere assay to enrich for melanoma stem cells in a collection of primary and metastatic human melanoma samples obtained from a broad spectrum of sites and stages [27]. We have found that spheres display extensive *in vitro* self-renewal ability and that as few as 10 melanoma cells can sustain tumor growth *in vivo*, generating human melanoma xenografts that recapitulate the phenotypic composition of the parental tumor. We have shown that spheres express high levels of HH pathway components and embryonic pluripotent stem cell factors, such as *SOX2*, *NANOG*, *OCT4* and *KLF4*. In addition, we have demonstrated by FACS and immunocytochemistry that human melanomas contain a subset of cells expressing high ALDH activity ($ALDH^{high}$), which is endowed with higher self-renewal and tumorigenic abilities than the $ALDH^{low}$population. Moreover, we found a good correlation between the number of $ALDH^{high}$ cells and sphere formation efficiency *in vitro* [27]. In addition, we discovered a potential clinical correlation between ALDH expression and melanoma progression. In fact, primary and metastatic melanomas contained more cells expressing

ALDH than normal and dysplastic melanocytic nevi. We also detected more ALDH-positive cells in thick primary melanomas and melanoma metastases than in thin primary melanomas. Interestingly, in most of the thick melanomas ALDH was highly expressed in tumor cells at the tumor-host interface, suggesting that the ALDH-positive melanoma stem cells might be mainly localized at the invading tumor front. These results led us to hypothesize that ALDH-positive cells might therefore be involved in the formation of metastatic melanoma, whereas less aggressive tumors might originate from other types of melanoma-initiating cells. Notably, the aggressive biological behaviour demonstrated in mice by isolated ALDH^{high} cells and the high percentages of these cells detected in some rapidly lethal melanomas of our collection suggested that ALDH might be a melanoma stem cell marker that correlates with a poor prognosis [27].

To demonstrate the role of the HH signaling pathway in regulating self-renewal and tumorigenicity of human melanoma stem cells, we knocked-down the transmembrane protein SMO and the transcription factor GLI1 by using specific short hairpin RNA (shRNA). Silencing of SMO and GLI1 drastically reduced self-renewal of melanoma spheres and the number and self-renewal of putative ALDH^{high} melanoma stem cells. Similar results in terms of decrease in self-renewal were obtained by pharmacological inhibition of SMO with the antagonist cyclopamine and of GLI with GANT61, a novel GLI inhibitor [27].

To test the role of the HH signaling in ALDH^{high} melanoma stem cells *in vivo*, we engrafted subcutaneously into athymic nude mice ALDH^{high} melanoma cells transduced with shRNA specific for SMO and GLI1. Cells transduced with the control yielded rapidly growing tumors, whereas silencing of SMO and GLI1 greatly reduced tumor growth. These results strongly suggest that the HH signaling is required for growth *in vivo* of ALDH^{high} melanoma stem cells [27].

8.5 Conclusions

During the past decades, numerous reports have portrayed melanoma as an aggressive cancer with an exceptionally high degree of heterogeneity and plasticity. Today, a growing body of experimental data provides direct evidence that many characteristics of melanoma might be found on the existence of a cell population with stem cell–like properties. Recent data support the existence within human melanomas of a subpopulation of highly tumorigenic cells with features similar to embryonic stem cells, including the potential to self-renew and differentiate into a variety of tissue types. Our

study highlights the role of the HH signaling pathway in driving self-renewal and tumorigenicity of melanoma stem cells and points to SMO and GLI1 as novel and effective therapeutic targets for the treatment of human melanoma.

Although most reported findings seem to be highly promising, many unanswered questions still exist. We do not yet know how many subpopulations of melanoma cells with stem cell properties exist. First, is there a definite number of clearly distinguishable subpopulations, or is there a continuous spectrum of cells, that is passing through a state of trans-amplifying cells that gradually lose their stemness, to differentiated tumor cells? Second, although many cancers contain cells that display stem cell–like features, the identity of the normal cell that acquires the first genetic hit leading to the tumor-initiating cell remains elusive in melanoma. Normal cells that already have stem cell properties represent likely targets, but other mechanisms are conceivable. Third, almost nothing is known so far about the niche of melanoma stem cells. What is the impact of the niche on melanoma development, maintenance and metastasis? As a long-term perspective, melanoma stem cell research will certainly influence and improve the diagnosis, prognosis, and therapy of melanoma. Traditional treatments might be recalibrated and novel therapies need to be developed focusing on the ability to target the melanoma stem cell population and its specific signaling pathways.

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